Artificial Skin and Tissue Regeneration

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Artificial Skin and Tissue Regeneration

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Dedicated to my beloved ones
Frédérique, Jessica & Anthony
and to my parents

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by

Evert Nicolaas Lamme

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Chapter 1

General Introduction

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AIMS OF THE STUDIES

The general aims of these studies were to find ways to stimulate wound healing and tissue regeneration and prevent wound contraction and scar formation. Preceding to the studies in this thesis, a dermal substitute was developed by the Wound Healing Research Group (Department of Dermatology, Academic Medical Centre -Burns Centre, Red Cross Hospital and Dutch Burn Foundation, Beverwijk, The Netherlands). This dermal substitute proved to be capable to improve tissue regeneration and scar formation. Our objective was to further optimise the concept of dermal substitution to the ideal situation in which wound contraction and scar formation are completely prevented. To achieve this among other things fibroblasts were added to the dermal substitute.

The studies in this thesis were performed in full thickness wounds using a pig wound model. This animal was chosen because its skin resembles most the human skin. Wound healing was investigated in time by wound histology and the use of objective methods to measure changes in the wound histology. These results were correlated to a reduction in wound contraction and scar formation and allowed the identification of some mechanisms showing how dermal substitutes and the addition of fibroblasts are able to improve wound healing. These mechanisms are reviewed in this introduction, *chapter 1*. In the *2nd chapter*, the tissue regeneration of wounds treated with the dermal substitute was compared to tissue regeneration of the standard third-degree burn treatment: split-skin mesh grafts. The objective was to correlate the quality of healing to the distribution of extracellular matrix molecules and investigate how the dermal substitute altered and improved tissue regeneration. In the *3rd chapter*, the survival and proliferation of fibroblasts seeded in the dermal substitute were investigated and related to the rate of dermal substitute degradation in the wounds. In *chapter 4*, the rate and quality of tissue regeneration were investigated in relation to the seeding of different numbers of fibroblasts in the dermal substitute and the effects of pre-culture of the cellular substitutes were investigated. Different wound healing parameters were evaluated and correlated to the total

numbers of fibroblasts in the substitutes. *Chapter 5* compares the use of allogeneic fibroblasts to the use of autologous fibroblasts in the construction of a cellular dermal substitute. The use of allogeneic fibroblasts would be preferred to the use of autologous fibroblasts since patients could then be treated immediately without having to wait until autologous cells have multiplied in culture and generated sufficient cell numbers to treat large skin defects. However, the allogeneic fibroblasts induced inflammatory reactions which adversely affected the wound healing outcome. In *chapter 6*, we investigated whether the antimicrobial activity of a wound dressing could be combined with stimulation of healing and tissue regeneration. Deep burn wounds and chronic wounds are always contaminated with bacteria and have a high risk of infection. Dermal substitution in these wounds without antimicrobial therapy is an unacceptable concept. *Chapter 7* is a summary discussing the conclusions of the studies and their relevance for future patient treatments with dermal substitutes.

1. The skin and wound healing

1.1 The architecture of the skin

The architecture of normal skin shows the presence of three layers: the subcutis, dermis and epidermis. The epidermis can vary in thickness depending on the body location, but is in general about 0.2 mm thick. It consists of five layers, stratum corneum, lucidum, granulosum, spinosum, and basale. The two basal cell layers are in part slowly dividing cells serving as the germinate layer providing keratinocytes which terminally differentiate, forming keratohyalin granules, stratify and fuse forming the lipid basis of stratum corneum (chapter 1 in (1)). The epidermis is a highly specialized cell organ made out of keratinocytes, and residences melanocytes, Langerhans cells and Merkel cells. The dermis consists of 2 layers: papillary and reticular layer. The papillary layer and epidermis are connected by the basement membrane.

The keratinocytes are attached to the basement membrane with hemidesmosomes (reviewed in (2)) and by protein-glycosaminoglycan chain interactions mediated by transmembrane molecules like versican, CD44, syndecan and collagen XVII (further described throughout this review). The basement membrane consists of two layers, the lamina lucida and densa with the lamina lucida underlying the plasma membrane of the basal keratinocytes. The integrins in the hemidesmosomes bind members of the laminin family variants (3,4) and Bullous Pemphigoid antigen-230 (BP-230), which initiate the self-assembly of the structure of the basement membrane. The major proteins in the lamina densa zone of the basement membrane are collagen IV, nidogen (entactin), perlecan (heparan sulphate proteoglycan) and other less abundant molecules (described in chapter 10 in (1)). The lamina dense zone of the basement membrane is connected to the dermis by anchoring fibrils. These anchoring fibrils consist of collagen type VII or of fibrulin-2 possibly connected to fibronectin and/or fibrillin and they have a size of 800 nm. In the underlying tissue longer fibrils are present, but the anchoring, formation and structure of the these fibrils is less well understood. Components involved seem to be bamacan (basement chondroitin sulphate proteoglycan), elastin and possibly collagen V and VI. Fibrillin microfibrils are known to self-assemble and form elastin fibres. Furthermore, the focally distributed tenascin is likely to have a function in the epidermal-dermal junction (5,6). However, tenascin-C knock-out mice did develop normally without an apparent skin phenotype (7). The basement membrane structure has been reviewed in (8-12).

The papillary dermal layer contains numerous fine capillary loops, initial lymph vessels, and nerve endings. The reticular dermis is less vascularized and consists of a basket-weave pattern of collagen bundles intermingled with elastin fibres. The components and structure of the extracellular matrix (ECM) of the dermis were recently reviewed in (13). The fibril forming collagens are mainly of type I, III and V. At transient sites in between basement membrane (also of vessels) and the connective tissue an increase in type III collagen is found, which is less

cross-linked and less rigid than collagen type I. Type V collagen is also involved in regulating fibrillogenesis (collagen bundle diameter). Apart from fibrillar collagens, fibrils associated collagens are present, e.g. type VI, IX, XIV. They have a function in the spatial organization of collagen, interact with the attached glycosaminoglycan chains to other proteins, and regulate macromolecular assembly of the fibrillar collagens (e.g. type XIV collagen). The ECM also contains several proteoglycans. The small leucine-rich proteoglycans, e.g. decorin, fibromodulin, biglycan, lumican, epiphycan, have protein conformations suitable for protein-protein binding. Decorin and fibromodulin bind also to collagen I, thus forming bridges with other non-fibrillar collagens, fibronectin, and thrombospondin and regulate fibrillogenesis (14). Another abundant proteoglycan in the skin is versican. Versican is a member of the hyalectans or lecticans (aggrecan, brevican) and is able to bind hyaluronic acid (15). In between the ECM components, the interstitial fluid is present which mainly consists of hyaluronic acid (HA) and blood plasma proteins. Hyaluronic acid (HA) is a negatively charged high-MW polysaccharide (>1 MDa) with water binding capacity and high intrinsic visco-elastic properties. The highest concentration of HA is found in the connective tissues (16). It is synthesised by most cells, and the high turnover in the skin is mediated mainly by lymph drainage and subsequent degradation in the liver and in part by receptor mediated internalization (CD44) (17).

The cells that populate the dermis, apart from the cells in hair follicles and sebaceous and sweat glands, are mainly fibroblasts, vascular cells, nerve cells and some dendritic cells and mature basophils (mast cells), and a few monocytes, macrophages, and T cells (18). The resident fibroblasts are considered to be quiescent or involved in the normal turnover of the ECM. Some fibroblasts in the reticular dermis could be specially involved in the generation and/or turnover and organization of basement membrane proteins (19,20).

1.2 Skin injury and wound healing phases

The different phases in wound healing have been extensively surveyed in the books edited by R.A.F. Clark (21) and by I.K.Cohen, R.F.Diegelmann, and W.J.Lindblad (22). The different phases of normal wound healing initiated after skin damage will be briefly described in this section.

The first phase, the acute phase, is characterized by hemostasis, vasoconstriction and capillary pressure resulting in plasma and cell leakage into the wound bed. The next phase is the inflammatory phase in which granulocytes and monocytes infiltrate into the wound bed. They stimulate fibrinolysis and attract other and more mesenchymal cells to the wound bed. Consequently, the proliferative phase starts in which the wound epithelialises and dermal granulation tissue is formed. In the absence of bacteria, infection and excess tissue debris, the granulation tissue contains mainly vascular structures, fibroblasts and macrophages. The final phase in wound healing is the remodelling phase in which the ECM synthesized during the proliferative phase is remodelled into scar tissue.

In the acute phase blood cloth formation takes place. Platelets degranulate and secrete several active mediators and growth factors which in turn activate granulocytes present in the fibrin clot. The platelet mediators and granulocytes are the main effectors in recruiting more inflammatory cells, e.g., additional neutrophils and monocytes. These cells will not only protect the wound against bacteria and remove wound debris, but also start to resolve the fibrin clot. The activated fibrinolytic pathway will not only dissolve the fibrin cloth facilitating the ingrowth of cells, but also generate breakdown products of e.g. fibronectin, vitronectin and liberate and activate trapped growth factors. This forms a potent chemotactic signal for fibroblasts, endothelial cells and other monocytes. The infiltration of monocytes from the blood circulation is furthermore facilitated by upregulation of adhesion receptors on endothelial cells in microvessels at the wound edges. The inflammatory phase is likely to be responsible for the 'intensity' and amount of granulation tissue which will be formed.

The mesodermal cells migrate first through the connective tissue adjacent to the wound bed into the fibrin/fibronectin provisional matrix. The migration is mediated by the action of proteolytic enzymes and specific adhesion molecules, integrins. The neovascularisation and formation of the granulation tissue restores the blood circulation and metabolite homeostasis allowing the migration of keratinocytes from the wound edges, which will close the wound. The keratinocyte migration is also characterized by the specific upregulation and downregulation of several integrins and proteolytic enzymes. The keratinocytes migrate at the interface of blood cloth and granulation tissue using fibronectin and fibrillar collagens as substrates. Immediately behind the leading tip of the migrating epidermis, the restoration of the basement membrane starts. The basement membrane is a complex structure responsible for the attachment of the epidermis to the underlying connective tissue. The integrity of this structure is very important, and small alterations in basement membrane molecules and structure can result in epidermal abnormalities. The rate of basement membrane regeneration is often seen as a good parameter to study wound healing. However, the architecture of the regenerated dermal tissue is decisive for scar tissue formation and its elasticity and extensibility.

The granulation tissue can be characterized as a cell dense tissue consisting of a mixture of mesodermal cells which mediates tissue regeneration. The initiation of granulation tissue formation starts with angiogenesis and fibroplasia. Angiogenesis or neo-vascularisation not only restores the blood supply and metabolite homeostasis, but probably has an underestimated role in the regulation of tissue deposition and regeneration. Fibroplasia is characterized by fibroblast proliferation and increased ECM synthesis and deposition. The presence of macrophages and their regulating role in inflammation and in tissue regeneration is of renewed interest in the wound healing research field. In normal wound healing, the function of granulocytes and lymphocytes seems to be less important in time. Immune cell depletion studies in animals demonstrated that only macrophages were crucial for normal wound repair.

The function of the granulation tissue becomes more important as more dermal tissue is damaged or lost. The skin function needs to be restored rapidly, since infection remains an important risk factor. Large and deep wounds are closed by epithelialization and wound contraction, a process resulting in more scar formation. Till today, the phenomena of wound contraction and/or tissue fibrosis, skin hypertrophy and contractures are still problems with an intriguing complexity and unrevealed causes. The role of the myofibroblasts as effectors in these processes is however without any doubt. Nevertheless, the regulation and characterisation of fibroblast differentiation and the triggers leading to their disappearance in the granulation tissue by apoptosis are still unclear.

The last phase of wound healing is characterized by tissue remodelling. In the granulation tissue, the amount of synthesized and deposited ECM increases as the cellularity and vascularity decreases with time. The collagen fibrils will align, form bundles which become thicker and more mature. The ECM is characterized by changes in the composition of the different types of collagens and increased cross-links of the fibres in time. The increased levels of different proteoglycans will return to normal levels, the basement membrane will reintegrate with the dermis with the formation of the microfibrils, and finally elastin fibres will regenerate.

Each phase as described above is characterized by specific events. The different phases overlap in time and the time point of the start of the next phase is depending on multiple factors and the extent of overlap depends on complicating factors (23). The acute phase normally lasts no longer than 24-48 hrs, but may continue for several days in case of large wounds or lack of proper wound care. The inflammatory phase normally does not last longer than several days but may last much longer in the case of wound infection. The amount of granulation tissue formation can be insignificant when wound closure is rapid, but in the case of full thickness wounds, it may take more than three weeks before the cellularity decreases and ECM remodelling and maturation occur. The duration of tissue remodelling depends on the size and especially on the depth of the wounds. If wound contraction and scar formation have occurred, the remodelling of the ECM and the basement membrane are likely to take several years.

Wound healing research is a mixture of fields of cell biology, tissue physiology, biochemistry and molecular biology and is involved in a complex interplay of multiple events at different signal levels. Moreover, the cells encounter many signals during wound healing, which cannot be mimicked in culture models. New wound treatments can therefore only be properly investigated in animals models. The signals cells receive from their ECM surroundings consist of cell-cell contacts, cell-ECM adhesions, interaction with ECM degradation products, growth factors, eicosanoids, and chemokines. Depending on this information, the cell will decide how it will respond to their surrounding, e.g. start to migrate, proliferate or differentiate.

In the second section of the introduction, the events during early wound healing will be reviewed and an inventory will be given of all mediators involved in regulation of cell migration and initiation of wound repair. In the third section, an overview will be given of cell adhesion molecules and adhesions with its surrounding and the mechanisms involved in cell migration, which is consequently followed by a characterization of the cells that migrate into the wound. In the fourth section, the role of the fibroblast in tissue regeneration and wound contraction will be reviewed. During this introduction several intracellular signal transducing molecules and pathways will be mentioned, which are reviewed briefly in section 5 of this introduction. This section will help to understand cellular behaviour in response to different mediators present during wound healing.

2. The early phases in wound healing

2.1 The acute phase of wound healing

Skin damage results in bleeding and activates the process of hemostasis. Successful hemostasis is achieved by adhesion and aggregation of platelets to the endothelium. The endothelium is a monolayer of flat cells attached to the subendothelial structure existing of basement membrane extracellular matrix proteins, smooth muscle cells and pericytes. Endothelial cells normally prevent coagulation by multiple mechanisms: (I) secreting anticoagulant substances, e.g., endothelium-derived relaxing factor, prostaglandin I_2, NO, and tissue-type plasminogen activator (tPA); (II) expression of receptors which bind ADPase, trombomodulin and heparins; (III) by hiding the ECM of the subendothelium (IV); and by vasodilatation mediating dilution of procoagulant factors and reducing shear stress (reviewed in (24)).

After vessel damage platelets adhere to the endothelium and exposed ECM takes place, which initiates blood coagulation. Platelet adhesion to endothelium under higher shear forces is depending on von Willebrand Factor binding and subsequent activation of platelets (reviewed in (25)). Von Willebrand Factor is a multimeric protein present in plasma and subendothelium. It is synthesized by platelets and endothelium and the latter cells bind von Willebrand Factor through several integrins. Platelet adhesion to cells and ECM (fibrinogen, fibronectin, laminin, collagen and vitronectin) is mediated by multiple receptors, like E- and P-selectins, Platelet-endothelial cell adhesion molecule (PECAM), and GP-proteins (being mostly integrins). In addition, the activation of platelets induces conformational changes of adhesion receptors altering affinity and binding of ligands (reviewed in (24)). The adhesion and activation of platelets by activated endothelium is further mediated by secretion of platelet activating protein (PAF), a potent mediator of platelet aggregation and granule release, by phosphatidylserines present in the membrane of vesicles and calcium released from dense granules.

In the process of blood coagulation, the cleavage of fibrinogen by thrombin generates fibrin, which assembles into fibres and is the essential "glue" to complete the blood coagulation process and plug formation (reviewed in (24-26)). The endothelium also participates in the clotting cascade by producing and secreting von Willebrand Factor, Factor V and VII, tissue factor and kininogen (all co-factors in the production of thrombin). The activated platelets secrete several mediators. The platelets contain two main types of secretory granules, α–granules and dense granules. The α–granules are bigger and are present in higher numbers than the dense granules. The α–granules contain: Platelet Factor 4 (PF4), ß-thromboglobulin, platelet basic protein (PBP), albumin, kininogen, Factor V, von Willebrand Factor, thrombospondin, fibronectin, C1-inhibitor, α2-antitrypsin, α2-macroglobulin, α2-antiplasmin, insulin-like growth factor binding protein-3 and growth factors PDGF, EGF, IGF-I, TGF-ß's (26-29). The TGF-ß 1 is the predominant isoform and all isoforms are mainly secreted in the latent form (30). The dense granules contain predominantly serotonin, but also tromboxane A2, 5-HETE, ADP, ATP, Ca^{2+}, Mg^{2+} (31). These mediators are implicated in several effector functions, e.g., increasing platelet aggregation (ADP, PF4 and fibronectin), leading to vasoconstriction (5- and 12-HETE, serotonin, and TxA_2), are co-factors for the generation of thrombin (Factor V, kininogen), and stimulate chemotaxis and cell proliferation (PDGF, TGF-β's, EGF, IGF-1, tromboxane's and the chemokines PF-4, PBP (and its breakdown products CTAP-III and NAP-2). The induced vasoconstriction is mediated by the underlying smooth muscle cells, and is furthermore stimulated by exposure to other vasoconstrictors, such as endothelins, and angiotensins. The vasoconstriction is important for successful coagulation and stimulates collision of platelets with the vessel walls.

After a few minutes, vessels start to dilate due to the increased capillary pressure and the presence of several mediators ,e.g., histamine, 12-HETE, PGE-2 and serotonin (32). The adhesion of leukocytes to endothelial cells is stimulated by the upregulation of P-selectin expression (stored intracellularly) on endothelial cells. The following leukocyte diapedesis requires additional adhesion molecule interactions, PECAM-PECAM and CD11(b or c)/CD18-ICAM1, and drastic cytoskeleton reorganisations. The mechanisms involved in diapedesis are reviewed in (32,33). The leukocytes that infiltrate the wounds the first hours after wounding are mainly polymorphonuclear leukocytes, predominantly neutrophils, followed after a few days by mononuclear cells. The vessel dilatation not only results in plasma leakage into the wound bed but also increases the local temperature. The disturbed vascularisation in the wound edge furthermore leads to hypoxia, which will increase the CO_2 concentration in the tissue and lactate production. This leads to a reduction in the tissue pH causing alteration and to a certain extent denaturation of ECM proteins and structures, a process known as wound oedema. This local acidification could also be implicated in the activation of growth factors present in the ECM, such as TGF-ß's and FGF-2.

2.2 The ECM after fibrin clot formation

Small undeep wounds and incisional wounds have a different wound architecture and healing pattern than large partial and full-thickness wounds or burn wounds. The small and incisional wounds coagulate rapidly with minor eschar formation. The small wounds become hardly contaminated or infected, the fibrin clot will resolve fast and epithelialization from the wound edges will soon close the wound. The attraction and accumulation of inflammatory cells and need of wound contraction to close the wound are absent in this type of wounds. In large wounds, wound contamination is likely to be present as well as the risk of wound infection. The fibrin clot and eschar formation are a substantial part of the wound architecture and will hinder wound epithelialization from hair follicle remnants. The amount of proteolytic wound activity necessary to resolve the fibrin clot and eschar is increased and this will clear the way for migrating cells and granulation tissue formation. Wound closure by epithelialization from hair follicle remnants is absent as more dermal area is lost. As wound closure is delayed, the amount of granulation tissue and wound contraction will increase (34). In addition, the presence of necrotic tissue will further impede and delay the healing process. Its presence attracts more inflammatory cells and intensifies the proteolytic wound activity which will often result in the loss of viable tissue. Surgical or enzymatic debridement are required to positively influence the wound healing process.

The wound architecture of large and deep wounds can be characterised by the ECM at the wound edge and the fibrin clot. The wound edge will also be actively remodelled during the healing process. The fibrin clot consists of fibrin fibres with captured plasma proteins and blood cells. Important to realise is that several proteins are present or cross-linked to the fibres of the fibrin clot and that the fibrin clot provides a temporary ECM for infiltrating cells.

The role of fibronectin in the plasma clot has been extensively studied. Fibronectin is a dimeric glycoprotein which is crosslinked to fibrin by factor XIII transglutaminase. Fibroblasts are able to adhere, spread and migrate in fibronectin depleted fibrin clots, but migration is stimulated by fibrin cross-linking and the cross-linking of fibronectin to fibrin (35-37). Interestingly, fibronectin molecules are capable of spontaneous cross-linking and form multimeric proteins, which resemble fibrils (38,39). Fibronectin exists in multiple isoforms. The gene has two splicing sites ED-A and ED-B, and a splicing domain with multiple introns called CS domain. Plasma fibronectin is a fibronectin isoform that does not contain the splice domains ED-A and ED-B. The cellular fibronectin produced in wounds does contain the splicing domains ED-A and ED-B, and a variable proportion of exons of the multiple splicing domain CS (40). Cells can bind to fibronectin at multiple sites (at least six). The best characterized sites are the RGD-site (near the ED-A domain, integrin $\alpha 5\beta$ and $\alpha IIb\beta 3$ binding sites) and two binding sites in the variable CS domain (integrin $\alpha 4\beta 1$ binding sites), and the heparin binding domain. The latter site mediates cell attachment mainly indirectly through heparan sulphate binding. In

the fibrin clot the fibronectin form present is plasma fibronectin. Other predominant proteins in the fibrin clot derived from serum and platelets are thrombospondin and vitronectin. Their role in cell migration will be discussed throughout this review.

Within one day after wounding, mainly HA was shown to accumulate in the wound (41). The source of HA could be plasma and/or production by local cells, although the latter seems unlikely so early after wounding. In fetal wounds, scarless healing was associated with high levels of HA, and later in gestation stage the initiation of scar fibrosis was associated with increased hyaluronidase activity (42,43). The effects that HA exerts on wound healing seem to be dependent on the size and concentration of HA. Oligosaccharides and 4-25 dissaccharide HA units were shown to stimulate cell migration and angiogenesis, whereas high MW HA did not or even impaired angiogenesis (44,45). The expression of hyaluronidase activity by tumour cells also stimulated angiogenesis (46). In normal skin, 20%-30% of the total HA turnover occurs by receptor mediated internalisation and degradation, and the rest is removed by the lymphatic pathway (16). Interestingly, recently a hyaluronidase enzyme was identified in blood plasma (47,48), which also could be present and active early during healing. Furthermore, HA has been implicated in multiple cellular processes, e.g., cell migration, proliferation, and differentiation (49-52), preventing oxygen radical damage (53), inducing chemokine expression in macrophages (54), and in vitro HA was able to modulate fibroblast mediated collagen gel contraction (55). Cell division of adhering cells was stimulated by enhanced production and deposition of HA in the pericellular matrix. During cell division adhering cells almost completely detach from their substrate and the presence of HA is believed to facilitate cell detachment, which could explain the observed stimulation of cell proliferation (17). Nevertheless, opposite effects on fibroblast proliferation have been reported also (56). However, most data of the different in vitro studies are difficult to compare since different MW sizes, concentrations, and cell types were used. In addition, the sources of HA were not comparable and often a certain amount of contaminating proteins was present in the HA preparations (56,57).

During cell infiltration and granulation tissue formation, the fibrin clot is resolved. Degradation products of fibrin and fibronectin not only have chemotactic properties but also stimulate fibroblast proliferation and modulate protease expression (58-60). The upregulation and expression of other ECM proteoglycans in the granulation tissue, like tenascin, biglycan, decorin, versican, and collagens coincidence with the ingrowth of granulation tissue and declining presence of HA. Tenascin has been implicated in regulation of cell adhesion and de-adhesion during development and in wound healing (61-63). Tenascin expression in the dermis was upregulated after 3 days in partial thickness wounds in the rat and somewhat later in full thickness wounds (64). In humans, in punch biopsy wounds, tenascin staining was found in the papillary dermis after a few days and was abundantly present in the newly formed granulation tissue (5). In scar tissue, tenascin remained present in the dermal tissue up to months after

wounding (65). The proteoglycans, especially decorin, are important in collagen fibrillogenesis (14,66). The proteoglycans decorin, biglycan and versican all have variable chondroitin sulphate and/or dermatan sulphate chains attached to the core protein. The side chains have been implicated in control of cell adhesion and control of cell proliferation by binding growth factors (67,68). Versican seems to be associated with the elastin fibres in the dermis of normal skin (69,70) and is able to bind to HA (15). Versican has been implicated in the inhibition of cell-substratum adhesion (71). Studies describing versican expression in wound healing in time have yet to be published. Decorin and biglycan bind TGF-ß and are involved in the inactivation of TGF-ß (72-74). Decorin also modulates metallo-proteinase-1 (MMP-1) gene expression in fibroblasts growing on vitronectin and the 120 kDa fragment of fibronectin (75). This fibronectin breakdown product has previously been shown to induce MMP-1 and MMP-3 expression in fibroblasts (60). In human oral mucosa incisional wounds, decorin staining reappeared in between 3 and 7 days, whereas biglycan staining did not return within 7 days (41). However caution should be taken with the interpretation of these data, since it has been reported that proteoglycan glycosaminoglycosylation alters during wound healing and was shown to interfere with antibody recognition of decorin (76). The formation of the granulation tissue furthermore coincidences with the deposition of cellular fibronectin and collagen type I and type III whereas collagen type V and type VI deposition was more specifically related to angiogenesis (77-79).

2.3 The presence of different mediators in the wound

The infiltrating cells not only encounter multiple mediators present in the fibrin clot, but also produce mediators in response to the encountered signals. These signals will influence the behaviour of infiltrating cells and influence the differentiation pathways of monocytes and fibroblasts. In the following paragraphs the role and presence of some growth factors, eicosanoids and chemokines will be discussed. The signal transducing molecules these mediators activate in the cell will be mentioned and reviewed in greater detail in section 5.

2.3.1 Growth factors early in wound healing

The growth factors present early in wound healing are derived from platelets, neutrophils and plasma. The growth factors present in platelets were described above. The most abundant ones are TGF-ß isoforms and PDGF-AB. The production of growth factors by neutrophils requires de novo protein synthesis, and the most abundant ones are likely to be the pro-inflammatory cytokines IL-1 and TNF-α. The factors present in serum are IGF-I, insulin, angiotensins, endothelins, and possibly hepatocyte growth factor (HGF) and growth hormone. Discussing the role of all growth factors present in wound healing is beyond the scope of this review. Only PDGF, TGF-ß and IFN's will be discussed because of their central role in regulation of cell proliferation, differentiation and tissue regeneration.

Platelet-derived growth factor (PDGF) is one of the best studied growth factors in relation to signal transduction. PDGF exists as homodimers PDGF-AA and -BB or as a heterodimer PDGF-AB. The PDGF receptors are mainly localized on connective tissue cells and are implicated in chemotaxis, migration, and proliferation of fibroblasts, smooth muscle cells, neutrophils and macrophages. The PDGF receptor is a tyrosine kinase receptor consisting of two receptor chains α and ß. Tyrosine kinase activity is only induced after receptor homo- or heterodimerization. The PDGF receptor α chain has high affinity for PDFG-AA, whereas the PDGF-BB binds to both the PDGF-α and ß receptor chains with an equally high affinity. This implies a more efficient cell activation by PDGF-BB (ß-ß, α-ß, α-α receptor dimerization) than for PDGF-AB (α-ß receptor dimerization) and PDGF-AA (α-α receptor dimerization). Indeed, the proliferation of wound fibroblasts was stimulated more with PDGF-BB when compared to PDGF-AB and PDGF-AA (80).

In wound healing, topical application of PDGF, especially PDGF-BB, stimulated granulation tissue formation, but PDGF angiogenic effects were weaker than that of FGF-2 (81). Interesting data in relation to this are coming from PDGF-B and PDGF-ß-receptor knock-out mice. During embryogenesis, developing capillary sprouts secrete PDGF which stimulate the co-migration of smooth muscle cells and pericytes. In the knock-out mice, there was no co-migration of pericytes with blood vessels in the brain and no co-migration of connective tissue cells in the kidney (mesangial cells) and the lung (smooth muscle cells) (82,83). This suggests an important role for PDGF in the migration of pericytes, smooth muscle cells and fibroblasts. The PDGF induced membrane ruffling and rearrangement of actin cytoskeleton stress fibres and the association of the activated PDGF-ß receptor chain with the insulin receptor and with αvß3 integrin all seem to be related to the chemotactic activity of PDGF (84-86). The stimulation of actin cytoskeleton rearrangement might also be involved in the observed stimulation of collagen gel contraction by fibroblasts (87). In addition, PDGF also upregulated the expression of the α2 integrin chain on fibroblasts in stressed gels (88,89). The α2ß1 integrin mediates collagen binding and has been shown to mediate collagen gel contraction (90).

In humans, there are three isoforms detected for TGF-ß1, -ß2 and -ß3 (reviewed in (91-94)). They are synthesized as a preproform, cleaved intracellularly by furin, and normally secreted in the latent form. Outside the cells, TGF-ß's bind to the latent TGF-ß binding protein (LTBP), which interacts with multiple ECM molecules (95,96). TGF-ß also binds to decorin, biglycan, fibromodulin (72), and to other not yet identified molecules (97-99). Active TGF-ß1 was also shown to bind to α2-macroglobulin, which is believed to inactivate and clear TGF-ß from the site of injury (100). All this suggests a tight control for TGF-ß activation. Liberation of latent TGF-ß from the ECM has been shown to be mediated by the proteolytic enzymes chymase and elastase, thrombin and plasmin (101,102). Latent TGF-ß is activated after cell binding via specific receptors: TGF-ß binds via LTBP to the mannose-6-phosphate-insulin-like growth factor II (M6P-IGFII) receptor and via thrombospondin to the thrombospondin receptor,

CD36. Bound latent TGF-ß is subsequently activated by cleavage by plasmin or thrombin (91,94,103). It is noteworthy that latent TGF-ß's are also activated by acidification. As described at the end of paragraph 2.1, wound tissue pH reduction precedes wound oedema formation and it has been reported that cells are capable of reducing local pH at the plasma membrane. Collagen phagocytosis by fibroblasts for example showed local acidification in the environment of the phagosome mediated by V-ATPases, which denatures the collagen fibrils and facilitates collagen degradation by the gelatinase MMP-2 (104,105).

The effector activities of the TGF-ß isoforms on cells in vitro are similar in general and concern the regulation of cell proliferation, differentiation and ECM synthesis. These effects are dependent on cell type, TGF-ß concentration, cell density, the microenvironment, and the presence of other growth factors and ECM molecules (92). The strong induction of ECM synthesis in fibroblasts is not only mediated by upregulation of the synthesis of fibronectin, collagens, and other proteoglycans, but also by downregulation of MMP and upregulation of plasminogen activator inhibitor-1 (PAI-1) and tissue inhibitor of metallo-proteinases (TIMP) expression (92). In normal skin, TGF-ß2 is constitutively expressed in the dermis, whereas TGF-ß 1 and 3 are predominantly expressed in the epidermis. During wound healing, the expression, especially of TGF-ß1, is highly upregulated in the dermis (106,107). The expression of TGF-ß receptors RI and RII on fibroblasts in the normal physiological situation is low and becomes upregulated during granulation tissue formation (108,109). Several studies have investigated the expression of the TGF-ß isoforms during wound healing, but results were not always consistent. In general the antibodies used in these studies did not discriminate between the latent complex and the active TGF-ß molecules. Furthermore, antigen recognition can be hindered by TGF-ß binding to the multiple ECM proteins or altered during the fixation and embedding procedures of the tissues (personal observations). Topical wound treatment with TGF-ß1 accelerated collagen deposition and maturation (81,110,111). A different role for the TGF-ß isoforms during wound healing has been suggested and will be discussed in paragraph 4.2.

The interferons (IFN) are implicated in downregulation of collagen synthesis and inhibition of cell proliferation. In addition, in culture IFN-γ was able to reduce the expression of α–smooth muscle actin in myofibroblasts (112). However, IFN-γ seems to be a regulator of specific immune responses, since IFN-γ is mainly produced by a subset of differentiated T cells and induces MHC class I and II expression in nearly all cells (113). During wound healing, mainly CD4+ T cells infiltrate into the wound. A possible role of T cells in regulation of wound healing has only recently become a topic of renewed interest (65,114). The IFN–α and ß's are more likely to be implicated in silencing of granulation tissue formation. IFN-α is mainly produced by macrophages, whereas IFN-ß was identified as the fibroblast IFN. The IFNß-2b is better known as interleukin-6 (IL-6), and is strongly induced in fibroblasts by the pro-inflammatory cytokines TNF–α and IL-1, and multiple other growth factors (115,116). IFNß-2b

has been shown to inhibit fibroblast proliferation (117) and suggests that the balance between mitogenic and inhibitory signals regulates proliferation. IFNß-2b was also able to inhibit fibroblast mediated collagen gel contraction (118).

2.3.2 Eicosanoids and their role in wound healing

Lipid mediators or eicosanoids are derived from released arachidonic acid en generated by cyclo-oxygenases (prostaglandins and tromboxanes) and lipoxygenase (leukotrienes). Their synthesis is schematically given in figure 1.

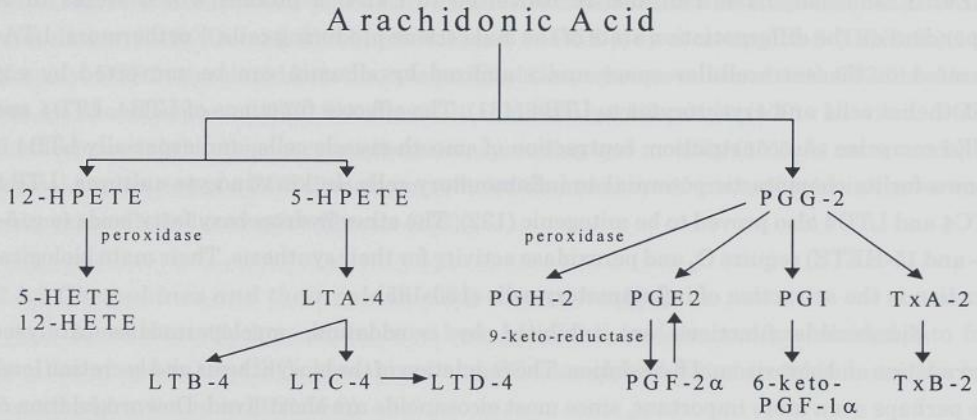

Figure 1. The eicosanoid pathways, describing the lipoxygenase and cyclo-oxygenase synthetic routes. Abbreviations: PG prostaglandin; HETE hydroxyeicosatetraenoic acid; HPETE hydroperoxyeicosatetraenoic acid;Tx tromboxane; LT leukotriene.

Arachidonic acid is generated in the cell by calcium-sensitive phospholipase A2 (c-PLA2) and calcium independent PLA2 (i-PLA2). C-PLA2 is activated by phosphorylation of mitogen activated protein kinases (MAPK), converts lysophosphatidate (LPA) into arachidonic acid and is translocated to the membrane in a calcium dependent manner (119). I-PLA2 activation mechanisms are less well understood, but the enzyme prefers plasmenylcholine as substrate and is transiently activated. Another group of PLA enzymes are platelet activating factor-phospholipases A2 (PAF-PLA2). They are both active intra- and extracellularly and hydrolyse PAF. The secreted form of PLA, s-PLA2, is only active at high calcium concentrations (mM) and generates arachidonic acid outside the cell (reviewed in (120,121)).

The prostaglandins are generated from arachidonic acid by cyclo-oxygenases (COX-1 and COX-2). The first metabolite is prostaglandin G2 (PGG-2) which is converted by peroxidase activity and oxygen into PGH2 and hydroxyradicals. PGH2 is the parent prostaglandin and can be converted into PGF-2α, PGE-1 and -2, PGI-1 and -2 (hydrolyses to 6-keto–PGF-1a) , PGD, TxA2 (hydrolyses to TxB2)(122). PGE-2 and PGF-2α are the most stabile prostanoids, can act in a paracrine manner and have opposing biological effects (Chapter 18 in (22)). PGE-2

is a vasodilator, and inhibits inflammatory responses, whereas PGF-2α induces vasoconstriction, platelet aggregation, chemotaxis, and cell proliferation. The identified receptors are coupled to heterotrimeric GTP-G-proteins (122-128). Effector functions of G-proteins are reviewed in paragraph 5.4.

The leukotriene (LT) biosynthesis depends on the availability of arachidonic acid and is mainly generated within membrane vesicles (129-131). In the vesicles, the generated LTA4 is converted to LTC4, which can be released outside the cells by an ATP-dependent export carrier. Extracellular LTC4 is rapidly converted by membrane bound enzymes into LTD4 and LTE4. In addition, LTA4 can also be converted to LTB4, a process which seems to be dependent on the differentiation state of the leukotriene producing cells. Furthermore, LTA4 secreted in the extracellular space and stabilized by albumin can be converted by e.g. endothelial cells and erythrocytes to LTB4 (131). The effector functions of LTB4, LTD4 and LTE4 comprise vasoconstriction, contraction of smooth muscle cells, and especially LTB4 is known for its chemotactic potential to inflammatory cells. In keratinocyte cultures, LTB4, LTC4 and LTD4 also proved to be mitogenic (132). The other hydroperoxy fatty acids (e.g. 5-, 12- and 15-HETE) require O_2 and peroxidase activity for their synthesis. Their main biological function is the attraction of inflammatory cells (133-135).

Eicosanoids functions are inhibited by ω-oxidation, myeloperoxidase-catalysed degradation and peroxisomal ß-oxidation. The regulation of the biosynthesis and secretion level are perhaps even more important, since most eicosanoids are short-lived. Downregulation of eicosanoids synthesis by PLA2 inhibition seems to be mediated by the induction of lipocortins also called annexins (Lipocortin I = annexin 1 = calpactin II). Lipocortins interfere with the substrate binding of PLA2 and inhibit the formation of arachidonic acid (136). Glucocorticoids are potent inhibitors of PLA2 activity. Glucocorticoids (e.g. cortisone, corticosterone and hydrocortisone) are derived from the renal cortex and induced systemically by stress. Their anti-inflammatory effects on inflammation are not only mediated through inhibition of eicosanoid synthesis, but also by other mechanisms. Glucocorticoid-receptor interactions negatively regulate the transcription of adhesion molecules (ICAM-1, ELAM-1), inflammatory cytokines (IL-2. IL-6, IL-8, IL-1 and TNF–α), intracellular NO synthetase, MMP-1, MMP-9 and keratinocyte growth factor (KGF) production in fibroblasts. In addition, they increase plasminogen activator inhibitor-1 (PAI-1) synthesis in keratinocytes and altered TGF-ß isoform expression in wound healing (137-140).

In early wound healing, the effects of eicosanoids range from stimulating platelet aggregation (tromboxane A2, 12-HETE), inhibiting platelet activation (PGF-2α, PGI-2), stimulate vasoconstriction (tromboxane A2, PGF-2α, LTC4, LTD4), chemotaxis (5- and 12-HETE, LTB4, PGE-2, and PGF-2α) or mediate vasodilatation (PGE-2, 12-HETE, LTC4 and LTD4)(141). The first hours after wound healing PGE-2 and PGF-2α are both present in equal amounts, thereafter the amount of PGE-2 diminishes as the cellular infiltration profile changes

to mononuclear cell types (142). Interestingly, PGE-2 can be converted to PGF-2α by PGE2-9-ketoreductase, an activity seen in proliferative disorders of the skin and in activated mice macrophages (135,143,144). Both prostaglandins are implicated in modulation of cell function and in the stimulation of synthesis of collagens and glycosaminoglycans, especially PGF-2α is a potent inducer of HA synthesis by fibroblasts (145,146). For mouse fibroblasts PGF-2α was mitogenic, PGF-1α and PGD were weak mitogenic, whereas PGE's and PGI's did not induce proliferation (147). The monocyte/macrophage is thought to be the main source of PGE-2 and PGF-2α in the later phases of wound healing, nevertheless PGE-2 can be produced by most cells (142,148). Keratinocytes in culture synthesize considerable amounts of PGE-2 after IL-1 stimulation (149). Dermal fibroblasts respond to PGE-1 by secreting IL-6 (IFNß-2b)(117). Notably, PGE2 stimulated keratinocyte proliferation, when co-cultured with 3T3 fibroblasts, but in the absence of 3T3 fibroblasts it inhibited proliferation (150). In vitro, PGE-2 furthermore inhibited monocyte adherence, spreading and motility (151). In rat wounds, the topical application of a PGE-2 analogue reduced the numbers of macrophages in the wounds, but eventually induced more fibrosis (152).

2.3.3 Chemokines and their role in early wound healing

More than 40 chemokines have been identified to date, and are divided into four families of which the CXC-chemokines and CC-chemokines, listed in table I, are the best described (reviewed in (153,154)).

CXC-chemokine family members	CC-chemokine family members
Epithelial-derived neutrophils chemoattractant-78 (**ENA-78**)	Thymus and activation-regulated protein (**TARC**)
Granulocyte chemotactic protein-2 (**GCP-2**)	**RANTES**, Regulated on activation, normal T cell expressed and secreted
Growth related oncogene (**GRO–α to -γ**)	Hemofiltrate CC-chemokines (**HCC-1 to -3**)
IL-8	**Eotaxin**
Interferon-inducible protein-10 (**IP-10**)	**I-309**
Monokine induced by IFN-γ (**Mig**)	Monocyte chemoactractant protein (**MCP-1 to -5**)
Platelet basic protein (**PBP**) and cleavage products	Macrophage inflammatory proteins (**MIP-1α and -1ß**)
Platelet factor 4 (**PF4**)	
stromal cell derived factor (**SDF-1α and -1ß**)	

Table I. Chemokines divided into their families based on the relative positions of their cysteine residues.

Most chemokines are not stored in cells and their production is stimulated by proinflammatory cytokines and bacterial mitogens (e.g. LPS). They are produced by a wide variety of cells, except for PF4 and PBP which so far were only found in platelets. In general, the CXC chemokines tend to attract neutrophils, and CC chemokines act preferentially on monocytes/macrophages. In solution most chemokines dimerize to form the biologically active

form, but recent data indicate that several chemokines are equally active in monomer forms. Their chemotactic properties are not solely mediated by a fluid concentration gradient. Chemokines bind to negatively charged molecules as heparan sulphate proteoglycans, which are present on cells and more importantly in the basement membrane and connective tissues. The implications of these observations are not yet fully understood. It implies the existence of a solid gradient but could also interfere with growth factor binding and change growth factor availability and/or activity.

The identified receptors for chemokines are members of the seven-transmembrane spanning (7-TM) receptor family and intracellular signalling comprises the activation of multiple signal pathways mediated by the activation of G-proteins (reviewed in (155,156)). The different effector functions of several family members of the G-proteins will be discussed in paragraph 5.4. The mechanisms that inhibit chemokine activity are not yet completely revealed. The mechanisms are sought on the level of down-regulating expression by anti-inflammatory cytokines (IL-4, IL-10, IL-13, and TGF-ß), neutralizing activity by autoantibodies present in the serum, and scavenging by receptors without signal transducing activities. The latter process involves a receptor called Duffy antigen receptor, which is highly present on erythrocytes and inducible on other cell types (157).

The general assumption is that chemokines play an important role in the stimulation and regulation of the inflammatory response after monocytes have infiltrated in the wound. This hypothesis is based on the observation that the production of chemokines is stimulated by pro-inflammatory cytokines and that they are produced in higher quantities by mononuclear inflammatory cells. In wound healing, the infiltrating monocytes are considered to be responsible for the attraction of vascular cells and fibroblasts into the wound. Only recently some reports have investigated the presence of chemokines early in wound healing (158-160). The role of chemokines in tissue injury is reviewed in (153,157).

The first chemokines present at the onset of wounding are PBP (or its breakdown products connective tissue activating protein-III (CTAP-III), ß-thromboglobulin, and neutrophil activating protein-2 (NAP-2)) and PF4, both are released by platelets and both attract predominantly neutrophils to the wound. MCP-1 was also identified early in wound healing (161). MCP-1 attracts solely monocytes, whereas MCP-2 to -5 also can attract and act on T cells, basophils and eosinophils (153,162). The latter MCP's have not yet been investigated in healing wounds. IL-8 and GRO–α, -ß and -γ are structurally related and are chemotactic for neutrophils and basophils, but the GRO proteins are less potent than IL-8 (162). Both IL-8 and GRO-proteins were present during early wound healing (161). Multiple cells have the ability to produce GRO and IL-8. Overexpression of IP-10 in mice delayed wound healing and inhibited angiogenesis (163). IP-10 and Mig attract predominantly IL-2 activated T-cells (153) and are both induced by IFN-γ. It is unlikely that these cytokines are involved in the early chemotactic signals during early wound healing since IFN-γ is selectively produced by a

subpopulation of T-cells. The same can be said of RANTES, a potent attractant for monocytes and activated T cells, predominantly produced by T cells and fibroblasts (153) and indeed proven to be absent the first days after wounding (161). MIP-1α and ß are predominantly secreted by activated macrophages, and were shown essential in recruitment of additional monocytes to the wound (160).

Although only a few studies have investigated the role of chemokines during early wound healing, it is more than likely that chemokines have a role in the attraction of mesenchymal cells into the wound tissue. A direct effect of chemokines on fibroblast migration has not yet been established, but in vitro fibroblasts were shown to respond to chemokines (164).

2.3.4. Proteolytic enzymes involved in wound healing

The metalloproteinases and serine proteases are the most investigated proteolytic enzymes in wound healing due their to specific roles in cell migration, ECM degradation and remodelling. However, other proteolytic enzymes also might have important roles in wound healing since they have been implicated in tumour metastasis and in pathological conditions leading to tissue damage and fibrosis. Moreover, in this field much effort has been put into the development of highly selective inhibitors to control the action of specific proteolytic enzymes involved in metastasis and tissue fibrosis (165,166). The activity of proteolytic enzymes can be classified in carboxypeptidases and endopeptidases and are subdivided into classes on the basis of catalytic mechanisms (167).

The family of cysteine proteases can be divided in enzymes of the calpain and papain family and enzymes related to the IL-1ß converting enzyme (ICE)(168). The ICE related enzymes are involved in apoptosis and release of IL-1ß. The calpain enzymes regulate membrane signalling and the papain enzymes are cathepsins B, H, K, L, O, S (and others). The cathepsins are mainly found in lysosomes and only active under reducing conditions and in an acidic pH range. In wound healing, their function and activity is related to phagocytosis and to the intra-cellular degradation and turnover of tissue proteins. However, during active neoplastic formation cathepsin activity is not only upregulated but is also found to be secreted extracellularly. In different carcinoma's, extracellular cathepsin B was found to degrade basement membrane proteins (169). Cathepsin K, identified in macrophages in inflammatory sites, has a strong degrading potential in an acidic pH range. The expression in macrophages has been correlated with a macrophage degrading phenotype. At neutral pH, the enzyme is only stable for a few hours. However, even at neutral pH cathepsin K is almost as potent in degrading elastin as pancreatic elastase and twice as potent as neutrophil elastase (168). In addition, cathepsin K has also a potent collagenase and gelatinase activity indicating a possible physiological role in macrophage mediated wound debridement. The aspartic acid proteinase cathepsin D has also been implicated in cancer metastasis. Nevertheless, its predominant

presence in phagosomes suggests that it has mitogenic activity instead of a direct involvement in ECM degradation and cell invasion. The mitogenic action involves growth factor activation and/or cleavage of inhibitors (reviewed in (170)).

The serine proteases include elastase, cathepsin A and G, proteinase 3, tryptase, complement activating enzymes, enzymes involved in coagulation and fibrinolysis like thrombin, tissue-type plasminogen activator (tPA), urokinase-type plasminogen activator (uPA), plasmin and multiple other enzymes (171). Cathepsin A or deamidase is a carboxypeptidase possibly involved in the extracellular regulation of angiotensin II activity (172). Cathepsin G is secreted by neutrophils and degrades fibrin and proteoglycans and is able to activate collagenases (173,174). Plasmin and thrombin also have other functions than generating and degrading fibrin which are mentioned throughout this review. The serine protease elastase involved in wound healing is neutrophil elastase. Neutrophil elastase has a broad range of substrates, e.g. elastin, fibronectin and other proteoglycans, collagens, and participates in fibrin fibrinolysis (174,175). In chronic wounds and burns elastase activity is responsible for the degradation of fibronectin (176-178), which in part could be explained by functional degradation of the elastase inhibitor α1-anti-trypsin (179).

Metallo-proteinases (MMPs) characterized by the necessity of a divalent cation for activity comprise collagenases (MMP-1, -8 and -13), stromelysins (MMP-3, -10 and -11), gelatinases (MMP-2 and MMP-9), metallo-elastase (MMP-12), matrilysin (MMP-7), membrane-bound MPP's (MT1-MMP to MT4-MMP or MMP-14 to -17) and carboxypeptidases (CPA, CPB, CPD, CPE, CPN and CPM) (reviewed in (180-183)).

The collagenases are unique in their ability to cleave fibrillar collagens in their triple helix conformation. The affinity of the different collagenases is variable for the different types of collagens, but they are capable as well to degrade other proteins such as aggrecan, serpins, tenascin, and the inhibitors α2-macroglobulin and inhibitor α1-anti-trypsin, or activate pro-MMP-2 and -9 (184). In chronic wounds and inflammation, MMP-13 expression was observed in fibroblasts and epithelial cells (185,186), but it has not yet been detected in normal healing wounds (187). Interestingly, dermal fibroblasts cultured in collagen gels did express MMP-13, but not when cultured on plastic, indicating an important role for ECM-fibroblast interactions in the upregulation of MMP-13 (187). In fibroblasts, TGF-ß1 downregulated the MMP-1 expression, but upregulated MMP-13 in a dose- and time-dependant manner (188). This suggests a role of MMP-13 in tissue remodelling since TGF-ß1 also upregulates ECM protein (collagens) and TIMP-1 expression (182).

The gelatinases first recognized for their degrading activity of denatured collagens proved to digest other substrates like several proteoglycans (e.g. tenascin, fibronectin) and type IV, V and XIV collagens. MMP-2 is constitutively expressed by most cell types and has an important role in collagen turnover and remodelling (104,105,189) and cell migration (190). MMP-9 activity seems to be more restricted. MMP-9 has been implicated in migration of

keratinocytes, and migration over the basement membrane of neutrophils and eosinophils (182).

The stromelysins have a very broad substrate specificity. MMP-3 and MMP-10 have potent degrading capacity towards ECM proteins, but this could be partly mediated by the functional activation of pro-collagenases and pro-MMP-9. In excess present, stromelysins impaired keratinocyte migration (191,192). MMP-11 activity is mainly found in stromal cells and inactivates serpins (193-195). Its expression has been associated with cutaneous scarring (194). However, the observed scarring was observed in the presence of high numbers of inflammatory cells, and scarring itself is not necessarily associated with excess inflammation.

The metallo-elastase (MMP-12) and matrilysin (MMP-7) both have the same substrate specificity as the stromelysins, but are also able to degrade elastin. Their expression seems to be restricted to a few cell types of which macrophages seem to be most relevant in wound healing (192,196). The membrane bound metalloproteinases seem to have an emerging role in the regulation of MMP activation and cell migration (190) and will be discussed later in paragraph 3.3.2.

Of the metallo-carboxypeptidases CPM seems to be the most interesting for wound healing. It is bound to the plasma membrane with the active site outwards and has multiple substrates such as EGF, growth hormone, and complement factor C5a. The removal of the C-terminal arginine of C5a abolishes the chemotactic activity of C5a (172). In addition, it has been suggested that the released arginine acts as a substrate for nitric oxide synthase in the generation of NO. Furthermore, it can be used as a marker of macrophage maturation (197). Other interesting membrane-bound proteases are adamalysin-related proteinases (ADAM) (reviewed in (198)). Although most of them are not yet described to have important functions during wound healing, a role for them in cell migration or protein shedding certainly seems likely. For example, the proteinase bone morphogenetic protein-1 (BMP-1 = procollagen C-peptidase, 70 kDa isoform), ADAM 10 and ADAM 17 are able to release cell-bound TNF–α, and BMP-1 can activate latent TGF-ß's (184). Moreover, the procollagen-N- and -C-proteinases have other biological functions than only processing procollagens and also cleave the precursor forms of lysyl oxidase and laminin 5 (199). Another interesting intracellular protease is furin, a member of the subtilisin/kex2 endoproteases involved in the processing of precursor proteins. Furin is present in the membrane of the Golgi apparatus and has interesting substrates specificity, e.g., growth hormone, TGF-ß1, IGF-1, endothelin-1, several receptors for growth factors, MT1-MMP and MMP-11 (200). Its expression is upregulated by TGF-ß1 (201).

The activity of proteinases is physiologically regulated by compartmentalization, pH, inhibitory proteins, rate of synthesis, secretion and activation of pro-enzyme forms, and proteolytic degradation or targeting to endosomes and lysosomes via mannose-6-phosphate-receptor pathway and by α2-macroglobulin/low-density lipoprotein receptor-related protein (202-204). The compartmentalization mediates limited degradation in the pericellular space

as evidenced by staining of the pericellular space by antibodies recognizing neo-epitopes on proteolytic degraded proteins. Furthermore, this is achieved by phagocytosis and lysosomal degradation. The inhibitory proteins can be divided in non-specific and specific inhibitors. Most non-specific inhibitors are abundantly present in serum and the extracellular space (205) and embody α2-macroglobulin, PAI-1 and PAI-2, α1- and inhibitor α2-anti-trypsin, complement-1-inhibitor (C1-inhibitor), protease nexin I (206). They protect the tissue against excess proteolytic activity. The specific proteinases inhibitors comprise tissue-derived inhibitors of MMP's (TIMP-1 to -4), cystatins, and serpins.

The TIMP proteins inhibit MMP's potently with a 1:1 stoichiometry. TIMP-1 inhibits most MMP's except MMP-2 and MT1-MMP. TIMP-2 also inhibits most MMPs except MMP-9. TIMP-3 is associated with the ECM and inhibits predominantly the collagenases and gelatinases. TIMP-4 is only recently discovered (180,182). The cystatins inhibit specifically the cysteine proteinases and are reviewed in (207,208). The serpins comprise most of the previously mentioned inhibitors present in the serum (209). An interesting aspect of PAI-1, inhibitor α1-anti-trypsin, and anti-thrombin is the existence of a latent form, which only after conformational changes obtains inhibitory activity (209). PAI-1 expression is strongly induced by TGF-ß1 (92) and inhibits both tPA and uPA. Strong PAI-2 expression was found in keratinocytes during wound healing and was correlated with keratinocyte proliferation and migration, and was downregulated in differentiating keratinocytes in an organotypic coculture system (210,211). The inhibitor α1-anti-trypsin is an elastase inhibitor, protease nexin I inhibits thrombin, plasmin, uPA and tPA, and C1-inhibitor inhibits complement activation (205,209). Notably, thrombospondin is a competitive inhibitor of elastase and cathepsin G (212). Other interesting inhibitory proteins are some members of the Trappin protein family, e.g., SKALP/Elafin. SKALP is expressed in activated and proliferating keratinocytes and protects against excess elastase activity (213-215).

3. Cell adhesion and migration into the wound

3.1 Cell-cell contacts and cell-ECM adhesions

The adhesion molecules can be divided into several groups on the basis of structural features (Fibronectin-type III, Ig fold domains, etc.) and homophilic or heterophilic binding modes. The cadherins and Ig family adhesion proteins (N-CAM, PECAM, VCAM) are predominantly homophilic, whereas integrins, and selectins (E-, L- and P-Selectin) (216) and other lectin family proteins are heterophilic. The cadherins, integrins, and selectins are dependent on divalent cations for their ligand binding (216-218). During cell migration the interactions with ECM are mainly mediated by integrins, and adhesion molecules which interact with the ECM via their covalently linked glycosaminoglycan chains. Most integrins bind their ligand with a relatively low affinity and they appear to be designed to function

coordinately in focal adhesion and not so much individually. Integrin binding is controlled by the cells in a diverse manner; regulation of ligand affinity (outside-in signalling), up and downregulation of expression, targeted expression on the plasma membrane and expression of truncated isoforms missing the catalytic domain as found for the ß1 integrin (219). In addition, adhesion sites and integrins are able to directly mediate signal transduction (220-222).

The strong contacts between epithelial cells and the basement membrane are established by tight junctions, desmosomes and hemidesmosomes (reviewed in (2,9,223-226)). The cell-cell contacts between mesenchymal cells during wound healing are established by gap-junctions, adherence junctions and single receptor interactions, the so called 'point' contacts. The gap-junctions are intercellular channels composed of six transmembrane connexin proteins forming a connexon that allows the diffusion of small molecules (< than 1kDa, Ca2+, cAMP, phosphatidylinositol-4,5-biphosphate (PIP2), and phosphatidylinositol-2,4,5-triphosphate (PIP3) (227,228). Adherence junctions are specialized forms of adhesive contacts based on cadherin homophilic cell-cell contact sites. The adherence junctions are stabilised by interaction of cadherin with the actin cytoskeleton. The bridging proteins involved are α– and ß-actinins, vinculin, $p120^{CAS}$ and proteins of the ERM family (ezerin, radixin, moessin) (226). The adherence junctions also participate in signal transduction and/or are regulated by signal transducing molecules, since several signal transducers colocalize in these sites. An example is the phosphorylation of $p120^{CAS}$ by c-Src kinase, which destabilizes adherence junctions (226). In addition, adherence junctions are important in the juxtacrine stimulation of neighbouring cells (229,230).

Adhesions to the ECM are more dynamic and established by focal adhesions and point-adhesion contacts. Specific integrins cluster into focal adhesions after ligand interaction like cadherins in adherence junctions. Focal adhesions differ from point-contacts intracellularly by the anchoring to actin fibres instead of actin filaments and therefore focal adhesions mediate stronger cell-ECM adhesion (217). In the next paragraph the molecular architecture of focal adhesions and the signalling pathways involved will be discussed briefly.

The ECM receptors which cluster in the focal adhesions are mainly integrins, although other receptor molecules might also interact with or be present in focal adhesion sites and stabilize and/or strengthen the adhesion with the ECM (218). The integrin receptors consist of an a and ß-subunit and the family includes more than 16 α–subunits and 8 ß-subunits. Both chains are involved in ligand binding and ligand specificity. An accepted model for multiple integrin receptors is that the α–subunit inhibits the function of the cytoplasmic domain of the ß-subunit preventing binding of specific proteins intracellularly. This inhibition is relieved after ligand binding and initiates subsequent conformation changes in the 3D-structure of the receptors (221,231). The ß1-subunit binds directly to α–actinin and is likely to interact with focal adhesion kinase (FAK), which is constitutively bound to paxilin and talin. The binding

of FAK to ß-integrin results in FAK autophosporylation, binding of $p130^{CAS}$ and exposure of a Src kinase homology (SH-2) binding site. The SH-2 site is recognized by c-Src kinase (and other Src-kinase family members), which phosphorylates FAK at other tyrosine localizations (see also figure 2 in paragraph 5.1). FAK can also bind via the SH-2 binding site to phosphatidylinositol-3-OH kinase (PI-3-K) and Grb-2 (reviewed in (218,222,232)). Possible effector functions of PI-3-K and Grb-2 will be discussed in paragraph 5.1.

The proteins α–actinin, talin and paxilin in the focal adhesion sites are regulated by phosphorylation, and bind to vinculin and actin. Their actin binding activity initiates actin filament bundle formation. Other proteins with a yet unknown function located within focal adhesions, which bind to integrins or FAK or other focal adhesion proteins are reviewed in (218,232). Another interesting signal transducer and mediator of focal adhesion formation seems to be the small GTP-binding protein Rho (233,234). The effector function of Rho proteins in signal transduction are multiple and Rho activity is of importance in actin fibre formation and actin mediated contraction and cell migration (discussed in the next paragraph 3.2). Nevertheless, it has not been excluded that Rho is also involved in the regulation of phosphorylation of focal adhesion components.

The mechanisms involved in focal adhesion disassembly are less well defined. The stability of focal adhesion sites is affected by agents stimulating actin depolymerization, which will also be discussed in relation to cell migration (paragraph 3.2.1). Focal adhesions could also disassembled in a rather drastic manner. Calpain II, a Ca2+ dependent protease and a substrate of c-Src, has been shown to be present in focal adhesion sites and is able to degrade talin and filamin (232). Dephosphorylation of FAK during cell detachment has also been observed and implies protein tyrosine phosphatase (PTP) activity. PDGF induces focal adhesion rearrangements and membrane ruffling and somewhat later the formation of stress fibres in fibroblast (84,235). The focal adhesion destabilisation by PDGF could be mediated by the activation of the PDGF receptor bound phosphatase PTP-1D (218) and possibly by the regulation the Csk kinase, a member of the Src kinase family (236,237). Other interesting proteins able to interact with integrins are membrane tetraspanners molecules (TM4 proteins, e.g. CD9, CD63, CD81). TM4 proteins normally reside outside focal adhesion sites, and have been suggested to play a role in cell motility (218). These molecules might be involved in controlling integrin recruitment to adhesion sites.

Focal adhesion sites are also involved in down stream intracellular signalling. Possible substrates for FAK phosphorylation are paxilin, tensin and $p130^{cas}$. Phosphorylated FAK itself is able to bind signal transducers, PI-3-K, GRAF (a GTPase inactivating protein), and phospholipase C (PLC)(218). FAK might also phosphorylate other not yet identified substrates since FAK activity has been found outside focal adhesions (222,238,239). The latter and the binding of signal transducers to FAK do suggest other roles for FAK than in adhesion regulation alone. Apart from FAK, integrins themselves could also be involved in signal

transduction. Several integrins have been implicated in direct activation of the MAP kinase pathway independent of FAK. Recently, an integrin linked kinase has been identified which is able to bind to integrin ß1 to ß3. In addition, the activation of a SHP phosphatase bound to ß4 in hemidesmosome clearly demonstrates that integrins are directly involved in regulation of signal transduction pathways. The role of integrins in signal transduction is reviewed in (218,220,238).

Cells also interact with the ECM by point adhesions. The adhesion molecules involved in these adhesions are integrins and membrane bound proteoglycans covalently linked to glycosaminoglycan chains. The integrins bind to multiple ECM molecules (reviewed in (221,240)). Since the focus of this review is cell migration and regeneration and remodelling of ECM, principally collagen, only the integrins able to bind to collagen will be discussed here. The integrins which are able to bind collagen are α1ß1, α2ß1 and αvß3. The αvß3 integrin binds preferably to collagen in the denatured form and is expressed on fibroblasts in culture (241,242). In vivo, a function of this integrin in relation to fibroblast migration on collagen or collagen phagocytosis has still to be proven. In addition, its affinity for vitronectin is much higher than for collagen (242). The α1ß1 and α2ß1 receptors also do not bind exclusively to collagen, but for example also to laminin (243). The basis for this specificity is not dependent on variations in the primary sequence, but more on conformational changes induced by the ß1 subunit, and the cellular environment (221,244). The α1ß1, and especially the α2ß1 integrin have been implicated in collagen phagocytosis (245-247). Surprisingly, in normal skin the expression level of α2ß1 on fibroblasts is weak when compared to other cells e.g. keratinocytes (248). Since collagens are the main components of the dermis and collagen turnover is regulated by fibroblasts, it is likely that other receptors could be involved. Indeed, recently, two new collagen receptors were identified, DDR1 and DDR2. Although they are expressed by multiple cells, expression of these receptors on fibroblasts in the skin has not yet been reported. The receptors, tyrosine kinases, are activated by different types of fibrillar collagen, and it appears that the triple helix structure is important for recognition (249). Other cellular interactions with collagen bundles are mediated indirectly by proteins interacting or integrated in the collagen fibres. These interactions often involve glycosaminoglycan-protein interactions. Two adhesion molecules which are expressed at high levels on multiple cell types and mediate cell-ECM contacts in which the attached glycosaminoglycan chains are important, are CD44 and syndecans.

CD44 is a transmembrane glycoprotein with variable N– and O-linked glycolysations and covalently linked to variable chains of chondroitin and heparan sulphate. The glycosaminoglycan moiety is responsible for the interaction with HA, but CD44 is also able to bind to fibronectin, laminin, and other glycosaminoglycans. CD44-H (sCD44) is the isoform without exons, but CD44 can also be expressed with the inclusion of a multitude of combinations of the seven exons. One CD44 isoform mediates cell migration on HA, CD44-M

(vCD44), and contains the exons v4 to v7. How this splicing is regulated is still unknown (250-252). CD44 is linked to the cytoskeleton via ankyrin or the ERM proteins. Phosphorylation of the cytoplasmatic tail interferes with actin binding and fibroblast migration, but not with HA interaction (253,254). CD44 is not the sole receptor which can bind to HA. Cells can also interact indirectly with HA through versican, an ECM chondroitin sulphate proteoglycan which binds to HA (15)

Other types of transmembrane proteoglycans are the syndecans. Syndecan-1 to -4 have multiple heparan sulphate side chains attached to the core protein. The syndecans are able to oligomerize (cluster) and can associate with the cytoskeleton. Syndecans mediate cell-cell and cell-ECM adhesions solely via their heparan sulphate chains. In cell-ECM interaction, they act as co-receptor for integrins, adhesion molecules of the Ig superfamily and selectins and bind to fibronectin, laminin, collagens, thrombospondin and tenascin. The functions in which syndecans are implicated are rather diverse. Apart from a stimulating activity in receptor clustering and promoting strength of adhesion sites, they can also bind multiple heparan binding growth factors (HB-EGF, HGF, PDGF, VEGF, FGF family members and growth factor binding proteins IGF-binding protein-3 and TGF-ß binding protein, (Chapter 15 in (21)). For certain growth factors, it has been reported that syndecans act as a cofactor promoting growth factor binding to their receptor. This effect is most apparent at submaximal concentrations of the growth factor and disappears at higher concentrations. For FGF-1 and FGF-2, there is evidence that heparan binding of the growth factor is necessary to promote receptor dimerization and tyrosine kinase activation (255). Syndecans also have been implicated in the internalization and subsequent degradation of bound proteins. Furthermore, cell activation can lead to membrane shedding of syndecans, which is probably mediated by membrane bound MMP proteolytic cleavage (230,256,257). The implications of these phenomena in cell migration will be discussed in paragraph 3.2.2. Alternatively, it has been shown that the released syndecans could be able to inactivate soluble growth factors and serine protease activity (e.g. cathepsin G, elastase). The inactivation of growth factors was reversed by degradation of heparan sulphate by heparanase, which liberates the active growth factor again (258,259)

3.2 Cell migration

Cell migration is a very typical process, and the understanding of cell motility regulation has progressed rapidly in the past few years. Directed cell migration is dependent on a stimulus (e.g. chemotactic signal) and ligands which can provide traction. The process of translocation and the tight regulation of cell membrane movement (protrusion), actin cytoskeleton and focal adhesion sites rearrangements, cell detachment and retraction has revealed interesting regulatory mechanisms. However, how the cell coordinates these mechanisms is still very unclear. Studying cell migration, one should not forget that cell

migration is often accompanied by cell division (260). Cell membrane movement (protrusion) and the regulation of actin cytoskeleton changes in relation to cell migration will be discussed in the next paragraph 3.2.1 and the role of membrane receptors and proteolysis in cell migration will be briefly described in paragraph 3.2.2.

3.2.1 Intracellular changes during cell migration

The cell has two options upon adherence to a surface, it can become immobilized or use the surface to migrate. The outcome seems to be dependent for a large part on the strength of adhesion. This was nicely illustrated by experiments in which integrin receptor α5ß1 overexpression and enhanced deposition of fibronectin reduced cell migration (39,261,262). Importantly, cell-cell contacts also seem to impair migration by the rapid formation of actin bundles at the contact areas generating stronger adhesions (217). Cell motility is linked to changes in the actin cytoskeleton. Typical morphological events observed in migrating fibroblasts are membrane ruffling and the formation of lamellipodia at the leading tip of the cell. The actin cytoskeleton consists of polymerized actin (F-actin), which is in equilibrium with unpolymerized actin, G-actin. Actin binding proteins e.g. α–actinin, profilin, filamin, gelsolin and radixin, are involved in promoting filament formation, branching, protein linking and the connection to the cell membrane. When actin filaments form contractile bundles, called actin fibres or stress fibres, myosin II proteins are integrated in the fibres. They hydrolyse ATP and translocate along the actin fibres generating the traction forces. Some myosin I subtypes are able to integrate in the plasma membrane. They have been observed in lamellipodia and could directly mediate membrane protrusion (263,264).

In lamellipodia, actin filaments are elongated, capped and/or nucleated. Cells having higher contents of capping proteins appear to move faster and the activity of actin depolymerizing factor (Cofilin/ADF) also correlates to actin based motility. The latter is important for the generation of a pool of free G-actin (265). Another actin binding protein, gelsolin, is involved in severing actin (cutting), regulation of capping and filament assembly of nucleated actin. Gelsolin activity is regulated by Ca^{2+} (+) and PIP2 (-) and has been found to be present in lamellipodia, focal adhesion sites and podosomes of fibroblasts (232,266). The severing activity of gelsolin has been related to fibroblasts migration (267). The forces necessary for protrusion are likely to be generated both by actin polymerization itself and/or by myosin I motor-driven membrane movement (260,263).

Traction forces are a necessary component of migration and move the cell body forwards. The requirements of energy (ATP) and the localization distant from lamellipodia are important secondary conditions. Agents that increase intracellular cAMP levels depleting the cell from ATP have been shown to interfere with cell migration, attachment, and can even induce cell rounding (268). The traction forces are generated by actin fibres bound to the cell membrane in focal adhesion sites. The signals from outside the cell involved in actin

cytoskeleton changes and actin mediated contraction are mediated by the small G-proteins of the Rho family, Rho, Rac and Cdc42 GTPases. The other small G-protein family members are: Ras GTPases (mediate mitogenic signal transduction (269)), Rab, Arf, Sar1, and Ran GTPases (involved in nuclear transport and membrane vesicle trafficking (270,271)). The members of the Rho small G-protein family are activated by GDP/GTP exchange proteins (GEPs). The activity of GEP proteins is negatively controlled by Rho-GDP dissociation inhibitors (GDIs) and positively by GTPase activating proteins (GAPs).

The small G protein Rho regulates cytoskeleton organization and actin contractility, whereas Rac mediates membrane ruffling and lamellipodia formation and Cdc42 induces filopodia (reviewed in (84,272-274)). The activation of Rac (within minutes) is often accompanied by Rho activation, but as a later response (20-30 min) (84). Rho directly interacts with p160ROCK (or its isoform ROCKa), which inhibits myosin-light chain phosphatase by phosphorylation, stimulating actin contractility (275,276). P140mDIA is another Rho-binding protein which can bind to profilin and might be involved in profilin accumulation in the plasma membrane. Profilin is involved in actin polymerization (233,277). Rho also is involved in actin anchoring to point contacts, like CD44, via the GDI binding protein and the ERM proteins (reviewed in (234)). Other proteins which have been reported to interact with Rho are protein kinase N (PKN), and PI-4P-5K (269,278). In non-adherent cells, there is a dramatic decrease in the level of PIP2 (269). Adhesion dependent synthesis of PIP2 by PI-4P-5K is positively regulated by Rac and Rho. PIP2 is an important cofactor for several enzymes and actin binding proteins containing a plextrin homology (PH) domain at the C-terminus (e.g. gelsolin, vinculin). PKN inhibits intrinsic and GAP-stimulated GTPase activity of Rho. PKN binds and phosphorylates α–actinin (for which it requires PIP2), vimentin, and glial fibrillary acidic protein (GFAP) (279,280). These proteins all associate with actin polymerization and anchoring of actin in focal adhesion sites. Furthermore, PKN regulates gene transcription by nuclear translocation and interaction with transcription factors, e.g., PCD17 (281,282).

Rac and Cdc42 can also interact with PI-3-K, which utilizes PIP2 to generate PIP3 (see also paragraph 5.1 and figure 2). Rac induced membrane ruffling is mediated by its downstream effector POR1 (283). Prior to the formation of filopodia, focal adhesions and actin fibres are disrupted. Cdc42 has been implicated in these rearrangements, but down stream effectors have not yet been identified. Notably, Cdc42 also has been linked upstream to the Rac induced formation of lamellipodia. Some studies suggested a role of p21(Cdc41/Rac)-activated kinases (PAK) in the regulation of actin and focal adhesion disassembly (84,272). In addition, Rac and Cdc42 stimulate the stress-activated protein kinase (SAPK) pathway through the activation of PAK (284). Interestingly, Ras activation is also implicated in the activation of both Cdc42 and Rac via Ral sG-proteins, but not in Rho activation (285) (for a schematic presentation of these signal pathways see figure 2).

During cell traction, the adhesion sites at the front of the cell should be stronger than

those at the tail in order to move the cell body forwards. Detachment or rear release contributes to the speed of migration (264). Migrating fibroblasts did leave a track behind of membrane ripped integrins. The other fraction of rear integrins re-appear in endocytic vesicles that accumulate in the perinuclear region (217). In migrating neutrophils, the αvß3 receptors is recycling to the leading front in a calcium and PTP-2B dependent manner (286). This also implicates important functions for the other cytoskeleton elements in cell migration, e.g. vesicle trafficking is regulated by microtubuli.

3.2.2 Plasma membrane receptors involved in cell migration

Outside the cell other processes also regulate cell migration and ECM invasion. Migration is stimulated by shedding of adhesion molecules, which decreases ECM-adhesion interactions. Cellular ECM invasion is mediated by membrane located proteolysis. To illustrate this the role of the uPA receptor (uPAR, CD87) and MT-MMPs in cell migration will be discussed briefly.

The uPA binds to uPAR, whereas tPA can bind to annexin II potentiating the enzyme activity. They both cleave plasminogen to form plasmin, which not only degrades multiple ECM proteins but also is involved in the activation of growth factors bound to the cell surface, like TNF–, HB-EGF and latent TGF-ß's. A role of plasmin in cell migration has been clearly established since wounds in the plasminogen knock-out mice, keratinocytes were incapable to degrade fibrin and close the wound (287). PAI-1 seems to play a key role in regulating uPAR-uPA activity and uPA mediated cell migration. PAI-1 binding to uPAR-uPA receptor stimulates the internalization and degradation of uPA-PAI-1 complexes by the α2-macroglobulin receptor (also called LDL-receptor related protein) (203,204). PAI-1 also binds to vitronectin preventing binding of the αvß3 and αvß5 integrins to vitronectin. This effect was more specific for the active form of PAI than for the latent or the cleaved form of PAI-1. uPAR-uPA binding to PAI-1-vitronectin inhibits uPA activity and uPAR-uPA receptor internalization and increased cell-ECM adhesion and inhibits migration of fibroblastic and smooth muscle cells (reviewed in (288-291)).

The shedding of adhesion molecules (L-Selectin and syndecan) occurs within minutes after cell activation and seems to be mediated by the activation of MT-MMPs (230). In addition, the MT-MMP's also bind TIMP-2 and TIMP-2 binds pro-MMP-2, thus resulting in the activation of cell-bound MMP-2, which mediates tissue invasion (184,190). Pro-MT-MMPs are activated intracellularly by furin or extracellularly by plasmin (184).

Migration on HA is mediated by the Receptor for HA Mediated Motility (RHAMM) (252,292). Its affinity for HA is lower than that of CD44 and the receptor has tyrosine kinase activity which is required for RHAMM mediated migration on HA. The receptor is linked to signal transducers c-Src kinase and activates MAPK signal pathway (293,294). TGF-ß1 upregulates RHAMM expression and stimulates tumour cell migration on HA (295).

3.3 Granulocytes early in wound healing

In large wounds, the fibrin clot size becomes more important and more blood cells will be trapped in the clot. The accumulation of waste products, lack of oxygen supply, and increased proteolysis, does not seem to favour cell survival and proliferation in this hostile environment. The main function of the trapped cells is probably the attraction of cells into the fibrin clot and the stimulation of fibrinolysis.

Blood leukocytes consists of 60-70% granulocytes of which 90% are neutrophils, making it the obvious granulocyte to be most involved in early wound healing. The emigration or diapedesis of neutrophils over the vascular cell walls into the wound seems to be largely dependent on the expression and binding of CD11/CD18 integrin to ICAM-1 and binding of integrin α4ß1 to VCAM-1 (296,297). The quiescent neutrophils are primed during withdrawal from the blood circulation by ATP, PF4 (298), PAF, lipopolysacharides, L-selectin and CD18 cross-links (reviewed in (299)). At the site of injury their primary function is the phagocytosis of injurious agents and protection against bacterial infections. Their priming is in this respect important since it also increases the intensity of respiratory burst, facilitates degranulation and stimulates de novo protein synthesis.

Neutrophils contain 4 different types of granules: azurophil, specific, and gelatinase granules and secretory vesicles. The azurophil granules contain multiple antimicrobial effectors, and fusion and degranulation is triggered by specific receptor interactions (300,301). The specific granules contain e.g. cathepsin G, elastase, collagenase (MMP-8), gelatinases, heparanases, lysozyme, myeloperoxidase, proteinase-3, uPA and azurocidin. The gelatinase granules contain e.g. gelatinases, uPA, lysozyme, and ß-microglobulin (302-305). The specific and gelatinase granules are implicated in neutrophil migration across the basement membrane into the underlying connective tissue. In the membrane of the granules multiple membrane bound adhesion proteins and enzymes are also present (reviewed in (304,306)). The secretory vesicles contain plasma proteins and membrane anchored alkaline-phosphatases and ß2-integrins. Secretory vesicles are mobilized after endothelial cell receptor contact (P-selectins) and transform the neutrophil into an integrin ß2 positive cell (304). Their granules do not seem to contain pro-inflammatory cytokines, chemokines or eicosanoids. Nevertheless, neutrophils are known producers of IL-1, TNF-α, chemokines IL-8, GRO-α -γ, and eicosanoids 5-HETE, leukotrines, and PGE-2, but apparently this requires de novo protein synthesis and/or enzyme activation (129,178,307,308). This implies that neutrophil activation and functions are regulated by receptors and their control of intracellular signal pathways.

3.4 Monocyte infiltration and macrophage maturation

The monocytes in the blood stream are evolved from monoblasts-promonocytes precursor cells. The migration into tissue in general induces monocyte maturation into macrophages. Monocytes differentiate into at least three macrophage subtypes with different phenotypes and

different functions characterised as secretory, cytocidal, and antigen presenting (309-311).

Monocytes are attracted to the wound bed by eicosanoids, chemokines, growth factors, fMLP and other bacteria derived products, and ECM degradation products. The eicosanoids and chemokines will first act at damaged vessel endings, where active platelets and granulocytes have generated PF4, PBP, NAP2, 5-HETE, LTB4, and PGE2. They stimulate monocyte emigration and maturation. In addition, the monocyte migration is strongly stimulated by PDGF and TGF-ß's. Notably, macrophages can not respond as well as monocytes to PDGF because on macrophages the PDGF receptors are downregulated (312). TGF-ß, in contrast, is a chemoattractant at femtomolar concentrations for monocytes and macrophages. At higher concentrations (picomolar), it induces the expression of cytokines, IL-1α and ß, TNF–α, PDGF-BB, and FGF-2 (313-315). TGF-ß is furthermore involved in regulation of differentiation. In activated macrophages, TGF-ß suppresses the respiratory burst capacity. LPS, however, downregulates on macrophages the TGF-ß receptor levels, desensitising the cell for TGF-ß activity (94). ECM proteins (collagens) and breakdown products of collagen, elastin and fibronectin are chemotactic for monocytes (316-318) and are probably generated by neutrophil secreted proteases.

Macrophages are essential in wound repair. Depletion studies showed a severe retardation of tissue debridement and ingrowth of cells (319). In general, macrophages are potent producers of cytokines, eicosanoids, chemokines and proteolytic enzymes in comparison to other cells (309), and their prolonged presence during tissue regeneration suggests a central regulatory role for macrophages during wound repair. The growth factors produced in large amounts by macrophages are TNF–α, IL-1, IL-6, PDGF, and TGF-ß, substantial amounts of FGF-1 and -2, TGF–α, IFN–α, and to a certain degree HB-EGF and IGF-I (Chapter 3 in (21)). The role of chemokines in the recruitment of more monocytes, and the selective attraction of lymphocytes, e.g. T cell subsets, B cells, and other granulocytes were reviewed in (153,157,319). Most proteolytic enzymes expressed by macrophages are summarised and described in paragraph 2.3.4. Interestingly, during monocyte maturation the activity of the serine proteases cathepsin G and elastase diminishes (320). Furthermore, it has been demonstrated that macrophages also can acidify their immediate local environment (321), which has implications for cathepsin mediated ECM degradation and growth factor activation (latent TGF-ß's). The involvement and role of macrophages during ECM remodelling and mechanisms involved in the clearance of macrophages (e.g. by apoptosis) are still very obscure.

3.5 Vascular structures and angiogenesis

The new formation of vascular structures is called angiogenesis and is characterized by the migration and proliferation of endothelial cells, smooth muscle cells and pericytes. In response to vessel damage endothelial cells will alter receptor expression for growth factors, cell-cell contacts and cell-ECM contacts. Secondly, they will produce blood clotting cofactors,

proteolytic enzymes and express de novo integrins mediating migration. The cell migration and direction of migration is depending on the balance of angiogenic and anti-angiogenic signals and the presence of a concentration gradient guiding the direction of migration.

After wounding, there is a delay of several days in granulation tissue formation and neovascularisation (322,323). Neovascularisation seems to be stimulated by macrophages forming the leading front of the cells infiltrating into the wound area. Vessel growth is directed to the hypoxia areas. In the excess of multiple metabolites, the increased presence of e.g. CO_2, biogenic amines, the redox environment and lactate are suggested to directly attract endothelial cells or modulate macrophage function into the production of angiogenic stimuli (324,325). Cell shape is correlated to cell growth and modulates differentiation (326), a phenomenon which seems to be especially important in angiogenesis. An increase in cell-ECM contacts and resistance to cell tractional forces was shown to be directly related to endothelial cell growth and tube formation (327). In this respect anti-adhesive proteins, i.e. secreted protein acidic and rich in cysteine (SPARC, osteonectin), thrombospondin and tenascin, present in the granulation tissue during early wound healing (5,328,329) could be important regulators of vessel morphogenesis. They could provide an ECM which does not anchor the endothelial cells too tightly and facilitates morphogenetic changes associated with capillary tube formation. Angiogenesis furthermore seems to be dependent on activation of protein kinase C (PKC) and is inhibited by cAMP inducing factors (330).

The immediate changes in endothelial cells after wounding are association with blood coagulation, binding of blood cells and cell diapedesis (described in previous paragraphs). On the cellular level several adhesion receptors are upregulated, e.g., ICAM-1, VCAM-1 and -2, E-selectin and PECAM. Furthermore, tight junctions and adherence junctions are disrupted (reviewed in (331-333)). The endothelial cell migration seems to be mediated by specific integrins, $\alpha v\beta 3$ and $\alpha v\beta 5$, only present at the leading tip of capillary sprouts (207,334-336). Although these integrins recognize other ECM molecules, the ligand involved in endothelial migration seems to be vitronectin. Interesting is the observation in models of angiogenesis using cornea and the chorioallantoic membrane that the $\alpha v\beta 5$ dependent migration is PKC dependent (VEGF, TGF–α), whereas the $\alpha v\beta 3$ mediated angiogenesis, stimulated by TNF–α and FGF-2, is not (336). Whether these receptors are also involved in fibroblast migration on vitronectin in vivo remains to be established, but in culture fibroblasts express functional $av\beta 3$ and $\alpha v\beta 5$ receptors (242). Furthermore, the membrane bound MT-MMP's and uPAR-uPA-plasmin binding are actively involved in endothelial migration (see paragraph 3.2.2).

The investigations on angiogenic factors are often difficult to interpret. Many factors mainly stimulate endothelial cell proliferation or induce the expression of angiogenic factors. Secondly, they are often tested for chemotaxis in the absence of appropriate ECM substratum and in the absence of smooth muscle cells and fibroblasts. In addition, in vivo often concentrations are used of which it is doubtful if these are physiologically relevant

concentrations. An example is a study with TGF-ß using the disc angiogenic system and subcutaneous injections with TGF-ß1 where the authors had to use dosages of more than 1 mg active TGF-ß1 before a stimulation of angiogenesis was observed (337,338). Angiogenic and anti-angiogenic factors have been reviewed extensively elsewhere (339-341). The most relevant and interesting growth factors for wound healing are probably VEGF, and FGF-1 and -2. VEGF is interesting since it is predominantly synthesized by epithelium (342), whereas FGF's are produced by multiple cells. Interestingly, some ECM proteins supporting angiogenesis, inhibited it when they were degraded. Breakdown of thrombospondin-1 and SPARC, two known mediators in angiogenesis with agonist and anti-agonist properties, resulted in the generation of protein fragments with anti-angiogenic properties (reviewed in (328,343)). Furthermore, a plasminogen fragment, angiostatin, also inhibited vessel growth by inhibiting endothelial cell proliferation (344). Angiogenesis is inhibited by multiple other mechanisms, e.g., downregulation of endothelial receptors, secretion of anti-angiogenic proteins, receptor-signalling interfering with cytoskeletal reorganisations involved in cell stretching and migration, inhibition of proteolytic enzymes and interaction with specific ECM proteins. The ECM synthesized and deposited behind the migrating tip of the vessels comprises multiple basement membrane proteins, e.g. laminin-1, collagen type IV, heparan sulphate proteoglycans, and nidogen. The interaction of endothelial and smooth muscle cells with these proteins by specific integrins initiates the neo-formation of the basement membrane structure and modulates endothelial and smooth muscle cell differentiation (345).

3.6 Fibroblasts in the granulation tissue

Fibroblasts are attracted to the wound-bed from the wound surroundings. In larger wounds, they mainly infiltrate from the underlying subcutaneous fat (346). The infiltration of fibroblasts seems to be a combined effect of migration and proliferation. In full-thickness wounds, migrating and proliferating fibroblasts are found until deep in the subcutaneous fat suggesting that alterations in the underlying tissue homeostasis and signals from the wound bed penetrate deep in the underlying tissue. The fibroblasts in the underlying tissue first start to proliferate and only start to migrate into the fibrin clot 3 days after wounding (323,347). This delay is dependent on the presence of stimulating signals and seems independent of maturation of the ECM in the fibrin clot (322). Moreover, the migration and formation of granulation tissue was accelerated by the continuing presence of stimulating signals (323). During the fibroblasts migration and proliferation, synthesized ECM proteins accumulate. After approximately 7 days the fibroblasts switch to a myofibroblast phenotype and will start to remodel the ECM. The time point of the switch from fibroblasts into myofibroblasts seems in large to be dependent on the extent to which the wound resists to contraction (348,349). The general hypothesis is that as contraction proceeds and resistence increases, fibroblasts and their actin cytoskeleton organize along the lines of greatest resistence promoting fibroblast

differentiation into myofibroblasts (348,350). Additional evidence for this hypothesis comes from a study which showed that scarring in adult tissue is significantly influenced by stretching and tensile forces (351). Relevant to mention is evidence from in vitro collagen gel contraction studies showing that gel contraction also occurred due to motile activity of fibroblasts trying to migrate through the floating gels. This so called 'traction remodelling' contraction occurred without fibroblast differentiation into myofibroblasts (352,353). Apparently, migrating fibroblasts already generate some of the tensile forces necessary to initiate tissue contraction. The differentiation of fibroblasts into myofibroblasts and the factors involved are reviewed in paragraph 4.2.

An interesting phenomenon is observed with the healing of deep burns. The surgical removal of eschar of deep burns often results in a wound bed with dermal remnants and fat protruding into the wound bed. The subsequent granulation tissue formation is mainly formed from subcutaneous tissue whereas the viable dermis remains relatively inactive. This rapid granulation tissue formation is associated with severe scar formation if re-epithelialization is slow (34). Apparently, the dermis contains substances which inhibit fibroblast migration, proliferation , and granulation tissue formation (354).

Apart from the surrounding tissues, a new origin of fibroblasts has been identified. These fibroblasts originate from the blood stream and are called fibrocytes. They are characterized by a specific phenotype and are positive for several immunological membrane marker (CD45, CD80, MHC-II). Although they readily infiltrate into injured sites and synthesize ECM molecules, it is more likely that fibrocytes primarily mediate inflammatory responses (355-358). It is unlikely that they will contribute substantially to the tissue regeneration process in normal healing wounds, since they are present in the blood circulation in very low numbers and have to cross the endothelial-basement membrane barrier.

The cellularity and density of myofibroblasts in the granulation tissue decreases due to apoptosis (359), and the signals leading to apoptosis are currently investigated (360,361). Notably, apoptosis was stimulated by covering granulation tissue by a vascularised skin flap (362). The latter not only indicates that tissue physiology is an important factor in the regulation of granulation tissue formation, but also shows that wound coverage or epithelialization is an essential component to achieve tissue homeostasis and downregulation of excessive granulation tissue formation.

4. The role of the fibroblasts in wound healing

4.1 Fibroblast heterogeneity

Several outstanding reviews have investigated and identified functional heterogeneity within fibroblast populations (363-366). These functional differences were characterized by the capacity of synthesizing different amounts of ECM molecules, differences in growth rates and

capacity of contracting collagen gels. The absence of markers to study fibroblast lineages and the apparent instability of some phenotypes in culture has made it difficult to study functional diversity of fibroblast populations in vitro and in vivo. Most evidence of the existence of functionally different fibroblasts is based on biochemical properties of fibroblasts isolated from highly specialized anatomical sites, e.g., periodontal ligament, ovarian follicles, and intestinal mucosa. Heterogeneity within one specialized tissue has also been observed and when studying fibroblast populations in culture one should be aware of this. In addition, the isolation and culture procedures could also favour the selection of certain fibroblast populations and therefore not be representative for the in vivo situation. For example, in the dermis fibroblasts derived from the reticular or papillary layers showed differences in growth potential (367), indicating the existence of different fibroblast subpopulations. This could however also be related to presence of stem or progenitor cells, which were observed to be enriched in locations adjacent to blood vessels in periodontal ligament of mice (368) and to be present in gingiva of hamsters (369). Interestingly, at different anatomical sites during tissue inflammation fibroblasts are also described as antigen presenting cells and could be identified by the expression of class II MHC and ICAM-1 molecules (141,365,370-372). One of the best characterized fibroblast differentiation phenotypes is that of the myofibroblasts and this will be discussed in the next paragraph.

4.2 Fibroblast differentiation into myofibroblasts

The ability of fibroblasts in the granulation tissue to differentiate into myofibroblasts is not only seen as the start of wound contraction but is also the cause of scar formation, contractures and hypertrophy. The myofibroblasts are morphologically similar to smooth muscle cells and are characterized by actin stress fibres linked to gap junctions (cell-cell) and fibronexus junctions (focal adhesions, cell-ECM), enlarged endoplasmatic reticulum, and indented nuclei (373). In the stress fibres often α–smooth muscle actin is co-localized. The latter leads to confusion, since in vivo α–smooth muscle actin positive fibroblasts are most certainly myofibroblasts, but fibroblasts negative for α–smooth muscle actin but positive for the other myofibroblast characteristics should also be considered as myofibroblasts. The origin of myofibroblasts, theoretically, can be from pericytes, smooth muscle cells or fibroblasts. In general, they are derived from fibroblasts, but in some skin diseases they also seem to be derived from smooth muscle cells and/or pericytes (363).

In culture, myofibroblasts are characterized by slower growth rates, presence of stress fibres, and a larger and stellate cell morphology. In comparison, fibroblasts have higher growth rates and a spindle shape cell morphology with predominant filamentous actin (350,374,375). Since myofibroblasts are larger than fibroblasts, the total intracellular actin can be used as marker (374). However, the total intracellular actin concentration does not seem to be related to the rate of collagen contraction. In several fibroblast cell lines from periodontal ligament and

gingiva in which a percentage of cells constitutively expressed α-smooth muscle actin, the rate of collagen gel contraction correlated with the amount of α-smooth muscle actin immunoreactivity, but not with that of total actin (376). In addition, this study also demonstrated the presence of pools of free α-smooth muscle actin molecules within the (myo)fibroblasts.

The myofibroblasts isolated from granulation tissue are likely to be contaminated with fibroblasts. Due to their reduced growth rates, it is likely that the myofibroblasts will be overgrown by the other fibroblasts, and this probably contributes to the observed instability of the myofibroblast phenotype in culture (377). In addition, the cloning of a fibroblast population with a certain percentage of α–smooth muscle actin positive fibroblasts never succeeded in generating fibroblast clones which were 100% positive or negative for α–smooth muscle actin (112).

The cell-cell contacts, adherence junctions and gap-junctions, observed in vivo between myofibroblasts and fibroblasts might also contribute to the formation of a contractile granulation tissue and the differentiation of fibroblasts into myofibroblasts. So far only circumstantial evidence is pointing in this direction. GAP junctions have been shown to be functionally active in cultured fibroblasts and myofibroblasts (378) and it is likely that the cells in the contractile granulation tissue communicate in a similar fashion. During early gestation wound healing in embryos showed that epidermal wound closure was achieved by an actin purse-string strongly interconnecting the keratinocytes via tight junctions and adherence junctions. This actin purse-string contracted and provided forces to draw the epithelium across the wound bed. This phenomenon demonstrates the existence of cooperation between cells in generating contractile forces. Interestingly, in the same embryo wound model in somewhat larger wounds, contraction of the mesenchymal tissue was also observed and contributed to wound closure. This contraction did not require the conversion of fibroblasts into α–smooth muscle actin positive myofibroblasts (reviewed in (379)). In full-thickness porcine wounds, a reduction in the total numbers of infiltrating fibroblasts was accompanied by a lower percentage of α–smooth muscle actin positive myofibroblasts and less wound contraction (380,381), which also indicated that cell density in the granulation tissue is an important factor in the formation of myofibroblasts. In contrast to this is the observation that fibroblasts isolated form cornea differentiate in vitro into myofibroblasts when seeded at low cell densities (382). In this study, it could however not be excluded that the isolated fibroblasts were for a large part smooth muscle cell progenitor cells and dedifferentiated smooth muscle cells.

The differentiation of myofibroblasts in vivo and in vitro could also be induced by some mediators. These factors are heparin, TGF-ß isoforms, and endothelin-1 (383-386).

The effect of heparin on fibroblasts could be direct or indirect since heparin is able to bind growth factors and potentiate their activity or inhibit it. The effect of TGF-ß's is more direct and probably mediated by the TGF-ß-receptors. The α–smooth muscle actin promoter does contain a specific TGF-ß control element (TCE), which acts in synergy with two CarG

elements (387). The signal transduction pathways activated by TGF-ß in the cell are the TAK1-p38 kinase pathway and the pathway mediated by SMAD proteins (described in detail in paragraph 5.2). SMAD proteins are able to interact with specific DNA sequences and it will be interesting to see if these SMAD proteins also interact with promoter sequences of the α–smooth muscle actin gene. Differences among the three TGF-ß isoforms seem to exist in vivo. In the rat subcutis, TGF-ß1 and 2 both increased significantly the number of α–smooth muscle actin positive myofibroblasts, whereas the TGF-ß3 only moderately increased the number of α–smooth muscle actin positive fibroblasts. In vitro, however, the activity of the TGF-ß isoforms was comparable (383). Differences between the TGF-ß isoforms in vivo is supported by data obtained with neutralizing antibodies and exogenous application of TGF-ß isoforms to incisional wounds in the control of scar formation (106,388). Positive effects on scar formation were shown with the administration of TGF-ß3 and neutralizing antibodies against TGF-ß1 and ß2 isoforms. It is noteworthy to mention that TGF-ß's induced the expression of α–smooth muscle actin in a percentage of cultured subcutaneous fibroblasts (383), which supports the existence of fibroblast subpopulations.

TGF-ß might also indirectly stimulate the formation of myofibroblasts by altering integrin expression and stimulating the synthesis of cellular fibronectin by fibroblasts. In wounds, TGF-ß1 induces the deposition of cellular fibronectin containing the ED-A domain which precedes the fibroblast differentiation into α–smooth muscle actin positive fibroblasts. ED-A fibronectin alone was also able to mimic TGF-ß effects on α-smooth muscle actin expression, whereas exogenous antibodies against the fibronectin ED-A domain and rec-fibronectin-ED-A domain protein were able to reverse the TGF-ß effects (389).

Endothelin-1 (ET-1) also induced myofibroblasts differentiation in vivo. The ET family consists of three endothelins, ET-1 to ET-3, are synthesized as preproform and secreted in the preform (big-ET). ET are activated by carboxypeptidase activity to generate the active form (suggested to be a membrane bound MMP). Three receptors have been identified, ET_A, ET_B and ET_C, being seven TM receptor coupled to G-proteins (Gq,Gs and/or Gi). ET-1 activates calcium influx, PLC-ß isoform, PLD, PKC, MAPK signal pathway and Rho, which is involved in actin contractibility (390,391). Fibroblasts seem to express mainly the ET_A and ET_B receptors and are capable of producing ET in culture (392-394). IFN-ß also has been shown to induce ET-1 expression in fibroblasts, especially at high fibroblast densities (394). In vivo, endothelial cells are considered to be the main producers of endothelins (391).

The administration of GM-CSF in vivo also induced myofibroblast differentiation, but this effect seems to be indirect by the activation of macrophages since in fibroblast cultures GM-CSF was unable to upregulate α–smooth actin mRNA and protein expression (395,396). However, normal gingival fibroblasts do express the GM-CSF-receptor and in culture low concentrations of GM-CSF (<80 ng) induced actin stress fibre formation and fibronectin production. Higher concentrations only induced cell proliferation (397).

5. Signal transduction pathways

Cell behaviour and differentiation and cellular effector functions are regulated by multiple mediators activating different signal transduction pathways. In table II, the most prominent factors in wound healing are listed and divided into four classes of signal transduction pathways: signal transduction by receptors with intrinsic tyrosine kinase activity (TK), receptors with serine-threonine kinase activity activating SMAD (stands for Sma and Mad gene proteins in Drosophila and C.elegans) protein signalling, receptor coupled to Janus kinase proteins (JAK) having tyrosine kinase activity and activating STAT proteins, and seven transmembrane spanning receptors (7-TM-receptor) coupled to heterotrimeric G-proteins activating phosphoinositide signalling cascades. In the next paragraphs these pathways will be described shortly and presented schematically.

Tyrosine kinase receptors/ mitogenic signal transducing molecules	Threonine-serine kinase receptor/SMAD proteins	Receptors coupled to JAK proteins/STAT proteins	7-Transmembrane spanning receptors/large heterotrimeric G-proteins
EGF, HB-EGF, TGF–α, VGF, insulin, IGFs, PDGF, FGF family members, c-Kit, amphiregulin, and epiregulin	Activins, Bone morphogenetic proteins, and TGF-ß isoforms	IFNs, HGF, IL-2, IL-3, IL-4, IL-6, IL-10, IL-12, IL-15 GM-CSF and EGF	Eicosanoid and chemokine receptors, complement factors (C3a, and C5a), fMLP and other bacteria peptides, and hormones, e.g., endothelins, adenosine, histamine, angiotensins

Table II. Soluble mediators in wound healing divided by receptor types and major signal transducing pathways activated after receptor-ligand interaction.
Abbreviations: SMAD stands for Sma and Mad gene proteins in Drosophila and C.elegans; JAK janus kinase; STAT signal transducers and activators of transcription; EGF epidermal growth factor; HB-EGF heparin-binding EGF like growth factor; TGF transforming growth factor; VGF vascular growth factor; IGF insulin-like growth factor; PDGF platelet derived growth factor; FGF fibroblast growth factor; IFN interferon; HGF Hepatocyte growth factor; IL interleukin; GM-CSF granulocyte-macrophage colony stimulation factor; fMLP N-formylmethionyl peptide.

5.1 Tyrosine kinase receptors mediated signal transduction

The growth factor receptors with tyrosine kinase activity comprise a family of more than 50 receptors, which are divided into 12 subclasses. They are characterized by intrinsic tyrosine kinase activity, which is often activated after receptor homo- or heterodimerization, and contain cytosolic domains with Src kinase Homology domains 2 and 3 (SH-2 and SH-3).

The activated receptors interact with multiple signal transducer substrates ranging from PLC-γ, phosphatidylinositol-3-OH kinase (PI-3-K), Grb2 and 7, Ras-GAP and other GAP proteins, SHC, Src kinase family members, and protein tyrosine phosphatases (PTP) (398). In figure 2, an overview is given of the PDGF-tyrosine kinase receptor induced signal transduction pathways leading to the activation of the pathways involving the kinases mitogen

activated protein kinase (MAPK), c-Jun N-terminal kinase/stress activated protein kinases (JNK/SAPK) and protein kinase 38 kDA (p38). PI-3-K activates Akt (an PKB), protein kinase C-ζ (PKC-ζ), and protein kinase 70 specific for ribosomal protein S6 (P70-S6K) (399). Akt and P70-S6K are both implicated in the activation of protein translation (400-402). PKC-ζ is an atypical PKC isoform (calcium and DAG insensitive), which translocates to the plasma membrane and the nucleus upon activation. PKC-ζ is implicated in the activation of NF-kappaB (403), P53 phosphorylation (404) and activation of other transcription factors (405). At the membrane, PKC-ζ was found in tight junctions (406).

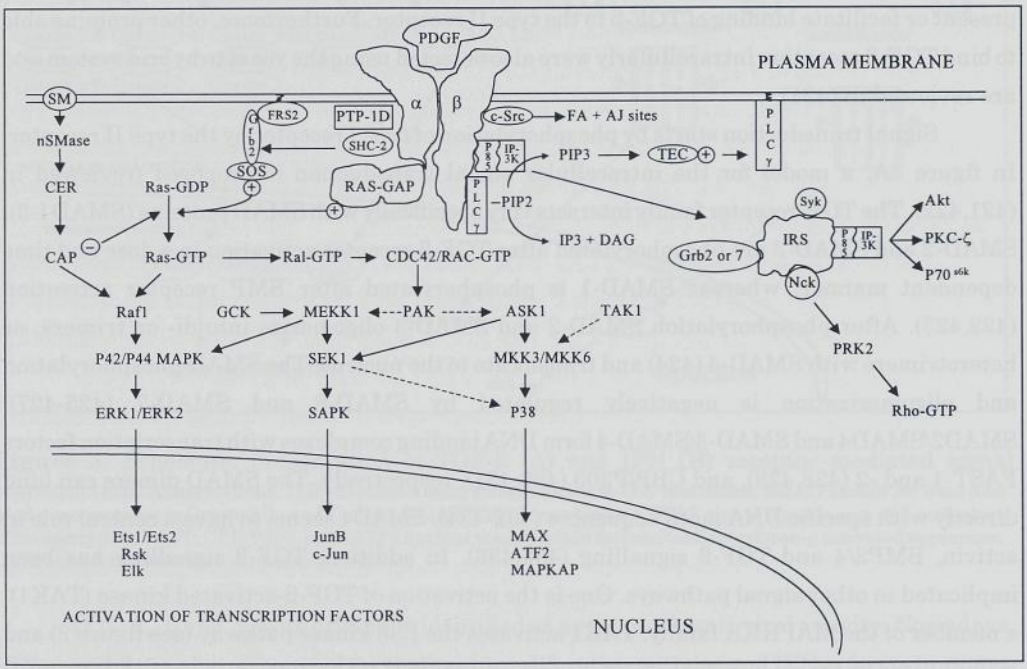

Figure 2. Schematic presentation of signal transduction by the PDGF tyrosine kinase receptor, adapted from the following reviews (283-285,398,407-413) and updated with information of the following publications (414-419). (Hatched lines represent possible or weak stimuli, thick lines indicate strong activation).

Abbreviations: PDGF platelet derived growth factor; PLC phospholipase C; FAS focal adhesion sites; AJ adherens junctions; PI-3-K phophatidylinositol-3-OH kinase; TEC Tec tyrosine kinase; PTP-1D protein tyrosine phosphatase-1D; FRS2 membrane bound Grb binding protein; RAS-GDP/GTP, Ral-GTP, Rho-GTP, Cdc42, and Rac: small GTPase protein bound to guanidine di- or tri-phosphate; IRS insulin receptor substrate protein; SM sphingomyelinase; nSMase neutral sphingomyelinase; CER ceramide; CAPK ceramide activated protein kinase; PKC protein kinase C; p70-S6K protein kinase (70 kDA) specific for ribosomal protein S6; Akt PKB isoform; TAK1 TGF-beta activated kinase; c-Src and Syk members of the Src kinase family; NCK Nck associated binding protein; PRK-2 Serine/threonine kinase Prk-2; PAK p21 activated kinase; GCK germinal center kinase; IP3 inositol trisphosphate; DAG diacylglycerol; PIP2 phosphatidylinositol 4,5 biphosphate; PIP3 phosphatidylinositol 3,4,5 trisphosphate; MAPK mitogen activated protein kinase; ERK extracellular signal-regulated kinase; MEKK MAPK/ERK kinase kinase; SEK SAPK/ERK kinase; SAPK stress-activated protein kinase; MKK MAP kinase kinase; P38 p38 kinase; Ets1/2, Rsk, Elk, JunB, c-Jun, MAX, ATF2, and MAPKAP (MAPK-activated protein) are transcription factors.

5.2 The TGF-ß receptor signal transduction pathway

The family of TGF-ß receptors are receptors for activins, bone morphogenetic proteins (BMP), and TGF-ß isoforms. The TGF-ß receptors belong to a family of receptors with intrinsic serine-threonine kinase activity consisting of type I and II receptors (420). They function as a heterodimer or higher-order multimer. The receptor types involved in TGF-ß receptor signalling are two type I receptors (TsK-7L/ALK2 and ALK-5) and one type II receptor both with a cytoplasmic tail with kinase activity. Two other cell-surface proteins betaglycan and endoglin (type III receptor) also bind TGF-ß, but lack kinase activity and are believed to present or facilitate binding of TGF-ß to the type II receptor. Furthermore, other proteins able to bind TGF-ß receptors intracellularly were also detected using the yeast to hybrid system and are reviewed in (421).

Signal transduction starts by phosphorylation of type I receptor by the type II receptor. In figure 3A, a model for the intracellular signal transduction is proposed (reviewed in (421,422). The TGF-receptor family interacts very specifically with SMAD proteins (SMAD1-3). SMAD-2 and SMAD-3 are phosphorylated after TGF-ß receptor activation in a dose and time dependent manner, whereas SMAD-1 is phosphorylated after BMP receptor activation (422,423). After phosphorylation SMAD-2 and SMAD-3 oligomerize into di- or trimers, or heterotrimers with SMAD-4 (424) and translocate to the nucleus. The SMAD phosphorylation and oligomerization is negatively regulated by SMAD-6 and SMAD-7 (425-427). SMAD2/SMAD4 and SMAD-3/SMAD-4 form DNA binding complexes with transcription factors FAST-1 and -2 (428,429), and CBP/P300 (430,431), respectively. The SMAD dimers can bind directly with specific DNA motif sequences (432-434). SMAD4 seems to have a central role in activin, BMP2/4 and TGF-ß signalling (435,436). In addition, TGF-ß signalling has been implicated in other signal pathways. One is the activation of TGF-ß-activated kinase (TAK1), a member of the MAPKKK family. TAK1 activates the P38 kinase pathway (see figure 2) and is implicated in the nuclear translocation and activation of transcription complex NF-kappaB (437). Some evidence also suggests that TGF-ß is implicated in the activation of heterotrimeric G-proteins involved in chemotaxis of natural killer cells (438). Other data also showed indirect evidence of TGF-ß 1 activation of the MAPK signal pathway in rat fibroblasts (439,440), or associated TGF-ß1 activity with an increase in PKA activity in mesangial cells (441).

5.3 The interferon JAK/TYK –STAT signal transduction pathway

The cytokines involved in antagonizing growth factor mitogenic actions or leading to attenuation of cell functions, and inducing differentiation seem to use a different biochemical signal transducing pathway activating STAT proteins. Interferons activate this signal pathway using the JAK, TYK and STAT proteins as illustrated in figure 3B. (442).

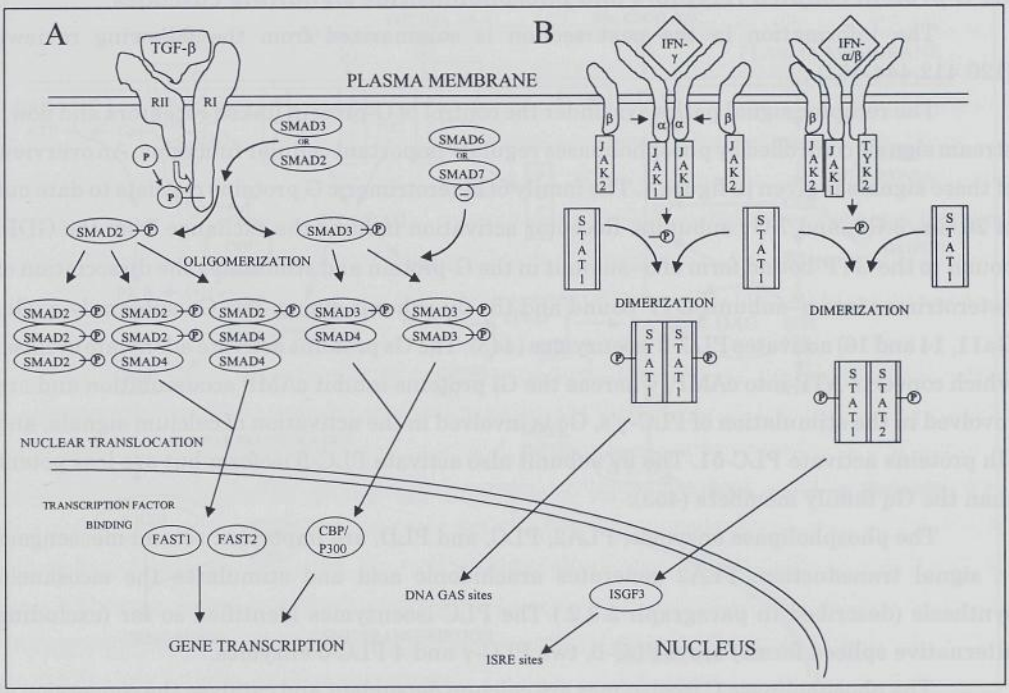

Figure 3. Schematic presentation of TGF-ß (**A**) and IFN (**B**) receptor mediated signal transduction. Abbreviations: TGF-ß transforming growth factor ß; IFN interferon; SMAD stands for Sma and Mad gene proteins in Drosophila and C.elegans; JAK/TYK Janus kinases; STAT signal transducers and activators of transcription; FAST, CPB/P300, and ISGF3 nuclear transcription factors; GAS interferon-g activated sequences; ISRE interferon stimulated response element.

IFN–α, -ß and -γ were originally identified as agents with anti-viral activity. Nowadays, IFN–α and ß are also recognized for their anti-proliferating activity and IFN-γ for its immunomodulatory activity (113). After IFN-γ binding and receptor complex assembly, the receptor activates the JAK1 and JAK2 kinases, which form a docking site for STAT1 molecules by exposing their SH-2 binding domains. After STAT1 is phosphorylated by JAK, it dissociates from the receptor as a dimer and translocates into the nucleus where it modulates gene transcription directly by binding to GAS response elements (113,411). The IFN-α/ß signalling pathway is somewhat different. Receptor activation also phosphorylates TYK-2 and for downstream signalling STAT2 is used next to STAT1. Furthermore, STAT1/STAT2 dimers need to interact with the transcription factor ISGF-3 for binding to the interferon stimulated response element (ISRE) and activation of transcription (411,443). The IFN induced factors involved in antiproliferative actions are less well defined. STAT1 can induce the expression of $p21^{WAF1}$, a cyclin-dependent kinase inhibitor (443). Receptor signalling is desensitized by dephosphorylation of JAK and TYK in part mediated by the phosphatase SHP-1 (409).

5.4 G-protein coupled receptors and phosphoinositide signalling cascades

The information in the next section is summarized from the following reviews (120,412,444-452).

The receptor signal pathways under the control of G-protein linked receptors and down stream signals controlled by phospholipases regulate important cellular functions. An overview of these signals is given in figure 4. The family of heterotrimeric G proteins consists to date out of 20 Gα, 3 Gß, and 7 Gγ subunits. Receptor activation induces the exchange from the GDP-bound to the GTP-bound form of α–subunit in the G-protein and stimulates the dissociation of heterotrimer into α–subunit-GTP bound and the ßγ-subunit dimer. The Ga subfamily q (Gq, Ga11, 14 and 16) activates PLC-ß isoenzymes (448). The Gs proteins activate adenylate cyclase, which converts ATP into cAMP, whereas the Gi proteins inhibit cAMP accumulation and are involved in the stimulation of PLC-γ's, Go is involved in the activation of calcium signals, and Gh proteins activate PLC-δ1. The ßγ subunit also activate PLC-ß isoform but are less potent than the Gq family members (453).

The phospholipase enzymes, PLA2, PLC, and PLD, are important second messengers in signal transduction. PLA2 generates arachidonic acid and stimulates the eicosanoid synthesis (described in paragraph 2.3.2.) The PLC isoenzymes identified so far (excluding alternative spliced forms) are 4 PLC-ß, two PLC-γ and 4 PLC-δ enzymes.

The phospholipase C isoenzymes are calcium dependent and catalyse the conversion of PIP2 into diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP3). The activity of PLC-ß enzymes so far seems to be solely stimulated by the Gq proteins and the ßγ-subunits. The PLC-γ enzymes are linked to tyrosine kinase receptors via their SH-2 and SH-3 domains and are activated after receptor tyrosine phosphorylation. Their membrane localization is calcium dependent and their activity is stimulated by H_2O_2 and Gi protein, arachidonic acid, Tau (a microtubuli associated protein) and PIP3-Tec tyrosine kinase (figure 2). In addition, c-Src and Syk kinases can phosphorylate PLC-γ, which seems to stimulate the translocation of PLC to the cytoskeleton (via SH-3 binding site). PLC-δ isoforms are activated by large G-protein Gh and small G-proteins of Rho family.

The inositol-1,4,5-triphosphate (IP3) and diacylglycerol (DAG) generated by the phospholipases activate calcium concentration upregulation and calcium dependent kinases (calmodulins) and PKC isoforms, respectively. The most abundant PKC isoforms (reviewed in (454)) are α, ß and γ (activity is dependent on calcium, PS and DAG), PKC-ϵ, -δ, -η (dependent on PS and DAG) and PKC ζ, θ (activity independent of calcium and DAG). Interestingly, the PKC-ϵ isoform has been implicated as the regulator of calcium independent contraction in vascular smooth muscle cells (455,456)

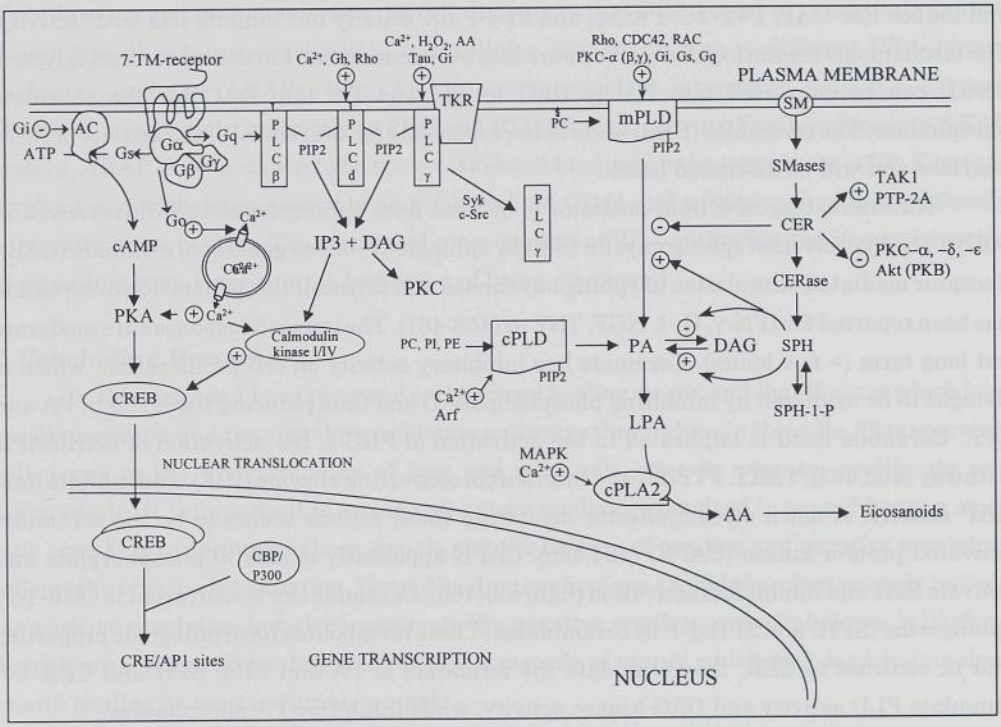

Figure 4. The G-protein coupled receptor signalling and phosphoinositide signalling summarized schematically presented and adapted from the following reviews (120,412,444-452).

Abbreviations: TM transmembrane; TKR tyrosine kinase receptor; AC adenylate cyclase; ATP adenosine trisphosphate; cAMP cyclic adenosine monophosphate; PKA and PKC protein kinase A and C; PLA2, PLC and PLD phospholipase A2, C and D; PI-3-K phophatidylinositol-3-OH kinase; PTP-2A protein tyrosine phosphatase-2A; Arf, Rho, Cdc42, and Rac: small GTPase proteins bound to guanidine di- or tri-phosphate; Tau microtubuli binding protein; SM sphingomyelinase; SMase sphingomyelinase; CER ceramide; CERase ceramidaase; SPH sphingosine; AA arachidonic acid; Akt Protein kinase B isoform; TAK1 TGF-beta activated kinase; c-Src and Syk members of the Src kinase family; PI phosphatidylphosphate; IP3 inositol trisphosphate; DAG diacylglycerol; PIP2 phosphatidylinositol 4,5 biphosphate; PIP3 phosphatidylinositol 3,4,5 trisphosphate; PC phosphatidyl choline; PE phosphatidyl ethanolamine; PA phosphatidate; LPA lysophosphatidate; CREB cyclic-AMP response element binding protein; CBP and P300 CREB binding proteins; CRE/AP1 cyclic-AMP response element.

The phospholipase D (PLD) activity generates phosphatidate (PA) and lysophosphatidate (LPA) from phosphatidylcholine. The PLD activity is stimulated by multiple proteins; PKC–α isoform, and possibly by the ß and γ isoforms, small (Rho, Cdc42, Rac) and large G proteins (Gi, Gs, Gq and G12). PIP2 is a necessary cofactor for PLD activity, and implicates that hydrolysis of PIP2 by PLC could inhibit PLD activity if the same PIP2 pool is used. Apart from membrane bound PLD, a cytosolic PLD (cPLD) exists. Cytosolic PLD has a broader substrate specificity (phosphatidylcholine, PI and PE), and it localizes to Golgi membrane vesicles where its activity is controlled by the small G-protein Arf. PA and LPA can be converted into arachidonic acid by PLA2. PA and LPA stimulate the MAPK signal pathway,

and induce Rac-GAP, PTP-1C, PKC-ζ, and PI-4-P-5K activity but inhibits Ras-GAP activity. The mechanisms mediating these actions are less well understood. Furthermore, diacylglycerol (DAG) can be converted into PA by DAG kinase and PA into DAG by phosphatidate phosphatase. The ceramides (CER) seem to be responsible for the control of the activity of these enzymes and will be discussed below.

An interesting new lipid mediator is derived from sphingomyelin (SM) (reviewed in (457)). The hydrolysis of sphingomyelin (SM) by sphingomyelinase generates ceramide (CER). Receptor mediated stimulation of sphingomyelinase and intracellular generation of ceramide has been reported for IFN-γ, IL-1, NGF, TNF–α (458-461). The induced responses are moderate but long term (> few hours). Ceramide has inhibitory activity on cell proliferation, which is thought to be mediated by inhibiting phospholipase D and thus reducing the levels of PA and LPA. Ceramide itself is implicated in the activation of PKC-ζ, the activation of JNK/SAPK pathway (462,463), TAK1, PTP-2A and interleukin converting enzyme (ICE), and inhibits Ras-GAP activity. A down stream effector mediating these actions seems to be the ceramide-activated protein kinase (CAPK) (464,465). CAPK apparently is able to phosphorylate and activate Raf1 and inhibit Ras activation (figure 2)(466). Ceramide can be converted to CER-1-P, sphingosine (SPH) and SPH-1-P by ceramidases. These metabolites have mitogenic properties and in contrast to CER, they stimulate the formation of PA and LPA. SPH and CER-1-P stimulate PLD activity and DAG kinase activity, while inhibiting PA phosphatase.

The proposed mode of action as presented in figure 4 shows that activation of sphingomyelinase and ceramidase activity generate sphingosine and stimulate cell proliferation, whereas the activation of only sphingomyelinase results in the accumulation of CER and leads to growth arrest (458,467,468).

An important pathway activating PKA also provides further evidence that external signals are transduced into the cell in a controlled and localized fashion. Several receptors (for glucagon, adenosine, PGE's) induce cAMP formation by activating the receptor coupled Gs-protein. Gs-proteins activate adenylate cyclase (AC) resulting in the formation of cAMP. cAMP activates PKA, which in turn phosphorylates cyclic-AMP response element binding protein (CREB) and its family members activating transcription factor-1 (ATF-1) and CREM. The latter interact and stimulate DNA transcription via CRE/AP-1 elements in the DNA. CREB dimers-DNA complexes bind CREB-binding protein (CBP) or P300, a necessary co-factor capable of binding Q2-TFIID, which allows effective RNA polymerase gene transcription (469) (illustrated in figure 4). CREB activity is coupled to the intensity of the stimulus. Intracellular calcium levels also modulate CREB activity. Calmodulin kinase stimulates the activation of PKA and calmodulin kinase I and IV are able to directly phosphorylate CREB.

Interesting in the cAMP-PKA signal pathway is the recent progress in the 'targeting hypothesis'. This hypothesis implies control of phosphorylation events depending on the location

of the kinases and phosphatases in the cell. It appears that PKA bound to the activity control R unit type II is targeted to certain intracellular sites by binding to different PKA–kinase anchoring proteins (AKAPs)(470). AKAPs are targeted to interesting sites e.g. calcium channels, microtubuli, indirectly to PKC and PTP-2B (calcineurin). Furthermore, one AKAP protein, AKAP78, was shown to be the cytoskeleton binding component ezerin (470). Ezerin is involved in cytoskeleton assembly and able to bind CD44 and possibly other Ig-superfamily adhesion receptors (218). The functional consequences of PKA anchoring and its participation in signalling complexes is in its beginning of being discovered.

6. Concluding Remarks

In this chapter, I have reviewed early wound healing events and the effectors which lead to cell migration and the signal transduction pathways they induce in the cells. This overview will assist in the understanding of how and why cells start to migrate, proliferate and differentiate. It is important to include all known mediators involved in wound healing since cells are likely to encounter these signals and the balance of positive and negative regulators reflects the true in vivo situation. Wound healing evaluations should therefore not only include the positive regulators but also investigate the negative regulators. An imbalance is likely to disturb normal wound healing and initiate a cascade of events which may lead to impaired wound healing as seen in chronic wounds.

The eicosanoids, chemokines and growth factors present in the microenvironment of wound cells seem to have important roles in regulating cell migration, proliferation and differentiation of inflammatory cells. However, their role in fibroblast behaviour has not been investigated in detail. This is in part caused by the absence of markers allowing proper characterisation of fibroblast differentiation phenotypes and the apparent instability of the myofibroblast phenotype in cell culture systems. Fibroblast phenotype characterisation and the regulation of fibroblast differentiation will be of major importance since fibroblasts are the main effector cells inducing tissue fibrosis and scar formation in the whole body.

In wound healing, interesting questions still remain to be answered. For example which signals result in the release of mediators implicated in the downregulation of inflammation and silencing of granulation tissue proliferation, and how is apoptosis triggered in the granulation tissue. Nonetheless, the most important question could be "How do fibroblasts sense that a wound is not closed and wound contraction is necessary". Answers to these questions might give us new tools in the control of skin fibrosis and scar formation.

Furthermore, it is advisable not to consider topical applications of new wound dressings or other wound treatments as a medical device. Any change in the wound environment, will lead to altered behaviour of wound cells. Cell toxicity studies alone for new products are not sufficient anymore. Additional experiments in appropriate model systems should be performed

to prove the efficacy of new treatments. The past already has taught us some expensive lessons in relation to wound treatment with growth factors. Most of them were rapidly inactivated by degradation due to proteolytic wound activity and/or the proteolytic activity of bacteria. However, if the proteolytic wound activity and bacterial growth could be controlled, growth factor therapy does seem a good choice to stimulate wound healing. Overstimulation of wound healing, nevertheless, could result in excessive granulation tissue formation and more tissue fibrosis and scar formation.

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Chapter 2

Extracellular Matrix Characterization During Healing of Full-Thickness Wounds Treated with a Collagen/Elastin Dermal Substitute Shows Improved Skin Regeneration in Pigs

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Abstract

We investigated the architecture of the extracellular matrix (ECM) during healing of full-thickness wounds in the pig. Two different treatments, one based on epidermal transplantation (split-skin mesh grafts, SP wounds) and one consisting of a combination of epidermal transplantation and a dermal matrix substitute (MA wounds) were compared. The dermal matrix consisted of native bovine collagen coated with elastin hydrolysate. The latter treatment reduced wound contraction and improved tissue regeneration. The expression patterns of fibronectin, von Willebrand Factor, laminin, chondroitin sulphate and elastin, detected by immunohistochemistry, were examined in time and indicated different stages of healing. During the early phase of healing the dermal matrix induced more granulation tissue, a different fibronectin expression pattern, and rapid vascular cell ingrowth (von Willebrand Factor). Furthermore, in the MA wounds chondroitin sulphate was detected earlier in the basement membrane and fibronectin staining disappeared more rapidly. During later stages of healing chondroitin sulphate expression was selective for areas in which ECM remodelling was active; in these specific areas elastin staining reappeared. ECM remodelling and elastin regeneration occurred both in the upper and lower dermis for the MA wounds but only in the upper dermis for the SP wounds. Electron microscopic evaluation of the wounds after two weeks showed many myofibroblasts in the SP wounds, whereas in the MA wounds cells associated with the dermal matrix had characteristics of normal fibroblasts. The results suggest that the biodegradable dermal matrix served as a template for dermal tissue regeneration, allowed a faster regeneration, and improved the quality of healing in large full-thickness skin defects.

Introduction

The healing of full-thickness skin defects, involving both dermis and epidermis, comprises complex processes leading to the formation of new tissue. Wound contraction and scar formation are still unavoidable components of the healing process. Scar tissue is less flexible than normal skin and can be cosmetically disfiguring, whereas wound contraction can lead to joint disablement (Clark, 1985). Wound contraction and scar formation are believed to be induced by poor dermal regeneration, extensive remodelling of the extracellular matrix (ECM), and differentiation of fibroblasts into myofibroblasts (Desmoulière et al., 1992; Gabbiani et al., 1971; Hinshaw et al., 1965).

The processes of cellular differentiation, dermal tissue degradation, synthesis, and remodelling are guided by cytokines, cell-cell contacts and cell-matrix interactions (Grinnell, 1994; Tooney et al., 1993; Ruoslahti, 1989). However, details of these mechanisms leading to wound contraction and scar formation are still unknown.

We have investigated the expression of several ECM molecules and the changes in the dermal architecture during different phases of wound healing to increase our insight into different cell-ECM interactions and the influence of ECM molecules on fibroblast behavior.

Fibronectin plays an important role in the early phase of wound healing. Its expression is highly upregulated after wounding and, together with fibrin, it acts as a provisional matrix and promotes e.g., keratinocyte and fibroblast migration (Kim et al., 1992; Knox et al., 1986; Clark et al., 1982; Postlethwaite et al., 1981).

The localization of von Willebrand Factor gives insight into the process of angiogenesis. The glycoprotein is tightly associated with Factor VIII, a cofactor in the blood clotting cascade that is present in blood, plasma, platelets, and endothelium (Ruggeri et al., 1993; Lollar, 1991). Angiogenesis has important regulatory functions in wound healing. Endothelial cells secrete cytokines and they control inflammatory cell infiltration (Albelda et al., 1994). Furthermore, angiogenesis has an important nutritive role, since split-skin mesh grafts and cultured grafts transplanted onto the wound site are unable to survive when vascularization of the wound bed is insufficient (Kamagai et al., 1988; Gallico et al., 1984).

Laminin is the major noncollagenous component of the basement membrane (BM). Laminin is important for cell attachment and maintenance of the differentiated state of epithelial and endothelial cell layers that are intimately associated with the BM (Yurchenco and Schnittny, 1990; Beck et al., 1990). In wound healing, BM regeneration is important for attachment and differentiation of epidermal grafts (Mommaas et al., 1992; De Luca et al., 1989).

Chondroitin sulphate is a disaccharide that forms glycosaminoglycan (GAG) chains of different lengths. These GAGs are covalently attached to core proteins forming chondroitin sulphate proteoglycans (CSPGs) (Hardingham and Fosang, 1992; Ruoslahti, 1989). In normal skin, CSPGs are mainly found in BM (Daugaard et al., 1991; McCarthy et al., 1989) but in healing wounds CSPG expression is upregulated throughout the granulation tissue (Yeo et al., 1991).

Elastin is a highly insoluble protein present as a fibrous network in connective

tissue and is responsible for the elasticity of the tissue. In human scars elastin fibres are only detected years after wounding (Compton et al., 1989).

Recently, we have described a standardized full-thickness wound model in the pig to test dermal substitutes (de Vries et al., 1993). One dermal substitute, composed of bovine Type I collagen with the addition of elastin hydrolysate, applied in combination with split-skin mesh grafts, allowed better healing of full-thickness wounds (de Vries et al., 1994) and improved dermal regeneration in a human punch biopsy wound model (de Vries et al., 1995).

In the present study, we analyzed in the pig wound model the ECM organization during the tissue regeneration process and compared these data to the architecture of normal pig skin. We compared two treatments, split-skin mesh grafts alone, or a combination of split-skin mesh grafts and the dermal substitute described above. The latter treatment reduced wound contraction by 10% in comparison to treatment without dermal substitute (24% versus 34%)(de Vries et al., 1994). Furthermore, at the electron microscopic level, we studied the biodegradation process of the dermal implant and differentiation of wound fibroblasts into myofibroblasts during the first two weeks of healing.

Materials and Methods

Materials

The dermal substitute, a non-crosslinked native bovine collagen matrix (Type I collagen, from bovine skin) coated with a 3% w/w α-elastin hydrolysate (80% MW 60 kDa and 20% MW 200 kDa, from bovine ligamentum nuchae) was kindly provided by Dr. P. Tewes-Schwarzer (Dr Otto Suwelack Nachf. GmbH & Co., Billerbeck, Germany). Exkin, a semipermeable polyether urethane membrane, was provided by Utermöhlen (Utrecht, The Netherlands).

Antibodies were purchased from Dako (Copenhagen, Denmark) and Sigma Chemical (St. Louis, Mo). The following matrix proteins were detected with polyclonal rabbit-antibodies using a three-step labeling procedure with biotinylated polyclonal swine-antibodies anti-rabbit IgGs (Dako, 1:400) as second antibody followed by streptavidin biotinylated horseradish peroxidase (Hrp) complex (Dako, 1:200) as third step: von Willebrand Factor (Dako, 1:1500), fibronectin (Dako, 1:800), and laminin

(Sigma, 1:150). Chondroitin sulphate and elastin were detected with mouse monoclonal antibodies (Sigma, 1:300 and 1:500, respectively) in a two step labeling procedure with second antibodies Hrp-conjugated goat anti-mouse (GAM) IgM (Sigma, 1:200) and Hrp-conjugated GAM immunoglobulins (Dako, 1:100), respectively. First antibodies were shown to crossreact with porcine antigens. Diaminobenzidine (DAB) was obtained from Sigma.

Operation procedures and skin biopsies

The operative procedures were performed as described previously (de Vries et al., 1994; de Vries et al., 1993). The protocol was approved by the University of Amsterdam Animal Use Committee. Briefly, 14 full-thickness surgical wounds (3.0 X 3.0 cm) were created on the back of female New Yorkshire pigs, weighing 20 kg at arrival. In the wound bed the dermal substitute was placed and covered with split-skin mesh grafts (0.20 mm thick) and Exkin. The latter is a polyetherurethane membrane that allows wound drainage but protects the wound against contamination (de Vries et al., 1993). Eight wounds were treated with both treatment modalities on six different pigs. Four mm punch biopsies for immunohistochemistry were taken from each wound after 1, 2, 3, 4, 5, 6, 8, 12, and 16 weeks post wounding and were fixed in 4% formalin-PBS solution for 7-9 hours at room temperature (RT) before embedding in paraffin. For electron-microscopy 2 mm biopsies were taken from each wound after 1 and 2 weeks and were fixed in 4% paraformaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4 for 24 hours at RT. They were post-fixed in 1% OsO_4 in PBS at RT, dehydrated through graded ethanols, and embedded in Epon LX 112 (Ladd Research Industries, Burlington, VT).

Immunohistochemistry

Sections of 5-6 µm thick were mounted on poly-lysine coated-glass slides. The sections were deparaffinized in xylol and hydrated through a graded series of ethanol. To remove endogenous peroxidase activity, the slides were incubated for 30 min in a 0.3% H_2O_2/methanol solution and then washed with water and PBS. Aspecific binding of antibodies was avoided by a 15 minutes preincubation with 10% normal goat serum (NGS) in PBS. The sections were incubated for 1 hour at RT with the first antibodies and washed three times with PBS. Subsequently, the appropriate second antibody was applied for 30 min diluted in PBS-10% NGS serum. If the second antibody was

biotinylated, a third incubation step was performed with the streptABComplex-Hrp for 30 min in PBS. After extensive washing to remove non-bound antibodies the color reaction was performed for 7 min in 0,05% DAB, 0.03% H_2O_2 50 mM Tris-HCl buffer, pH 7.8. Finally, the sections were counterstained with hematoxylin, mounted in glycergel, and examined.

As recommended by the manufacturers, the fixed tissues used for the fibronectin, laminin, elastin and the von Willebrand Factor stainings were predigested with a 0.25% pepsin, 10 mM HCl solution, pH 2,5, for 30 min at 37°C. Under these conditions, optimal dilutions of the antibodies were determined.

The sections were examined microscopically and photographs of representative stainings were taken using an Olympus SC35 camera (Tokyo, Japan) with 64T (EPY-135) Ektachrome film (Kodak, the Netherlands).

Positive and negative controls

Sections of human and porcine normal skin served as positive controls. As negative controls, adjacent sections of the wound biopsies were stained with nonimmune IgG from the same species at the same dilution as the primary antibody. No staining was noted in the negative controls.

Transmission electron microscopy

Semithin sections (2 μm) were stained with Richardson solution (2% methylblue, 2% azur II, and 2% dinatriumtetraborate in water) to locate the dermal area immediately above the subcutaneous tissue (area containing the dermal substitute). This area was selected for thin sections. The thin sections were collected on copper grids, double stained with uranyl acetate and lead citrate, and examined and photographed with a Philips 420 electron microscope.

Results

General Observations

During the first two weeks post-wounding (p.w.), a cell-dense granulation tissue was formed in the split-skin mesh grafts-treated wounds (SP wounds)(Figure 1A). In matrix-treated wounds (MA wounds), however, more granulation tissue was found and with lower cell density when compared to the SP wounds (Figure 1B)(de Vries et al.,

1994). After two weeks, matrix fibers were still clearly present in the lower dermis of the MA wounds. They could no longer be detected at the light microscopic level 4-6 weeks after implantation. Epithelization of both the SP and MA wounds was completed within two weeks. Wound contraction started after one week and lasted until six weeks p.w.. At this time point, the collagen bundles were organized more parallel to the epidermis in the SP wounds, which is typical for scar tissue, whereas the MA wounds showed a larger dermal layer with a more randomized collagen bundle organization. These observations were in agreement with earlier studies (de Vries et al., 1994, 1995).

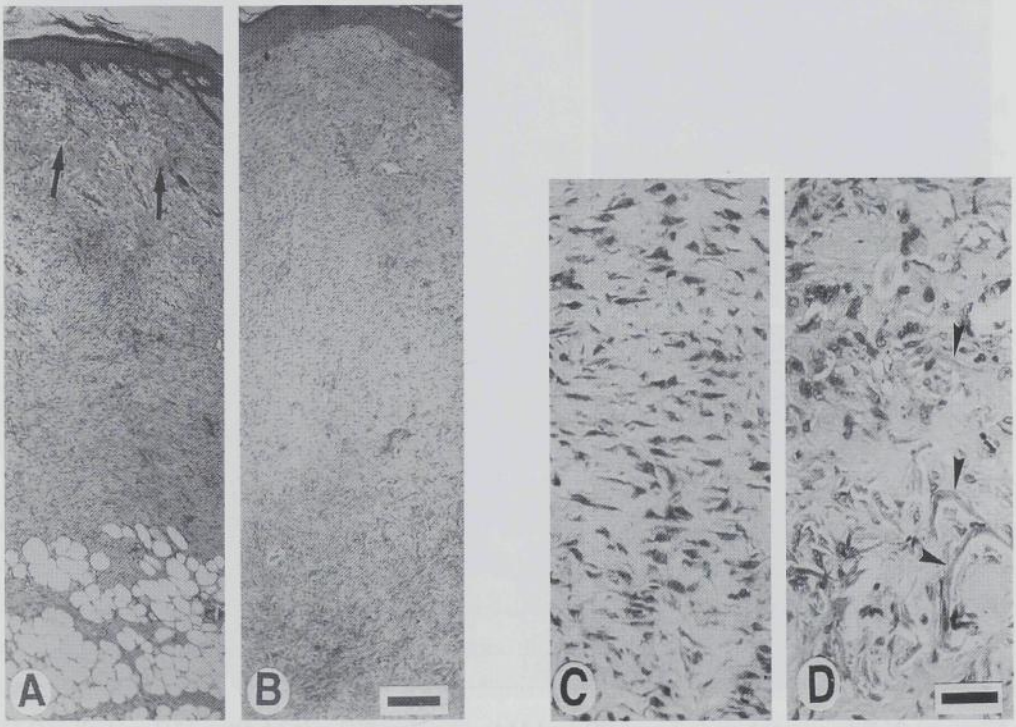


Figure 1. Hematoxylin and eosine stainings of the SP and MA wounds two weeks p.w. **A.** A cell dense granulation tissue was found in the SP wounds two weeks p.w.. Upper part of section represents a part of the split-skin mesh grafts (arrows). **B.** In the MA wound, more granulation tissue was formed with lower cell density compared to the SP wounds. A x5 magnification of the lower dermis illustrated the differences in cell density between both wounds. **C.** The SP wounds. **D.** The MA wounds, in which matrix fibers were still present in the lower dermis (arrowheads). Bars: A,B = 200 μm; C,D = 40 μm.

Fibronectin

In normal pig, skin fibronectin staining was detected only in blood vessels (results not shown). An intense fibronectin staining pattern was observed throughout the dermis for the SP wounds (Figure 2A). For the MA wounds, a less diffuse fibronectin staining was noted. Fibronectin staining was found in close association with the matrix collagen fibres (Figure 2B). In time, when the matrix was degraded the fibronectin staining became more diffuse. After six weeks fibronectin staining was still present in the lower dermis of the SP wounds (Figure 2C), but was similar to normal skin in the MA wounds (Figure 2D).

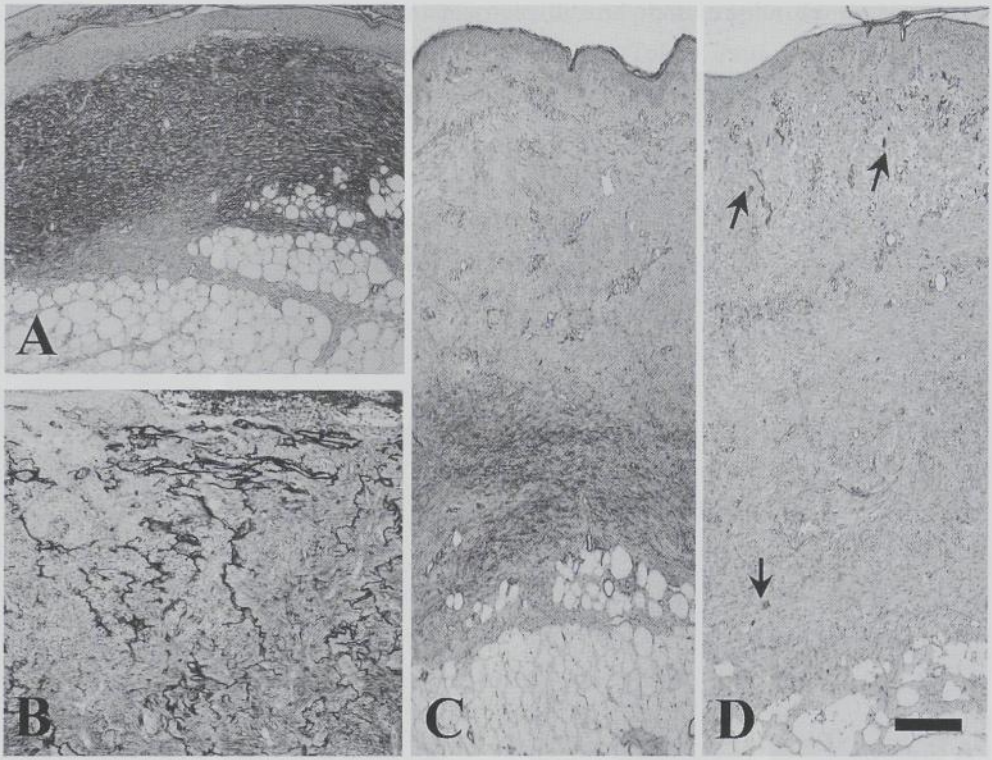


Figure 2. Fibronectin staining of SP and MA wounds after one (**A,B**) and six weeks (**C,D**) p.w.. **A.** After one week the SP wounds showed fibronectin staining throughout the dermis. **B.** In the MA wounds fibronectin was localized in close association with the matrix collagen fibres. **C.** After six weeks fibronectin staining was still abundant in the lower dermis of the SP wounds. **D.** In the MA wounds the diffuse fibronectin staining pattern had disappeared. Only some fibronectin staining was detected in association with vascular structures (small arrows). Bar = 200 μm.

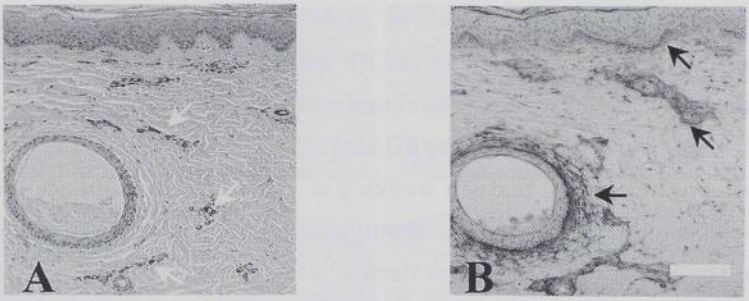


Figure 3. Normal pig skin stained for von Willebrand Factor (**A**) and chondroitin sulphate (**B**). Von Willebrand Factor staining showed endothelial cells (open arrows), while chondroitin sulphate labeling was found in the periphery of vascular structures and in the BM (solid arrows). Bar = 70 μm.

Laminin

Laminin was already present in the BM at one week p.w. and was also found associated with vascular structures. Only at the leading edge of migrating epidermis, laminin was not yet present (results not shown). No differences in laminin staining were detected in the two types of wounds.

Von Willebrand Factor and chondroitin sulphate

In normal pig skin, endothelial cells stained positive for von Willebrand Factor (Figure 3A), indicating vascular structures. Chondroitin sulphate labeling of normal pig skin was found in the BM and in the periphery of vascular structures (Figure 3B).

One week p.w., in SP wounds (Figure 4A), less granulation tissue was detected with only a few vascular structures compared to MA wounds, in which neovascularization was already abundant (Figure 4B). In time, more granulation tissue was formed in the SP wounds, and differences in vascularization between both wound types disappeared after three weeks. After sixteen weeks, in both wounds more vascular structures were still present compared to normal pig skin (not shown).

In the BM zone of the SP wounds, no newly formed chondroitin sulphate could be detected after three weeks p.w. (Figure 5A). However, in the MA wounds this could be readily detected (arrows, Figure 5B).

In the dermal tissue the chondroitin sulphate staining was present throughout the tissue during the first two weeks. Thereafter, chondroitin sulphate staining diminished in the upper dermis of the wounds and in time this area enlarged.

Differences were observed in the lower dermis. After six weeks the chondroitin sulphate staining remained diffuse for the SP wounds (Figure 5C and 5E), whereas in the lower dermis of the MA wounds the chondroitin sulphate labeling co-localized with newly formed collagen bundles (Figure 5D and 5F). Over time this difference persisted between both wound types. Sixteen weeks p.w., chondroitin sulphate staining was present in both wounds in the BM and around the collagen bundles in the mid-dermis. However, in the SP wounds the lower dermis still showed a diffuse staining pattern (Figure 5G) whereas the lower dermis of the MA wounds labeled as normal pig skin (Figure 5H). At this time point the regenerated dermis in the MA wounds was clearly larger than in the SP wounds.

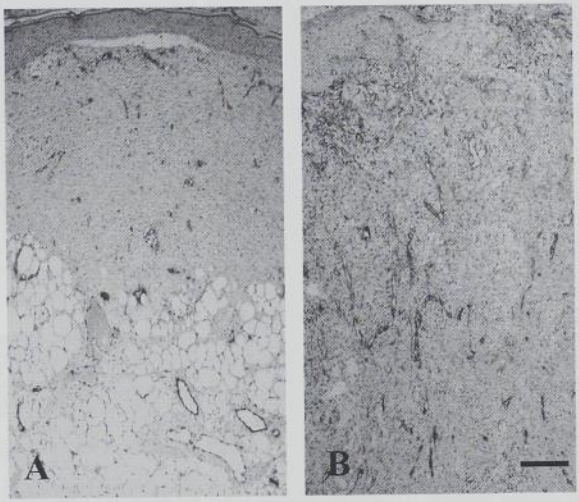
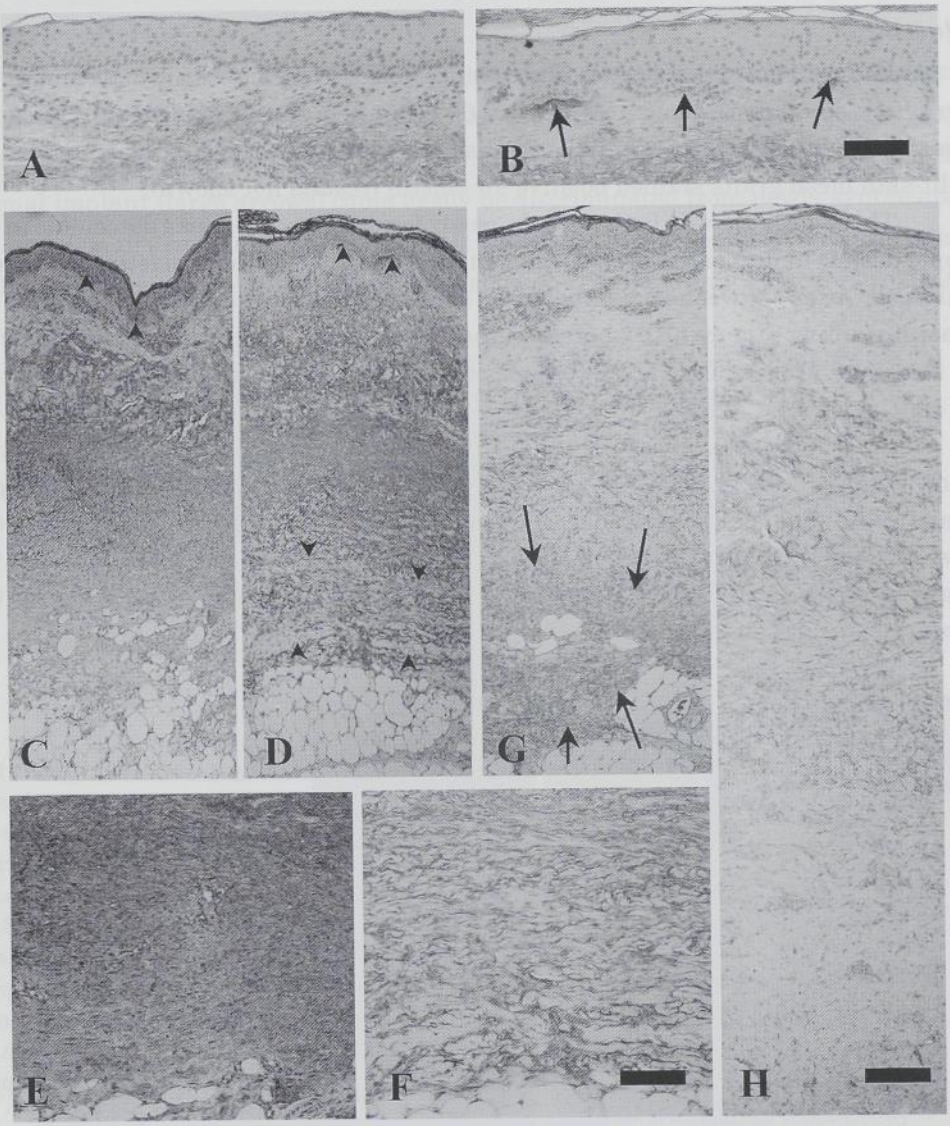


Figure 4. Staining for von Willebrand Factor of the SP and MA wounds one week p.w.. **A.** In the SP wounds some vascular structures stained positive for von Willebrand Factor. **B.** In the MA wounds more vascularization was observed. (Arrows indicated examples) Bar = 200 μm

> **Figure 5.** Chondroitin sulphate staining of the SP and MA wounds three (**A,B**), six (**C,D,E,F**) and sixteen weeks (**G,H**) p.w.. **A.** After three weeks SP wounds showed no chondroitin sulphate staining in the BM of newly regenerated epidermis; the upper part of the dermis was negative for chondroitin sulphate. **B.** The MA wounds showed positive chondroitin sulphate staining in the BM (arrows). **C.** After six weeks, chondroitin sulphate labeling was found in the BM (arrowheads) and throughout the lower dermis of the SP wounds. **D.** In the MA wounds, the BM stained positive for chondroitin sulphate (arrowheads) and in the lower dermis the labeling co-localized with collagen bundles (arrowheads). **E.** Higher magnification (x2,5) of the lower dermal area presented in figure 5C clearly showing diffuse overall positive staining for chondroitin sulphate. **F.** Higher magnification (x2,5) of the lower dermal area



presented in figure 5D clearly showing positive chondroitin sulphate staining in between the newly formed collagen bundles. **G.** After sixteen weeks, SP wounds showed less regeneration of dermal tissue and the lower dermis still stained diffusely positive for chondroitin sulphate (area between arrows). **H.** In the MA wounds a larger dermis was formed, and the upper and lower part of the dermis resembled the chondroitin sulphate labeling of normal pig skin. Bars: A,B = 70 μm; C,D,G,H = 175 μm; E,F = 70 μm..

Elastin

In normal pig, skin elastin fibres were found throughout the dermis and in the periphery of vascular structures (Figure 6A). In early granulation tissue no elastin staining could be detected. Only the thin layer of dermis attached to the transplanted split-skin mesh grafts stained positive for elastin (results not shown).

Three weeks p.w. in both wounds, newly formed elastin became detectable in the upper dermis underneath the epidermis. In time, the area at which elastin was formed enlarged. After six weeks p.w., the lower dermis of the SP wounds was negative for elastin staining (Figure 6B), whereas in the MA wounds elastin staining was detected in this area (Figure 6C). This difference persisted over time until the last evaluation at sixteen weeks.

Immunohistochemical staining of the matrix material before implantation showed the presence of the elastin hydrolysate (Figure 7A), in contrast to a collagen matrix without elastin hydrolysate coating (Figure 7B). In the MA wounds one week p.w., the elastin hydrolysate coating was still detected. Two weeks p.w., however, the matrix material was negative for elastin, indicating ongoing biodegradation of the material (results not shown).

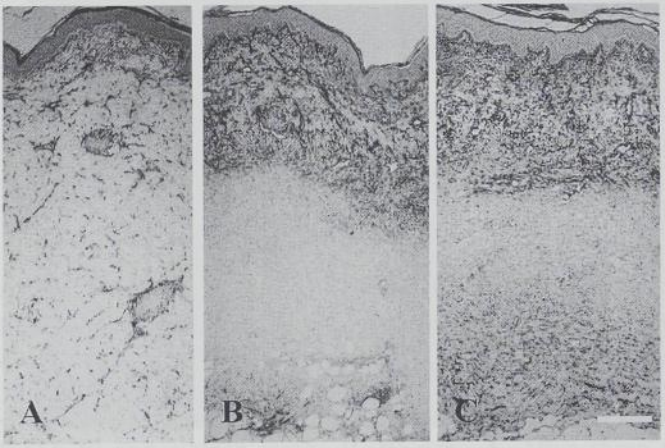


Figure 6. Elastin localization in normal skin and six weeks p.w. in the SP and MA wounds. **A.** Normal skin elastin was detected throughout the dermis. **B.** In the SP wounds elastin reappeared only in the upper dermis. **C.** In the MA wounds elastin labeling reappeared in both the lower and upper dermis. Bar = 175 μm.

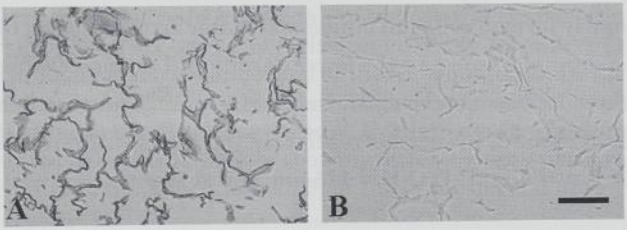


Figure 7. Elastin staining of dermal matrix materials before implantation. **A.** The collagen matrix coated with elastin hydrolysate. **B.** Collagen matrix without elastin hydrolysate coating. Bar = 40 μm.

Electron microscopy

In the SP wounds (Figure 8A and 8B) many fibroblasts contained microfilament bundles (arrows) and other morphological characteristics of myofibroblasts (e.g. indented nuclei) (Desmoulière et al., 1992). In the MA wounds, the collagen from the dermal implant (large arrows) could easily be discriminated from newly synthesized collagen (small arrows)(Figure 8C and 8D). Fibroblasts were seen in close association with the matrix collagen fibres and did not show the characteristics of myofibroblasts.

Figure 8. Transmission electron micrographs of the dermal architecture of the SP and MA wound two weeks after wounding. **A.** In the SP wounds myofibroblasts were present, containing microfilament bundles (arrows) and indented nuclei. **B.** Enlargement of area indicated in figure 8A clearly showing stress fibres (arrows). **C, D.** In the MA wounds, collagen of the dermal matrix (large arrows) was easily distinguished from newly synthesized collagen (small arrows). The fibroblastic cells in close association with matrix collagen showed no myofibroblast characteristics. Bars: A = 0,9 μm; B = 0,25 μm; C = 0,4 μm; D = 0,3 μm.

Discussion

Full-thickness skin defects are often treated with split-skin mesh grafts or epidermal sheets. Several studies have shown that a dermal component is essential for improved epidermal graft succes and for improved quality of healing (Kangesu et al., 1993; Nave, 1992; Cuono et al., 1986). Therefore, we have developed a dermal substitute which in combination with a split-skin mesh graft, improved the dermal regeneration of full-thickness wounds in the pig compared to treatment with split-skin mesh grafts alone. Physical structure of the dermal matrix was visualized previously (Middelkoop et al.,1995). The matrix fibers did not provoke foreign body reactions and were biodegraded within 4 - 6 weeks after implantation (de Vries et al., 1994).

Early Matrix Formation

At one week p.w., fibronectin staining in MA wounds was mainly co-localized with collagen fibres of the matrix, in contrast to the SP wounds, in which fibronectin was present as a diffuse signal throughout the granulation tissue (Vande Berg et al., 1993; Grinnell et al., 1981). In wound healing, fibronectins and fibronectin peptides play an important role. They are chemotactic for inflammatory cells and fibroblasts (Postlethwaite et al., 1981) and serve, together with fibrin as a template for cell migration and adhesion. For example, fibroblasts could not migrate into plasma clots when fibronectin was depleted (Couchman et al., 1990; Knox et al., 1986). Earlier cell density studies performed in our wound model showed that cell density in MA wounds after two weeks p.w. was significantly lower compared to SP wounds (Figure 1A and 1B)(de Vries et al., 1994). The fibronectin staining pattern in the MA wound was visualized as a more localized expression and probably also a reduced expression which could result in reduced chemotactic activity. This hypothesis might explain why fewer cells have migrated into the wound during the early healing process.

Angiogenesis

Staining for von Willebrand Factor revealed that early vascularization of the granulation tissue was stimulated in the MA compared to the SP wounds. The presence of the collagen matrix, acting as a deposit for growth factors, probably facilitates cell adherence and migration, resulting in a more rapid vascularization. The deposition of fibronectin along the collagen fibers might play an role in this, since it was demonstrated in vitro that endothelial cells grew and differentiated more rapidly

when the fibronectin concentration in the substrate was higher (Ingber et al., 1989).

BM Regeneration

BM regeneration was studied with the markers laminin and chondroitin sulphate. After one week laminin was already detected in the area under the migrating epidermis. Furthermore, laminin was associated with vascular structures and this labeling pattern corresponded to the stainings found for von Willebrand Factor. The early presence of laminin in regenerating BM was described in other studies in humans (Tooney et al., 1993; Clark et al., 1982), but its presence is not necessarily correlated with a well-differentiated epidermis or with the formation of anchoring filaments. Chondroitin sulphate labeling proved to be a more discriminative marker for BM regeneration. In general, chondroitin sulphate reappeared approximately one week earlier in the MA wounds, indicating more rapid BM regeneration.

New Tissue Formation

In normal pig skin, chondroitin sulphate labeling was not limited to the BM but was also found in the dermis in the periphery of vascular structures (Daugaard et al., 1991). It has been reported that CSPG expression was upregulated in pig granulation tissue using antibodies to the core protein (Yeo et al., 1991). The monoclonal antibody used in this study recognized the chondroitin sulphate GAG chains attached to the core protein and stained all the CSPGs present in the wound bed.

During the first weeks p.w., fibronectin and CSPG expression were strongly upregulated throughout the granulation tissue. After three weeks fibronectin and chondroitin sulphate started to disappear in the upper dermis and in the same area elastin staining reappeared. Fibronectin staining was reduced to control levels within six weeks in the MA wounds and after approximately 12 weeks for the SP wounds. The same observations were made for the lower dermis of the MA wounds. Apparently, the matrix induces faster maturation of the granulation tissue and dermal regeneration in the lower dermis. This is an important observation which explains the reduction of scar formation and wound contraction in these wounds.

ECM Remodelling

The regeneration of elastin fibres indicates a more final stage of the healing process. Elastin fibres regeneration in the pig was found to be much faster than in humans, in which scar tissue elastin was regenerated only after several years (Compton et al.,

1989). In addition, punch biopsies in humans treated with the same dermal substitute did not show elastin regeneration three months after wounding (de Vries et al., 1995). In our animal wound model, we have not investigated elastin regeneration at a later time point than sixteen weeks. Whether the reduced elastin expression found in the SP wounds at this time point represents a final stage of the scar tissue or merely indicates a slower healing than in the MA wounds is presently not clear.

The elastin hydrolysate present on the dermal matrix was found to be rapidly degraded after implantation in the wound. This is not surprising, since many proteolytic processes take place in the early stages of wound healing (Stricklin et al., 1994). Apparently, the beneficial effect of the elastin hydrolysate on the reduction of wound contraction and stimulation of dermal regeneration (de Vries et al., 1994) is executed in the early healing period (within two weeks p.w.). The exact nature of this effect, e.g., by modulation of the proteolytic activity (Gminski et al., 1991; Rinehart et al., 1993) or by cytokine mobilization, is now under investigation.

Myofibroblasts are believed to play an important role in wound contraction. It was shown that after two weeks p.w. in MA wounds fewer myofibroblasts were present compared to SP wounds (de Vries et al., 1994). Our electron microscopic observations were in agreement with these results. Cells in close association with the matrix had no myofibroblast features. Apparently, the dermal substitute reduced the differentiation of fibroblasts into myofibroblasts. This effect might also be mediated by cytokines able to bind the collagen-elastin dermal substitute.

In conclusion, the present study indicates that immunohistochemistry is an adequate method to visualize the beneficial effects of a dermal substitute on the tissue regeneration process. The effects of the dermal substitute on ECM deposition and remodelling are multiple: both early and late wound healing processes were influenced, and the regeneration in the lower dermis was especially stimulated. Furthermore, macroscopical observations such as wound contraction and scar formation, which were more pronounced for the SP wounds, were correlated with microscopic observations of synthesis and turnover of specific ECM molecules.

Acknowledgements

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Chapter 3

Living Skin Substitutes: Survival and Function of Fibroblasts Seeded in a Dermal Substitute in Experimental Wounds

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ABSTRACT

The healing of full-thickness skin defects requires extensive synthesis and remodeling of dermal and epidermal components. Fibroblasts play an important role in this process and are being incorporated in the latest generation of artificial dermal substitutes. We studied the fate of fibroblasts seeded in our artificial elastin/collagen dermal substitute and the influence of the seeded fibroblasts on cell migration and dermal substitute degradation after transplantation to experimental full-thickness wounds in pigs.

Wounds were treated with either dermal substitutes seeded with autologous fibroblasts or with acellular substitutes. Seeded fibroblasts, labeled with a PKH-26 fluorescent cell marker, were detected in the wounds with fluorescence microscopy and quantified with flow cytofluorometric analysis of single-cell suspensions of wound tissue. The cellular infiltrate was characterized for the presence of mesenchymal cells (vimentin), monocytes/macrophages and vascular cells. Dermal substitute degradation was quantified by image analysis of wound sections stained with Herovici's staining.

In the wounds treated with the seeded dermal substitute, fluorescent PKH-26 labeled cells were detectable up to six days and were positive for vimentin but not for the macrophage antibody. After five days, flow cytofluorometry showed the presence of 3.1 (±0.9) $x10^6$ (mean ±SD, n=7) PKH-26 positive cells in these wounds, whereas initially only $1x10^6$ fluorescent fibroblasts had been seeded. In total, the percentage of mesenchymal cells minus the macrophages was similar after five days between wounds treated with the seeded and the acellular substitutes. In the wounds treated with the seeded substitute, however, 19.5% of the mesenchymal cells was of seeded origin. Furthermore, the rate of substitute degradation in the seeded wounds was significantly lower at 2-4 weeks after wounding than in wounds treated with the acellular substitute. Vascular ingrowth and the number of infiltrated macrophages were not different.

In conclusion, cultured dermal fibroblasts seeded in an artificial dermal substitute and transplanted onto full-thickness wounds in pigs survived and proliferated. The observed effects of seeded fibroblasts on dermal regeneration

appeared to be mediated by reducing subcutaneous fibroblastic cell migration and/or proliferation into the wounds without impairing migration of monocytes/macrophages and endothelial cells. Moreover, the degradation of the implanted dermal substitute was retarded, indicating a protective activity of the seeded fibroblasts.

INTRODUCTION

Treatment of full-skin defects has changed rapidly in the last decennia and several new treatments have been developed. Nowadays, wound infections can be controlled by proper wound care and rapid wound closure is obtained by treatment with epidermal grafts, e.g. meshed split skin or cultured epidermal sheets. Nevertheless, problems occurring with deep skin defects such as wound contraction, scar formation and hypertrophy are as yet unsolved (Hinshaw and Miller, 1965; Herd *et al*, 1987; Matsuzaki *et al*, 1995). For such wounds it has been recognized that application of a dermal template can induce more dermal tissue than epidermal grafts alone (Murphy *et al*, 1990; Cooper *et al*, 1991; Kangesu *et al*, 1993; de Vries *et al*, 1994). Since the early eighties several artificial dermal substitutes have been developed (Bell *et al*, 1981; Boyce *et al*, 1990; Shahabeddin *et al*, 1990; Hansbrough *et al*, 1992; Koide *et al*, 1993; de Vries *et al*, 1994; Geesin *et al*, 1996), and several were tested on burn patients (Heimbach *et al*, 1988; Hansbrough *et al*, 1989; Suzuki *et al*, 1990; Boyce *et al*, 1993; Burke *et al*, 1993; Hansbrough *et al*, 1995). Two dermal substitutes, Dermagraft^{R} (Advanced Tissue Sciences, La Jolla, Ca) and Integra^{R} (Integra Life Sciences Corp., Plainsboro, New Jersey), and one full skin equivalent, Apligraf^{R} (Novartis, Basel, Switzerland), have recently been launched into the market for clinical use. The cellular component of Apligraf^{R} consists of allogenic fibroblasts and keratinocytes, Dermagraft^{R} contains allogenic fibroblasts and is directly applied in combination with an autologous epidermal transplant (Hansbrough *et al*, 1992), whereas Integra^{R} is acellular and the autologous epidermal transplant is only applied after vascularization of the dermal substitute (Stern *et al*, 1990). We developed an acellular dermal substitute which improved dermal regeneration in a porcine full-thickness wound model (de Vries *et al*, 1994; Lamme *et al*, 1996) and in a clinical pilot

study (de Vries *et al*, 1995b). The addition of autologous fibroblasts to the dermal substitute resulted in a significant additional reduction of myofibroblast formation and wound contraction (de Vries *et al*, 1995a). Our concept is based on artificial skin grafting with autologous cells, as this is safer with regard to transmittable diseases and no risk of induction of inflammation and rejection reactions (Hultmann *et al*, 1996). The dermal substitute is seeded with fibroblasts ($1x10^5/cm^2$) using short culture times (7-10 days) and is directly applied in combination with an epidermal transplant. We believe that rapid wound treatment is advantageous because wound contraction processes are very potent in the early wound healing phase (Clark, 1993; de Vries *et al*, 1994). Using this concept, full-thickness wounds can be treated at the earliest intervention possible, especially when fibroblasts are isolated from adipose tissue (de Vries *et al*, 1995a).

Fibroblasts play a central role in the regeneration of new skin tissue. Several studies showed that the presence of fibroblasts in a dermal equivalent stimulated epidermal differentiation (Cooper *et al*, 1993; Okamoto and Kitano, 1993; Saintigny *et al*, 1993; Maruguchi *et al*, 1994; Tuan *et al*, 1994) and dermal regeneration (Murphy *et al*, 1990; Stern *et al*, 1990; Boyce *et al*, 1991; Marks *et al*, 1991; de Vries *et al*, 1995a). It is assumed that these fibroblasts accelerate the healing process by reducing the time needed for fibroblasts to invade the wound tissue and by early synthesis of new skin tissue. In this study, we investigated survival of fibroblasts seeded in a dermal substitute and their effects on formation of granulation tissue, tissue regeneration and substitute degradation in the porcine wound model.

MATERIALS AND METHODS

Materials

The dermal substitute was a non-cross linked native bovine collagen matrix (type I collagen from bovine skin) coated with a 3% (w/w) α-elastin hydrolysate (80% MW 60 kDa and 20% MW 200 kDa from bovine ligamentum nuchae) and was kindly provided by Dr. P. Tewes-Schwarzer (Dr Otto Suwelack Nachf. GmbH & Co.,

Billerbeck, Germany). Exkin^R, a semipermeable polyether urethane membrane allowing wound drainage but protecting the wound against contamination, was provided by Utermöhlen (Utrecht, The Netherlands).

The monoclonal antibody (clone MO107, 1:10.000) specific for porcine monocytes/ macrophages was a kind gift from Dr. J. Pol (DLO, Lelystad, The Netherlands).

Operation Procedures, Wound Treatments and Evaluations

The operation procedures were performed as described previously (de Vries *et al*, 1993). The protocol was approved by the University of Amsterdam Committee of Animal Welfare and for this study three female Yorkshire pigs (20 kg at arrival) were included. Two weeks prior to operation, porcine dermal fibroblasts were isolated from punch biopsies and cultured in DMEM supplemented with 10% (v/v) FCS, 1 mM L-glutamine (Life Technologies, Breda, The Netherlands) and antibiotics (penicillin (100 IU/ml), streptomycin (100 mg/ml), Life Technologies). On the day of operation, full-thickness wounds (3.0 x 3.0 cm) were created on the back of the pigs using a dermatome. Dermal collagen substitutes were placed in the wounds, and subsequently covered with split-skin mesh grafts (0.2 mm thick) and Exkin^R. Three treatments were applied: acellular substitutes (n=7), substitutes seeded with fluorescent fibroblasts ($1x10^5$ cells/cm^2, n=7), and substitutes seeded with non-viable fluorescent fibroblasts ($1x10^5$ cells/cm^2, n=2). Dermal substitutes were seeded with cultured porcine fibroblasts passage 2 by inoculating the upper side of the matrices with culture medium containing the fibroblasts (1 ml/10 cm^2 of substitute). Before seeded substitutes were implanted, the fibroblasts were allowed to attach to the substitute matrix for 4h. During this period substitute contraction did not occur. Fibroblasts were labeled with PKH26-GL according to the manufacturer's protocol (Sigma, St. Louis, Mo) and in 2 cases made non-viable by 3 repeated freeze-thawing cycles. The histology of seeded substitutes before implantation showed fibroblasts attached to matrix fibres throughout the substitute but with a higher density in the upper part.

After 5 days, at an identical place from all wounds two 4 mm punch biopsies were taken for immunofluorescence and immunohistochemistry, one snap-frozen in liquid nitrogen and the other fixated in 4% formaldehyde PBS solution. Split-skin

mesh grafts were removed and the wound tissues were excised with a scalpel at the interface of the granulation tissue and subcutaneous fat. The wound tissues had an identical area and thickness, as wound size and depth remained the same until 5 days after operation. Blood coagulates attached to the wound tissue during the surgical removal were removed without damaging the wound tissue. The tissues were enzymatically digested at 37°C for 1.5 h with a phosphate-buffered saline (PBS) solution containing 0.25% collagenase (Boehringher Mannheim, Mannheim, Germany) and 0.25% dispase (Boehringher Mannheim)(3 ml/g of tissue). The digest was washed with PBS and sieved through a 10 μm nylon membrane (Schleicher & Schuell, Den Bosch, The Netherlands) to obtain single-cell suspensions.

Immunofluorescence and Immunohistochemistry

Unfixed cryostat sections of 10 μm thickness were used to detect the presence of PKH-26 fluorescent cells and stained for vimentin as follows. After 15 min of preincubation with PBS containing 10% human ABO serum (Central Laboratory for Blood Transfusion Services, Amsterdam, The Netherlands), sections were incubated for 1 h with the anti-vimentin monoclonal antibody (clone V9; Dako, Copenhagen, Denmark) diluted in PBS (1:100). After two washes in PBS, they were incubated for 30 min with FITC-conjugated $F(ab)_2$ fragments of rabbit anti-mouse antibodies (1:50, Dako). The excess of $F(ab)_2$ fragments was removed by two wash steps in an excess of PBS. Finally, sections were covered with Vecta-shield (Sigma) and immunofluorescence analysis was performed with a LSM confocal scanning laser microscope (Leica Lasertechnic, Heidelberg, Germany) equipped with an argon-krypton laser. Double excitation and detection were performed with the 488 nm and 568 lines for vimentin and PKH-26, respectively. The images were corrected for cross-talk and matched with the multi-color analysis software package.

For immunohistochemistry, 4% formaldehyde fixated wound biopsies embedded in paraffin were sectioned at 5 μm. The sections were deparaffinized, hydrated through a degrading series of ethanol solutions, and incubated for 10 min in 0.02% H_2O_2/methanol solution. Sections were predigested at 37°C for 30 min with a solution containing 0.25% pepsin and 10 mM HCl (pH 2.5) and incubated with the next solution

at room temperature in the following order: PBS solution/10% AB serum for 15 min; for 60 min with the polyclonal rabbit anti-von Willebrand factor antibody (1:1500, Dako) diluted in PBS; for 30 min with biotinylated swine anti-rabbit IgGs antibodies (1:400, Dako) diluted in PBS 10% AB serum; and for 30 min with streptavidinABcomplex/horseradish peroxidase (1:50, Dako) diluted in PBS. Between incubations, the sections were washed twice in PBS. Color reaction was performed for 7 min in 50 mM Tris-HCl buffer (pH 7.8) containing 0.05% DAB and 0.03% H_2O_2. Finally, the slides were washed in water, counterstained with hematoxylin, mounted in glycergel (Dako) and examined microscopically.

Sections of porcine normal skin served as positive controls. As negative controls, adjacent sections of the wound biopsies were stained with nonimmune IgG from the same species in the same dilution as the primary antibody. No specific signal was noticed in the negative controls.

Flow Cytofluorometry Analysis

The single-cell suspensions derived from the wounds were analyzed for PKH-26 fluorescent positive cells without prior fixation. In addition, part of the cell suspension was labeled intracellularly with monoclonal antibodies anti-α-smooth muscle actin (clone 1A4, Dako), anti-vimentin, and anti-monocyte/macrophage antibodies as follows. Cells ($5x10^5$) were fixed for 10 min in 0.25 ml PBS containing 1% formaldehyde, washed twice with PBS and resuspended in 0.25 ml PBS containing 0.1% saponin and 0.5% BSA. After 10 min, first antibodies were added at saturating concentrations and incubated for 30 min. After two washing steps, a second incubation of 30 min was performed with FITC-labeled $F(ab')_2$ fragments of rabbit anti-mouse IgGs (1:50). After two additional washes, cells were sampled in PBS containing 0.5% BSA. All incubations and washings were performed at 4°C in the presence of 0.1% saponin and fluorescence analysis was performed on at least 1×10^5 cells per sample using a FACScan (Becton Dickinson, Mountain View, Ca). Negative controls were labeled identically with control isotype-matched antibodies diluted in the same way as the first antibodies.

Herovici Staining and Image Analysis

For the picropolychrome staining of Herovici formaldehyde fixated biopsies were used. The biopsies were embedded in paraffin and cut into 5 μm sections. The wound biopsies were taken at 1, 2, 3 and 4 weeks after wounding from wounds treated with the acellular (n=7) and seeded substitutes (n=6) (identical treatments procedures as described in the operation procedures) obtained from previous studies (de Vries *et al*, 1995a). After deparaffination and hydration, wound sections were stained as described by Herovici (Herovici, 1963). At three and four weeks post-wounding, the substitute fibers were mainly localized in the middle and lower dermal area of the sections. This area was chosen for image analysis.

Absorbance measurements of Herovici stained sections were performed with the use of white light, an infrared blocking filter, and a monochromatic 543 nm filter (Chieco *et al*, 1994). Digital images were recorded with a Cohu CCD camera (768x575@8 bits, Cohu Systems, San Diego, Ca) attached to a Olympus AH2 microscope (Tokyo, Japan) with a stabilized power supply. The resolution of the sample area was 512x768 pixels, corresponding to a section area of 0.65 x 0.96 mm. Images were captured with a Scion NuBus framegrabber (Scion Corporation, Frederick, Maryland) using a Macintosh Power PC. Greyscale images were corrected for background and transformed into absorbance images using a density-step tablet (Jonker *et al*, 1997). Image grabbing and analysis were performed by the public domain image processing program NIH-Image software (written by W.Rasband, US National Institutes of Health). Area measurements were performed after segmentation using a threshold at 2/3 of the maximum absorbance in each image. An example of a digital image is shown in **Fig 1*A***. The signal detected along the bar across substitute element is shown graphically as an absorbance profile in **Fig 1*B*** (hatched line indicated 2/3 of maximum absorbance recorded). **Fig 1*C*** shows the areas which were measured as substitute-matrix areas (threshold at 2/3 of maximum absorbance).

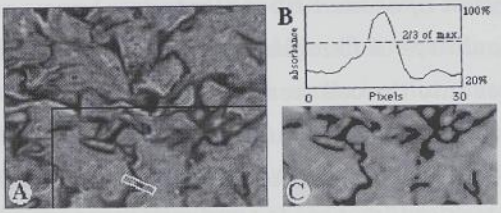


Figure 1. Evaluation of a digital image and localization of substitute matrix by absorbance measurements. (**A**) Unmodified digital image of Herovici-stained wound section, treated with seeded substitute, at 1 week post-wounding; (**B**) Graphical representation of absorbance profile detected along the bar shown in Fig. 1A. Hatched line in graph indicates two-third of the maximum absorbance recorded; (**C**) Same image as outlined in Fig. 1A in which absorbances above two-thirds of maximum absorbance density were selected using NIH software. These areas were selected as substitute-matrix surfaces.

RESULTS

General histological observations

In hematoxylin/eosin stained sections of the wounds treated with either seeded or acellular dermal substitute the start of granulation tissue formation was noted 5 days after wounding (**Fig 2*A***). The infiltrating cells had migrated approximately half-way into the dermal substitute. In the upper half mainly granulocytes were located. Vascular in-growth was assessed with von Willebrand Factor staining and evaluated by the percentage of dermal substitute which was vascularized (Fig 2*B*). The wounds treated with the acellular or seeded substitute showed no differences in vascularized area.

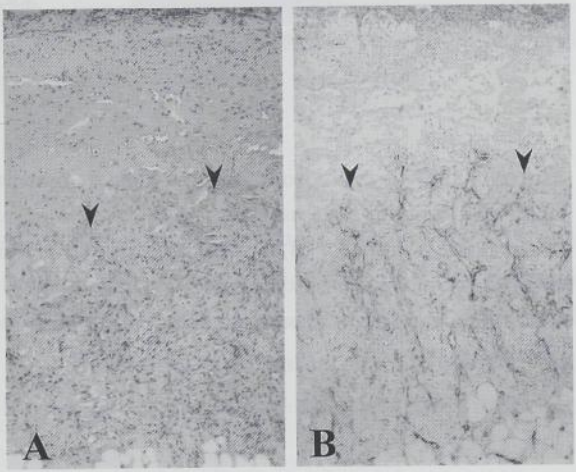


Figure 2. Hematoxylin Eosin (**A**) and von Willebrand (**B**) stainings of a wound treated with the seeded substitute at 5 days after wounding. The figures show the presence of the substitute in between the subcutis (lower part of photomicrograph) and the Exkin^{R} membrane at the top. Biopsies were taken in between the interstices of the split skin mesh grafts and showed no signs of reepithelialization. Cell migration (arrow heads, A) and vascularization (arrow heads, B) had proceeded up to half way into the dermal substitute. *Scale bar*, 130 μm

Fluorescence detection of seeded fibroblasts

Subconfluent cultures of fibroblasts were labeled with PKH-26, a fluorescent label which integrates into the cell membrane. The labeling procedure was checked with flow cytofluorometry (**Fig 3*A***) and fibroblasts were used in vitro for different tests: cell attachment and proliferation on plastic and collagen I coated culture dishes, and in the dermal substitute. Significant differences between labeled and unlabeled fibroblasts were never observed (data not shown) indicating that the labeling procedure and the label did not influence fibroblast behavior. After 5 days of culture, fluorescent intensity of the fibroblasts decreased (**Fig 3*B***). The degree of this decrease was correlated with the number of cell divisions that had occurred in this period. Flow cytofluorometry also showed that the seeded fibroblasts at the moment of seeding contained a small percentage of cells (2-8%), which was positive for α-smooth muscle actin (data not shown).

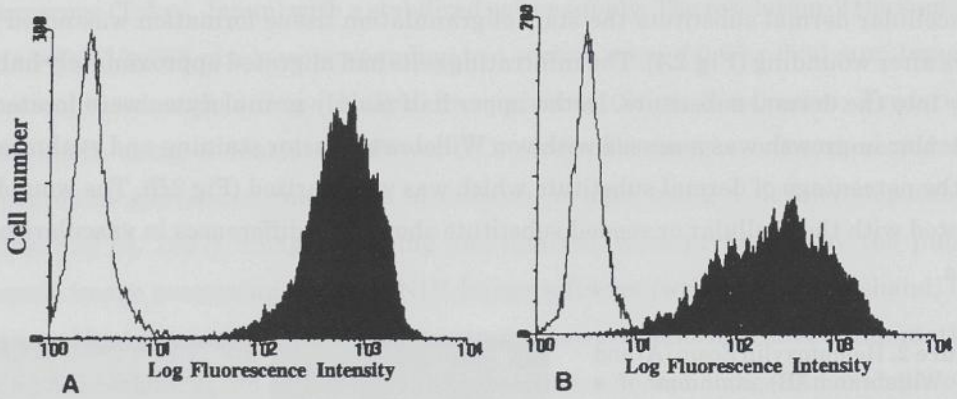


Figure 3. Flow cytofluorometric analysis of identical numbers of fibroblasts labeled with the PKH-26 cell membrane marker. (**A**) PKH-26 labeling of cultured fibroblasts before seeding in the dermal substitute; (**B**) PKH-26 positive fibroblasts, cultured for 5 days *in vitro* showing a reduced mean fluorescent intensity when compared with freshly labeled fibroblasts (3A). The non-shaded curves represent baseline fluorescence profiles of identical populations of unlabeled fibroblasts.

In unfixed frozen wound sections PKH-26 fluorescent elongated cells (bright red) were detected with confocal microscopy at 5 days after wounding (**Fig 4**). In addition, most cells were labeled for vimentin (green), a marker for mesenchymal cells. Most PKH-26 positive cells showed colocalization with vimentin (yellow)(**Fig 4**). After 5 days, the number of cells with a progressively weaker PKH-26 fluorescence increased rapidly, which made further investigations of seeded fibroblasts at later time points unfeasible. In wounds treated with the acellular substitute, PKH-26 fluorescence was not detected.

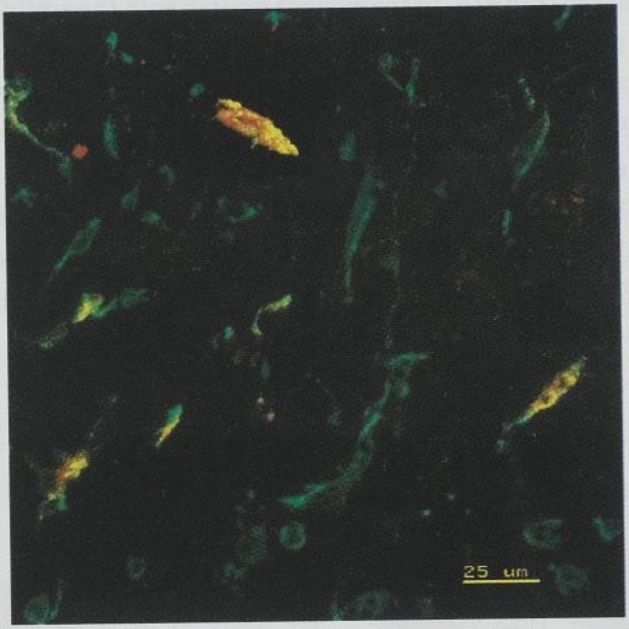


Figure 4. Confocal scanning laser microscopy of wounds seeded with fibroblasts showing PKH-26 positive cells (red) and vimentin-positive cells (green). Most PKH-26 positive cells also labeled for the mesenchymal cell marker vimentin (yellow). *Scale bar*, 25 μm.

Quantification of seeded cells and cell infiltrates

Flow cytofluorometric analysis of single-cell suspensions obtained from wounds (indicated with gate **Fig 5*A***) showed no PKH-26 positive cells in the unseeded wounds (**Fig 5*B***), whereas in the seeded wounds a cell population was positive for PKH-26 (gate **Fig 5*C***). The PKH-26 cells did not label with propidium iodide, indicating that PKH-26 cells were viable (data not shown). In **figure 6A** the light scatter properties

of fixated intracelullar labeled single-cell suspensions is shown. Particles with reduced scatter properties were excluded from the gate. For both wounds, the labeling for vimentin (**Fig 6*B***) showed that approximately 96-97% of the cells was vimentin-positive. Alpha-smooth muscle actin labeling (**Fig 6*C***) of the single cell suspensions was not different between the seeded and the acellular substitute treated wounds. Most cells were moderately positive, but only a small population of the cells (6%+/-1%) was clearly positive. The latter population probably represents isolated smooth muscle cells. In addition, both wounds labeled similar percentage of cells (19%) with the macrophage antibody (**Fig 6*D***). In the wounds treated with the acellular substitute no double labeling for PKH-26 with vimentin or the macrophage marker was observed (**Fig 6*E*** and **6*G***). In the seeded wounds all PKH-26 positive cells were positive for vimentin (**Fig 6*F***) and a few PKH-26 cells double labeled with the macrophage marker (**Fig 6*H***). The number of macrophages that double labeled for PKH-26 was <2.2% of the total cell suspension isolated. As a control, two wounds were seeded with non-viable labeled fibroblasts. In these wounds the PKH-26 label was only detected in macrophages which did not take up propidium iodide. Non-viable labeled fibroblasts were not detected after 5 days (data not shown).

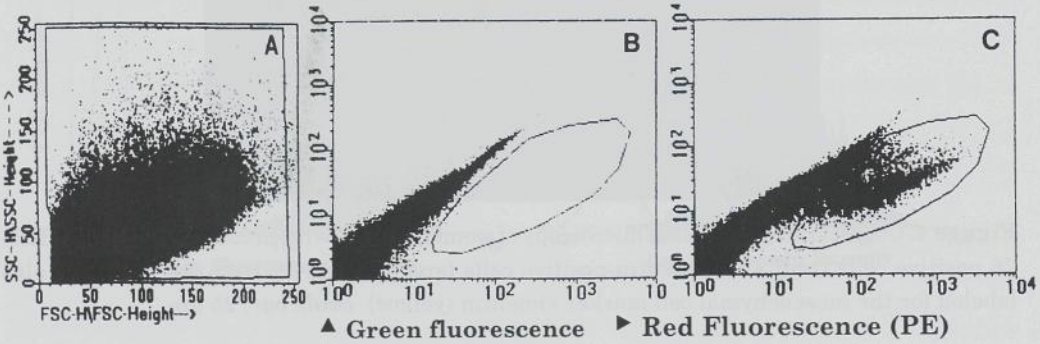


Figure 5. Flow cytofluorometric analysis of single-cell suspensions of wounds after 5 days of treatment with the seeded or with the acellular substitutes. (**A**) Forward(FCS)-Sideward(SCC) scattered dot plot of a cell suspension showing the gated cells; These results are shown as dot plot green fluorescence (y-axis, FL-1) against red fluorescence (x-axis, FL-2), and not as histograms due to autofluorescence of the single-cell population; (**B**) Dot plot showing no PKH-26 positive cells within the gate for the wounds treated with the acellular substitute; (**C**) Dot plot of the wounds treated with the seeded substitutes showing a PKH-26 (FL-2) positive cell population within the gate.

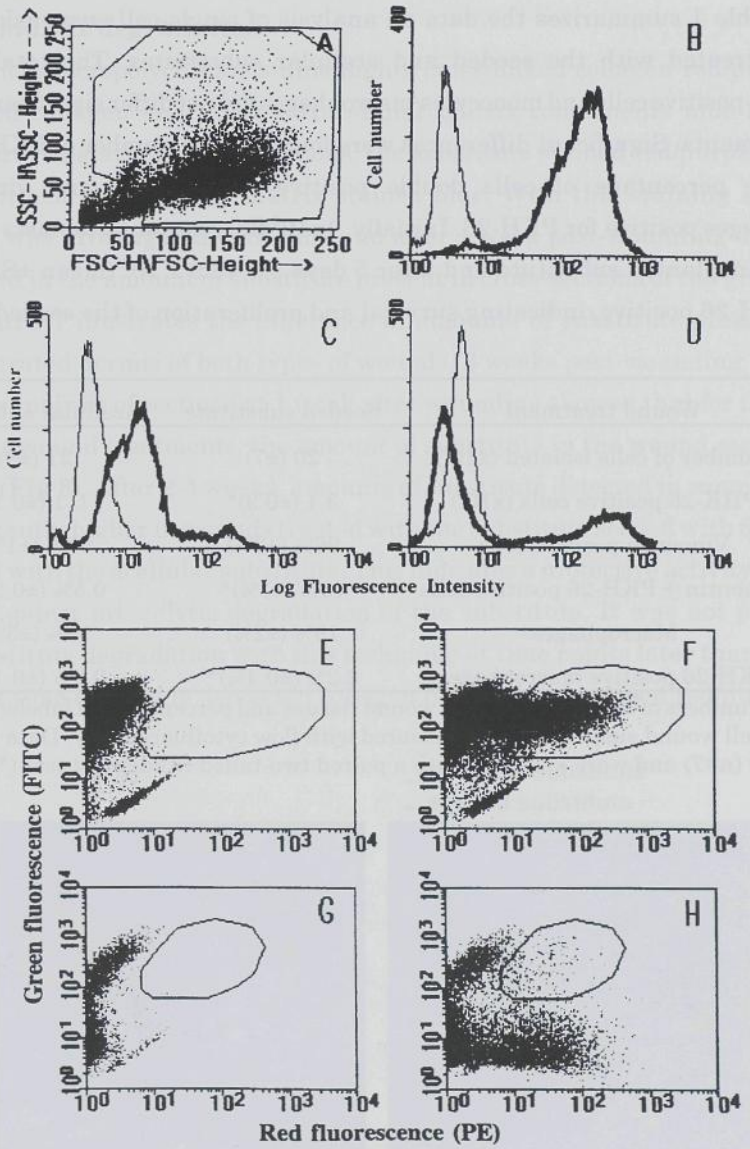
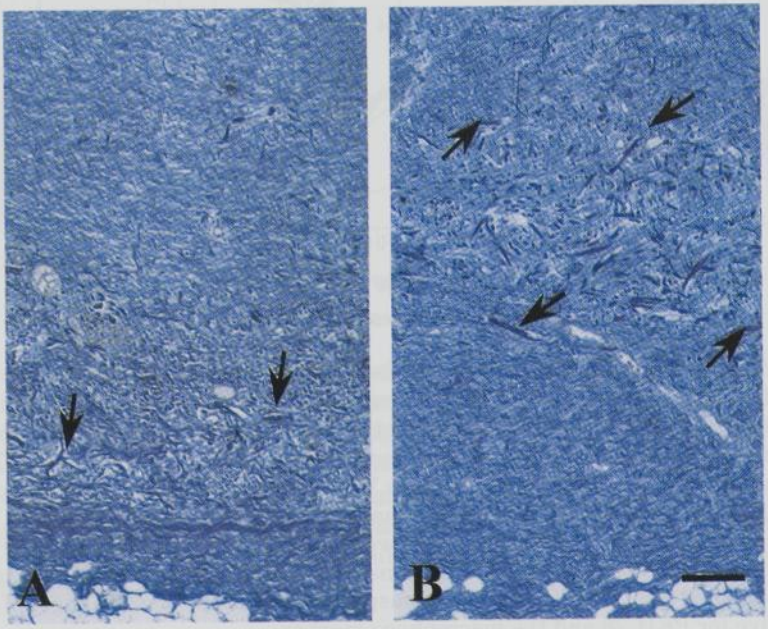


Figure 6. Flow cytofluorometric analysis of fixated and permeabilized single-cell suspensions of wounds after 5 days of treatment with the seeded or with the acellular substitutes. (**A**) Forward(FCS)-Sideward(SCC) scattered dot plot of a cell suspension showing the gated cells; Histogram of cell suspension labeled with vimentin (**B**), α-smooth muscle actin (**C**), and for macrophages (**D**)(thin line = iso-type control). Vimentin labeling of cell suspension of the unseeded (**E**) and seeded (**F**) wounds showed that most cells were positive (FL-1); (**F**) In the seeded wounds, the vimentin labeling also showed that all PKH-26 positive cell (FL-2) were positive for vimentin. (**G** and **H**) Cell suspensions from both wounds labeled for macrophages of which 19% labeled positive (FL-1); (**H**) Cell suspension labeled for macrophages of which 2% labeled positive for PKH-26 and the macrophage marker.

Table 1 summarizes the data on analysis of single-cell suspensions from the wounds treated with the seeded and acellular substitutes. The total number of vimentin-positive cells and monocytes/macrophages did not differ significantly between the treatments. Significant differences were found for the number of PKH-26 positive cells, the percentage of cells double positive for PKH-26 and vimentin, and macrophages positive for PKH-26. Initially, $1x10^6$ fluorescent fibroblasts were seeded in the transplanted substitute and after 5 days $3.1 \pm 0.9 \times 10^6$ (mean ±SD, n=7) cells were PKH-26 positive, indicating survival and proliferation of the seeded fibroblasts.

Wound treatment	Seeded substitute	Acellular substitute
Number of cells isolated ($x10^6$)	20 (±7)	21 (±9)
PHK-26 positive cells ($x10^6$)	3.1 (±0.9)*	0.1 (±0.1)
Vimentin positive cells	96% (±2%)	97% (±1%)
Vimentin + PKH-26 positive cells	15% (±3%)*	0.5% (±0.2%)
Macrophages	19% (±2%)	19% (±3%)
PKH-26 positive macrophages	2.2% (±0.4%)*	0.2% (±0.1%)

Table I. Numbers of cells isolated from wound tissues and percentages of labeled cells present in single-cell wound suspensions as measured with flow cytofluorometry. Data represent the mean ±SD (n=7) and were analyzed with a paired two-tailed Student's *t* test (*, $p<0.01$)



Substitute matrix degradation

Herovici's picropolychrome stains highly cross-linked collagen red/purple and newly formed collagen and other extracellular matrix components blue (Herovici, 1963). In Herovici-stained wound sections, the substitute stained red/purple whereas the newly formed extracellular matrix stained blue. With this staining substitute degradation was investigated with time and after 1 week post-wounding differences were observed in the amount of substitute present in cross-sections of the granulation tissue. **Figure 7** illustrates the difference in amounts of substitute present in the lower regenerated dermis of both types of wounds, 3 weeks post-wounding.

Image analysis of sections at 1 week after wounding showed that for the seeded and acellular wound treatments, the amount of substitute in the wound sections was comparable (**Fig 8**). After 2-4 weeks, amounts of substitute detected in wound sections were significantly higher in wounds treated with the substitute seeded with fibroblasts as compared with the acellular substitute. This indicates a protective activity of seeded fibroblasts against proteolytic degradation of the substitute. It was not possible to analyze substitute degradation with this technique at time points later than 4 weeks, since at that time the newly deposited collagen bundles also stained red/purple.

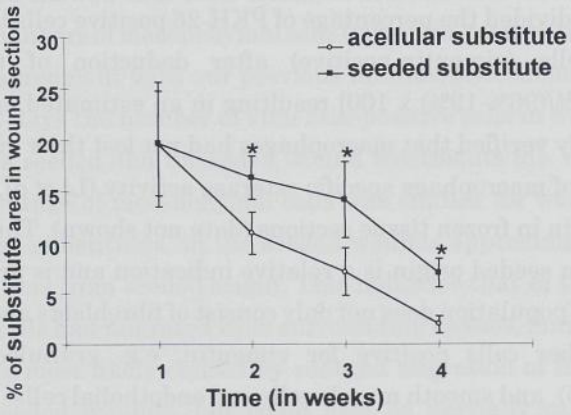


Figure 8. Image analysis of Herovici-stained wound sections at 1 to 4 weeks after wounding. In wounds treated with the seeded substitute (n=6; -■-) matrix degradation was significantly slower as compared with wounds treated with the acellular substitute(n=7; -o-). Results were analyzed with a paired two-tailed Student's t-test (* $p<0.05$) and represented as the mean ±SD.

< **Figure 7.** Herovici's picropolychrome staining of wounds at 3 weeks after wounding. In the wounds treated with the acellular substitute (**A**) clearly fewer substitute fibers (arrow heads) were present as compared with the wounds treated with the seeded substitute (**B**). Scale bar, 130μm.

DISCUSSION

We have previously shown that seeding of autologous fibroblasts in a native collagen/elastin dermal substitute improved tissue regeneration as compared with an acellular treatment (de Vries *et al*, 1995a). The present study provides evidence that fibroblasts seeded in this dermal substitute at a few hours prior to transplantation survive and start to proliferate in the wound tissue. Upon transplantation, 1×10^6 fluorescent fibroblasts were seeded and after 5 days $3.1 \pm 0.9 \times 10^6$ (mean ±SD, n=7) PKH-26 fluorescent viable cells were detected. In the cell suspensions of seeded wounds some PKH-26 fluorescence was associated with macrophages, which was more evident when non-viable fluorescent fibroblasts were seeded. Apparently, macrophages became PKH-26 positive due to phagocytosis of fluorescent-cell remains.

To investigate the influence of the seeded fibroblasts on the wound healing process, we characterized cellular infiltration profiles at 5 days after wounding. Total cell numbers isolated from wounds represented cell populations without red blood cells and vascular structures which were removed during the isolation procedure. Characterization of cell suspensions for fibroblasts was not possible due to lack of specific antibodies. In order to estimate the percentage of fibroblasts which was from seeded origin, we divided the percentage of PKH-26 positive cells by the percentage of mesenchymal cells (vimentin-positive) after deduction of the percentage of macrophages [15%/(96%-19%) x 100] resulting in an estimated percentage of 19.5%. It was additionally verified that macrophages had not lost their vimentin expression, by colocalization of macrophage specific esterase activity (Li *et al*, 1973; Ramos *et al*, 1992) and vimentin in frozen tissue sections (data not shown). This percentage 19.5% of fibroblasts from seeded origin is a relative indication and is probably higher. The mesenchymal cell population does not only consist of fibroblasts and macrophages, but also contains other cells positive for vimentin, e.g. granulocytes, lymphocytes (Tiirikainen, 1995), and smooth muscle cells and endothelial cells. From the FSC-SSC scatter plot, we were not able to distinguish granulocytes from other cells, although they normally have different scatter properties than most other cells. This is due to the broad scatter properties of the overlapping population of fibroblastic cells and macrophages and because the fixation and permeabilisation procedure resulted in a reduction of SSC scattering properties. With regard to smooth muscle cells and endothelial cells, the sieving step with the 10 μm membrane, used to isolate the single cells, clearly removed vascular structures. Nevertheless, the labeling with α-smooth

muscle actin showed in all wounds a small percentage (6%+/-1%) of the cells clearly positive for α-smooth muscle actin which probably represented smooth muscle cells. Apparently, the enzymatic digestion also liberated to a certain extent smooth muscle cells from vascular structures. In addition, most cells seemed to be weakly positive for a-smooth muscle actin. This weak expression might be explained by the early time point of cell isolation. Five days after wounding, fibroblasts are only in the process of becoming myofibroblasts expressing α-smooth muscle actin stress fibres. To investigate whether indeed the number of α-smooth muscle actin-positive cells or the expression levels are different between both wound treatments would require measurements at later time points. Immunohistochemical staining of identically treated wounds however showed for both wounds no significant differences in the number of a-smooth muscle actin-positive cells at later time points (de Vries *et al*, 1995a). The seeding of low numbers of cultured fibroblasts apparently does not alter the fibroblast differentiation into myofibroblasts in particular. Differences in the contractile forces generated or ECM synthesized by the myofibroblasts might still exist and depend on signals derived from the direct surrounding of the myofibroblasts.

Analysis of single-cell suspensions of wounds treated with the seeded and acellular substitutes showed that total numbers of isolated cells were identical, and contained similar numbers of mesenchymal cells (vimentin-positive) and macrophages. These data are in agreement with our previous immunohistochemical results which showed that after 7 days the number of vimentin-positive cells in wound sections was not different for the seeded and unseeded wound treatments (de Vries *et al*, 1995a). Although the percentage of mesenchymal cells was similar for wounds treated with acellular and seeded substitutes, in the seeded wounds approximately 19.5% of the mesenchymal cells was from seeded origin. This indicates that in the seeded wounds less mesenchymal cells had migrated from surrounding tissues, mainly from subcutis. This difference was most likely caused by reduced migration of fibroblasts into the wound tissue of seeded wounds, but could also be partially caused by a higher proliferation rate of fibroblasts in the wounds treated with the acellular substitute. In view of the observed proliferation of fluorescent labeled cells, it seems unlikely that fibroblast proliferation is disturbed in the seeded wounds. The early presence of fibroblasts in the healing process and liable reduced migration may cause a reduction in the time and/or intensity of the early phases of wound healing.

Cellular migration is associated with expression and consecutive action of

proteolytic enzymes (Mignatti and Rifkin, 1996; van Leeuwen, 1996). Inhibition of fibroblast migration might be mediated through inhibition of proteolytic activity in the wound tissue. Interestingly, in wounds treated with seeded substitutes, substitute degradation was retarded during the first four weeks of the healing process. The prolonged presence of the dermal substitute could be beneficial for the tissue regeneration process, since the substitute matrix serves as a template for cell ingrowth, proliferation and neo-matrix deposition. Furthermore, increased proteolytic activity leads to the fragmentation of more proteins and activation of larger numbers of latent growth factors (Mignatti and Rifkin, 1996) resulting in a higher chemotactic activity of the wound tissue. For example, peptide breakdown products of different types of collagens (Postlethwaite *et al*, 1978), fibrin (Laevell *et al*, 1996) and fibronectin (Postlethwaite *et al*, 1981; Seppa *et al*, 1981) proved to be chemotactic for fibroblasts. Moreover, some peptides upregulate synthesis of proteolytic enzymes in fibroblasts. Integrin binding to e.g. fibronectin peptides but not to intact protein induced synthesis of matrix metalloproteinases (Werb *et al*, 1989; Huhtala *et al*, 1995) whereas elastin peptides (generated during substitute degradation) increased elastase-like activity (Gminski *et al*, 1991). In addition, the latter peptides were also shown to be chemotactic for monocytes (Bisaccia *et al*, 1994).

Wound immunohistochemistry showed identical vascular ingrowth for both types of treatments. Seeding of fibroblasts in the substitute apparently did not affect angiogenesis. This is an important observation, since inhibition of angiogenesis would interfere with epidermal graft survival and outgrowth (Gallico *et al*, 1984; Kamagai *et al*, 1988). Moreover, wound macrophages originate from peripheral blood monocytes, which might explain why the number of macrophages was similar in the wounds treated with the acellular and seeded substitutes.

In conclusion, fibroblasts seeded in a dermal substitute and transplanted to full-thickness wounds in pigs survived and proliferated in the newly formed wound tissue. The seeded fibroblasts seemed to exert their beneficial action on the wound healing process by reducing migration and/or proliferation of subcutaneous fibroblasts in the wound tissue and by inhibiting degradation of the dermal substitute. Presently, clinical studies are ongoing using the dermal substitute seeded with autologous fibroblasts for treatment of full-thickness wounds on burn victims.

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Chapter 4

Higher Numbers of Autologous Fibroblasts in an Artificial Dermal Substitute Improve Tissue Regeneration and Modulate Scar-tissue Formation

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Abstract

Cultured skin substitutes are increasingly important for the treatment of burns and chronic wounds. It is a general assumption that wound healing is accelerated when more fibroblasts are present in a living-skin equivalent. Therefore, we investigated the quality of dermal tissue regeneration in relation to the number of autologous fibroblasts seeded in dermal substitutes, transplanted instantaneously or precultured for 10 days in the substitute.

A full-thickness porcine wound model was used to compare acellular dermal substitutes (ADS) to dermal substitutes seeded with fibroblasts at two densities, i.e. $1x10^5$ (0-DS10) and $5x10^5$ cells/cm^2 (0-DS50), and to dermal substitutes seeded 10 days before operation at the same densities (10-DS10 and 10-DS50) (n=7 for each group, 5 pigs). After transplantation of the dermal substitutes, split-skin mesh grafts were applied on top. Additional dermal substitutes (n=5) were simultaneously prepared for each group, fixed on the day of operation, and analysed for the number of fibroblasts and myofibroblasts present in the matrix. Wound healing was evaluated blinded for six weeks, both macroscopically and microscopically (biopsies taken after 1, 3 and 6 weeks). Cosmetic appearance was scored for skin colour, smoothness of wound surface and tissue elasticity/suppleness. Wound contraction was measured by planimetry. The wound biopsies taken after 3 weeks were stained for myofibroblasts (α-smooth-muscle actin). The percentage of dermal area positive for myofibroblasts and the intensity of staining in this area were determined by image analysis. Six weeks post-wounding, scar tissue in wound cross-sections was identified by thin immature collagen bundles organized parallel with the epidermis, and by the absence of elastin staining. Collagen maturation was investigated with polarized light.

The overall wound cosmetic parameter scores and wound contraction showed an improvement in the following order: 10-DS50>10-DS10=0-DS50>0-DS10>ADS. For wound cosmetic parameters, the 10-DS50 and 0-DS50 treatments scored significantly better than the ADS treatment, as the 10-DS50 treatment did for wound contraction ($p<0.05$, paired *t*-test). Three weeks after wounding, a similar trend was observed in the granulation tissue showing a reduction in cell density and early deposition of extracellular matrix (ECM). In addition, an area with myofibroblasts positive for α-smooth-muscle actin was observed in the granulation tissue, which was significantly smaller for 0-DS50, 0-DS10 and 10-DS50 than for the ADS treatment ($p<0.04$, paired *t*-test). The intensity of staining became less as the size of the area diminished. After 6 weeks, the wounds treated with 0-DS50, 0-DS10 and 10-DS50 had significantly less

scar tissue and significantly more mature collagen bundles in the regenerated dermis. This observed improvement of wound healing was correlated with the numbers of fibroblasts present in the dermal substitute at the moment of transplantation.

In conclusion, dermal regeneration of experimental full-skin defects significantly improved by treatment with dermal substitutes containing high numbers of (precultured) autologous fibroblasts.

Introduction

Patients with full-thickness burns suffer from a substantial loss of dermal tissue. In the past, these wounds were successfully grafted with autologous split-skin grafts and cultured keratinocytes.[1-3] Nevertheless, the lack of a dermal component often negatively influences the outcome, because split-skin grafts alone often fail to correct contour defects, are prone to contractures, and can lead to a poorly developed dermal-epidermal junction.[4,5] In addition, cultured epithelium grafts are also known to regenerate slowly the dermal-epidermal junction and to blister and ulcerate for several months after grafting.[3,4,6] Several composite grafts, comprising both cultured fibroblasts and keratinocytes applied in an one-stage transplantation procedure, were tested clinically and were able to prevent this blister formation.[7-9] It is now generally accepted that a dermal component is needed to improve the final outcome of cultured epithelial grafts. Most artificial skin substitutes include fibroblasts to promote dermal-epidermal regeneration and to stimulate epidermal growth and differentiation[10-15], but so far little attention was paid to the influence of the fibroblasts on dermal tissue regeneration. Nowadays, for practical reasons more effort is put into the use of allogeneic fibroblasts.[16-21] The current view is that allogeneic cells provide and maintain graft integrity while they are being replaced by host cells.[22] Whether or not this substitution causes inflammatory and immunological reactions[23] and contributes to or prolongs the wound-scarring process remains to be elucidated. Pathological conditions of the skin associated with inflammatory responses in general result in tissue fibrosis and do not mediate true tissue regeneration.[24]

In our opinion, the use of autologous fibroblasts in artificial skin is a feasible concept. In the practice of Dutch burn centres, if the patient is stable and fluid loss is under control, further surgical treatment is postponed for two to three weeks in order to observe healing of partial-thickness wounds.[25] In addition, usually a temporary wound coverage such as cadaver skin conserves a good wound environment for future grafting.[26,27] Normally, if skin biopsies or split skin are used for the isolation of

fibroblasts, culture periods longer than 2 weeks are required to obtain sufficient cells to treat large wound areas. However, cell yields could be improved by the use of subcutaneous fat as a source for fibroblasts[28], which is easily obtained by liposuction or lipectomy[29].

With respect to skin substitution with autologous cells and efforts to obtain sufficient numbers of fibroblasts, we felt it was important to investigate in which quantity and in which fashion fibroblasts contribute to dermal tissue regeneration. Therefore, we studied dermal tissue regeneration in relation to the number of autologous fibroblasts in dermal substitutes. Fibroblasts were seeded at different densities and transplanted instantaneously or after a precultured period of 10 days in the dermal substitute. This relatively short culture period was chosen because of the limited time available for cell amplification in a clinical setting. For allografted substitutes, long pre-culture periods are employed to increase the numbers of fibroblasts, the deposition of ECM, and the amount of growth stimulatory proteins in the substitute.[16,22] The general hypothesis is that wound healing is accelerated by the presence of more fibroblasts and ECM in the dermal substitute. However, in earlier experiments we found that high numbers of fibroblasts in the early granulation tissue were associated with the presence of higher numbers of myofibroblasts, which finally resulted in wound healing with more wound contraction.[28,30]

In this study, we used the porcine full-thickness wound model and an elastin/collagen dermal matrix as dermal substitute.[31] One week and 3 weeks post-wounding, the cellularity of the granulation tissue and the presence of ECM were scored, and the percentage of dermal area containing myofibroblasts was determined. Six weeks post-wounding, we investigated ECM remodelling, maturation of collagen, and elastin regeneration, and then correlated the histological data with macroscopic appearance and contraction results.

Materials and methods

Preparation of dermal substitute grafts

After 1 week of acclimatization and 4 to 6 weeks prior to operation, dermal fibroblasts were isolated from 5-10 punch biopsies (4 mm) from each pig. The epidermis was cut off, dermal tissue was minced and digested for 1-2 h at 37°C in a phosphate buffered saline solution (PBS) containing 0.25% (w/w) collagenase A and dispase (Boehringer Mannheim, Mannheim, Germany) (1ml/biopsy). The digest was sieved

over a 70 μm cell strainer (Falcon, Becton Dickinson, Mountain View, CA), washed in culture medium and subsequently cultured in DMEM supplemented with 10% (v/v) FCS, 1 mM L-glutamine and antibiotics (penicillin (100 IU/ml), streptomycin (100 mg/ml)) (all from Life Technologies, Breda, The Netherlands). The dermal substitute was a non-cross-linked native bovine collagen matrix (type I collagen from bovine skin) coated with a 3% (w/w) α-elastin hydrolysate (80% MW 60 kDa and 20% MW 200 kDa from bovine ligamentum nuchae) and was kindly provided by Dr. P. Tewes-Schwarzer (Dr Otto Suwelack Nachf. GmbH & Co., Billerbeck, Germany). Dermal substitutes were seeded with cultured porcine fibroblasts by inoculating the upper side of the pre-moistened matrices with culture medium containing the fibroblasts (1 ml/10 cm^2 of substitute). The seeding volume was absorbed by the dermal substitute. Two groups were treated with dermal substitutes seeded 10 days before the operation with $1x10^5$ and $5x10^5$ fibroblasts/cm^2 (10-DS10 and 10-DS50) respectively. The dermal substitutes were cultured in petri-dishes (non-TC-coated; d=20 cm) and 4 h after seeding 20 ml of medium supplemented with 25 mg/ml ascorbic acid (Sigma, St.Louis, MO) was added. Ascorbic acid was added to stimulate the deposition of collagens and glycosaminoglycans.[32,33] The media were changed every day and supplemented with freshly prepared ascorbic acid. The other two groups were treated with dermal substitutes seeded with $1x10^5$ and $5x10^5$ fibroblasts/cm^2 (0-DS10 and 0-DS50) 4-16 h prior to grafting. As controls, the dermal substitutes without cells (ADS) were also incubated in culture media for 4-16 h. For each group, extra dermal substitutes were prepared which were fixed on the day of operation with 4% paraformaldehyde PBS solution for 16 h at RT.

Operation and grafting procedures

The protocol was approved by the University of Amsterdam Committee of Animal Welfare and five female Yorkshire pigs (15 kg at arrival) were included in the study. Before each surgical procedure, wound evaluation and bandage changing, the pigs were sedated by intramuscular injection with azaperon 4 mg/kg (Stressnill^R, Janssen-Cilag, Gent, Belgium). Complete anesthesia was induced with a face mask with 5% isoflurane and a 50:50% mixture of nitrous oxygen and oxygen. Anesthesia was maintained with 1%-2% isoflurane and the same air mixture. If necessary post-operative pain was treated with a subcutaneous injection containing flunixine (50 mg/50 kg/day ; Finadyne^R, Schering-Plough, Segré, France).

For the operation in which the full-thickness wounds were created, 0.01 mg/kg atropine was included as additional sedative and to loosen muscle tension. After

complete anesthesia was induced, animals were intubated and artificial respiration was applied. During operation, all vital functions were monitored and fluid loss was compensated by an intravenous infusion of Ringers solution. Anesthesia was antagonized with 0.005 mg/kg sufentanil (Sufenta^R, Janssen-Cilag, Gent, Belgium) and postoperative analgesia was provided with 0.05 mg/kg buprenorphine (Temgesic^R, Reckitt & Colman Products Ltd, Hull, UK), both administered intravenously.

One week before the operation, the hair was clipped from both flanks and the skin was disinfected with hibiscrub, 70% alcohol solution containing 0.6% chlorohexidin, and with 2% iodine solution. A grid was tattooed by cutting the skin with a scalpel till subepidermal depth and applying tattoo paste. On the day of the operation, the skin was disinfected as described above and full-thickness wounds (3.0 x 3.0 cm) were created on the back of the pigs using a dermatome. First split-skin mesh grafts (0.2 mm thick) were harvested from the wound sites, moistened in PBS, and expanded at a ratio of 1:3. The wounds were re-excised to a depth of 2.5 mm up to the subcutaneous fat layer. In total, 14 wounds per animal were created, which were grafted with the dermal substitutes and covered with the split-skin mesh grafts. The wounds were protected against dehydration and bacteria with a polyether urethane top layer (Exkin^R, X-Flow, Twente, The Netherlands). Protection against mechanical trauma was achieved by wound coverage with one layer of hydrophilic gauzes (5x5 cm) fixed with adhesive tape, two layers of hydrophilic gauzes (20x20 cm) fixed with elastic adhesive tape from the back to the midriff/abdomen, and elastic stockings (Tubigrip). The pigs were housed individually and fed twice a day. At the moment of the operation the pigs weighed 40-45 kg and 6 weeks later 75-85 kg.

Evaluation of wound healing

The wounds were evaluated weekly. Wound contraction was followed by tracing the wound edges and the tattooed grid on transparent film. Wound contraction was measured by planimetry and expressed as percentage of reduction of original wound area and was corrected for the local growth of the animal. The final wound evaluation included a blinded score for three wound cosmetic parameters by two independent experienced observers. They scored on a 1-5 scale wound colour (pink - purple/red), smoothness and skin level of the epidermis, and wound suppleness or stiffness. After 1 and 3 weeks, 4 mm punch biopsies were taken from similar locations in each wound. After 6 weeks, a cross biopsy was taken from the middle of the wounds (0.5 x 4.0 cm). The biopsies were fixed in 4% formaldehyde PBS solution for at least 16 h at RT, and subsequently embedded in paraffin according to standard procedures.

Immunohistochemistry, image analysis, quantification of dermal regeneration

For immunohistochemistry, the 4% formaldehyde fixed wound biopsies were embedded in paraffin and sectioned at 5 μm. The sections were deparaffinized, hydrated through a graded series of ethanol solutions, and incubated for 30 min in 0.02% H_2O_2/methanol solution. Sections were subsequently incubated at RT with the following four solutions: (1) PBS solution/10% AB serum for 15 min; (2) anti-α-smooth-muscle actin monoclonal antibody (clone 1A4, Dako) diluted in PBS (1:100) or anti-elastin monoclonal antibody (1:1000, Sigma) for 60 min; (3) with biotinylated rabbit anti-mouse IgGs antibodies (1:400, Dako) diluted in PBS 10% AB serum for 30 min; (4) and with streptavidin-AB-complex/horseradish peroxidase (1:100, Dako) diluted in PBS for 30 min. Between these incubations, the sections were washed twice with PBS. Colour reaction was performed for 7 min in 50 mM Tris-HCl buffer (pH 7.8) containing 0.05% diaminobenzidine and 0.03% H_2O_2. Finally, the slides were washed in water, counter-stained with haematoxylin, mounted in glycergel (Dako) and examined with bright-field microscopy. Sections of normal porcine skin served as positive controls. As negative controls, adjacent sections of the wound biopsies were stained with nonimmune IgG from the same species in the same dilution as the primary antibody. No specific signal was noticed in the negative controls.

The dermal substitutes that were fixed on the day of operation were sectioned at 10 μm, deparaffinized, hydrated and stained for α-smooth-muscle actin as described above, except that for the second and third steps were replaced with an incubation with goat anti-mouse IgG2a antibodies conjugated with Texas Red (Southern Biotechnology Associates, Birmingham, AL). To identify nuclei, adjacent sections were stained with a 5 μg/ml propidium iodide PBS solution for 15 min. Slides were washed in PBS and mounted in vecta-shield.

Image analysis of sections stained for α-smooth-muscle actin was performed using white light, an infrared-blocking filter, and a low-magnification objective (2x). The sections of the dermal substitute, which were stained for α-smooth-muscle actin and cell nuclei were analysed with a 10x objective. Images were recorded with a colour CCD camera (1024x1280@24 bits) attached to an Olympus AHBT3 microscope (Tokyo, Japan) and saved as a TIF file. The images were analyzed blinded with the Leica Qwin-colour analysis software package (Leica Imaging Systems Ltd., Cambridge, UK). For the α–smooth-muscle actin staining, the total dermal area and the area with myofibroblasts were measured. Subsequently, the staining intensity in the area with myofibroblast was measured as the percentage of stained area relative to the total

positive dermal area. Colour selection was performed by exclusion of the highest colour intensities, resulting in the selective loss of most of the vascular staining. For an estimate of the numbers of cells in the dermal substitutes, images of fluorescent nuclei of one dermal substitute were merged together, giving a complete overview of the cross-sectioned dermal substitute. This image was analysed for the total number of fluorescent areas corresponding to the size of a nucleus.

Six weeks post-wounding, the maturation of collagen fibres was analysed with polarized light in H&E stained wound sections. The thickness of the regenerated tissue was measured at 4 different places at similar intermediate distances with a microscope with built-in calliper. The area with mature collagen fibres was expressed as the percentage of the total regenerated dermal tissue. The elastin stainings at six weeks were measured and analysed as described above using bright-light microscopy.

Statistical analysis

The different treatments were all applied at one side of each animal in a randomized fashion. The treatments were tested for significant differences with a paired Student's *t*-test, except for the evaluation of cosmetic parameters, for which the non-parametric Wilcoxon signed rank test was used. Correlation and correlation significance were tested with the Spearman Rank correlation test. $P<0.05$ was considered to be statistically significant.

RESULTS

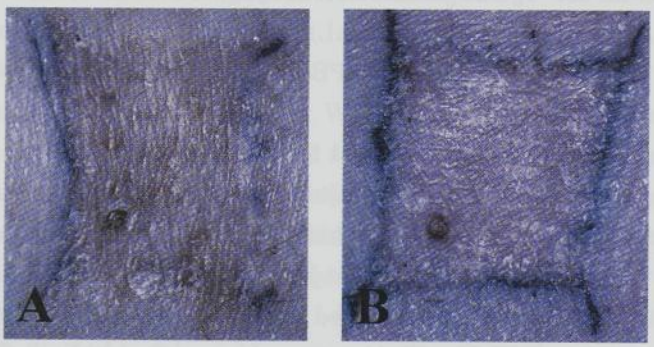

Figure 1. Appearance of wounds treated with ADS (acellular dermal substitute) (**A**) and with 10-DS50 (dermal substitute seeded with $5x10^5$ fibroblasts/cm^2, precultured for 10 days) (**B**), six weeks post-wounding. The ADS-treated wound had contracted more, showed a rougher skin surface, and was not uniform in wound colour (red areas). The colour of the 10-DS50-treated wound was almost comparable to the surrounding skin.

Macroscopic evaluation of wound healing

For all wounds the epithelium out-growth from the split-skin mesh grafts was similar and the wounds closed within two weeks. The wound colour changed from initially purple/red to pink, a colour comparable to the surrounding skin. The speed of wound-colour transition was different for the treatment groups. In general, the wounds treated with the cultured dermal substitutes (10-DS10, 10-DS50) and dermal substitutes seeded with the highest concentration of fibroblasts (0-DS50) became sooner pink. In addition, the wound tissue was more supple, and the epidermal upper layer was smoother compared to treatment with the acellular dermal substitute (ADS; controls). Representative examples for the ADS (A) and 10-DS50 (B) treatments are shown in figure 1.

Six weeks post-wounding, these different parameters (wound colour, skin elasticity, and surface smoothness) were scored blinded on a scale of 1-5 (good - worse) by two experienced observers. The scores were totalled for each wound, and averaged per treatment group. The best score for a wound was 3, whereas the wounds which healed the worst could have a maximal score of 15. The latter, however, did not occur, and the maximum score given for a wound was 12. The average scores for the different treatment groups are shown in figure 2A. The 10-DS50 and 0-DS50 treatments showed a significant improvement in cosmetic appearance compared to the ADS treatment ($p<0.03$, unpaired Wilcoxon rank test). The cosmetic parameters of wounds treated with the 0-DS10 were comparable to those of wounds treated with ADS.

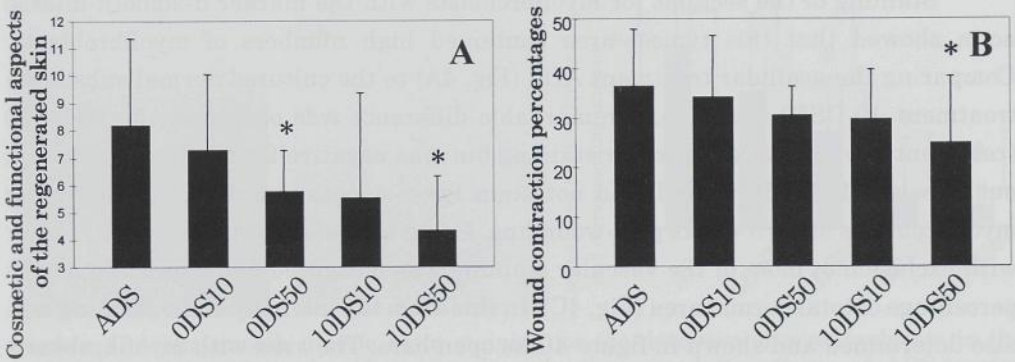

Figure 2. The average cosmetic scores and average wound contraction per treatment after 6 weeks of healing. **A**. The 0-DS50 and 10-DS50 wounds scored significantly better for cosmetic appearance compared to those treated with ADS ($*p<0.04$). **B**. The 10-DS50 treatment significantly diminished wound contraction compared to the ADS treatment ($*p<0.05$). SD is indicated by bars.

The percentage of contraction was determined as reduction in original wound area and was corrected for the local growth of the pigs. Wound contraction started after 5 to 7 days, was most pronounced for another two weeks, and continued slowly or was arrested after 3 weeks. After 6 weeks, the wound contraction for the 10-DS50 treatment was 24.8 (SD±13.4%) and was significantly reduced compared to the ADS treatment with an average contraction of 36.4 (SD±11.4%) (Fig. 2B).

Histological observations after one and three weeks of healing

After 1 and 3 weeks, 4 mm biopsies were taken from the wounds. One week post-wounding, most wounds showed a completely infiltrated dermal substitute in the H&E stained wound sections. Although the cell densities varied, this could not be attributed to one specific treatment. After 3 weeks, a clear difference in cell density was observed between treatments. For the cultured dermal substitutes 10-DS10, 10-DS50 and 0-DS50, the granulation tissue had not only a lesser cell density but it also contained more ECM in between the cells when compared to the ADS and 0-DS10 treatments. In figure 3, this is illustrated for the ADS (A and C) and the 10-DS50 treatments (B and D). In addition, the thickness of the granulation tissue was especially thick in wounds which showed the most contraction. Furthermore, in the middle of the granulation tissue of the ADS and 0-DS10 treatments and to a lesser extent for the 0-DS50 and 10-DS10 treatments an area in the section was observed with cells orientated parallel with the epidermis. This area was absent in the wounds treated with 10-DS50.

Staining of the sections for myofibroblasts with the marker α-smooth-muscle actin showed that this typical area contained high numbers of myofibroblasts. Comparing the acellular treatment ADS (Fig. 4A) to the cultured dermal substitute treatment 10-DS50 (Fig. 4B), a remarkable difference was observed: the 10-DS50 treatment showed strong vascular staining but was negative for myofibroblasts. Six out of seven 10-DS50 wounds did not stain for α-smooth-muscle actin containing myofibroblasts at all 3 weeks post-wounding. Using image analysis, the positive area with exclusion of most of the vascular staining was measured and expressed as the percentage of total dermal area (Fig. 4C). In this area, the intensity of the staining was also determined and shown in figure 4C as open bars. The area with myofibroblasts positive for α-smooth-muscle actin was significantly reduced for the cultured dermal substitute treatments (10-DS10 and 10-DS50, $p<0.001$) and the 0-DS50 treatment ($p<0.04$) compared to the acellular treatment (ADS). In addition, as the size of the positive area diminished, the intensity of the staining in the area also decreased. The

percentage dermal area containing myofibroblasts correlated significantly with the percentage of wound contraction after 6 weeks of healing (correlation coefficient of 0.72, $p<0.001$, Spearman Rank correlation test).

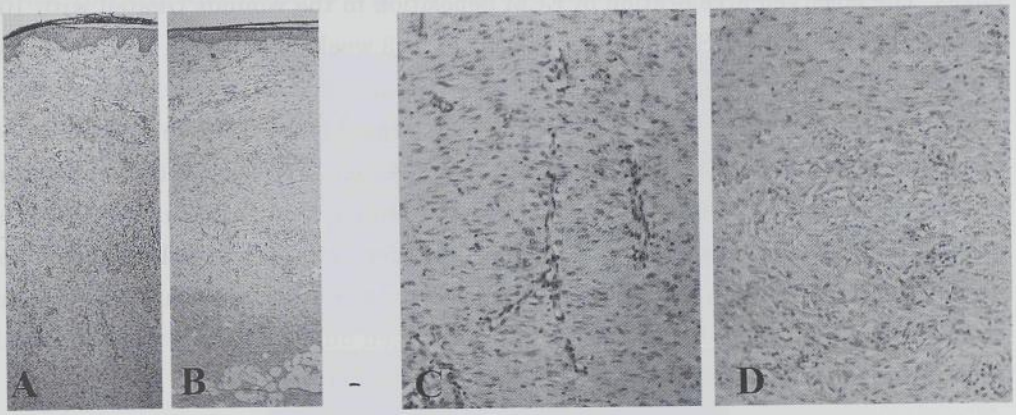

Figure 3. Granulation tissue of wounds treated with ADS (**A, C**) and 10-DS50 (**B, D**) substitutes, 3 weeks post-wounding. In the ADS-treated wounds more granulation tissue was formed with higher cell densities and less deposition of ECM than in the 10-DS50-treated wounds. Underlying subcutaneous fat is indicated with arrowheads and is not visible in A due to the thickness of the granulation tissue. A and B are shown at identical magnifications (x 55), C and D show a 2.5-times higher magnification of the granulation tissue in figure A and B, respectively.

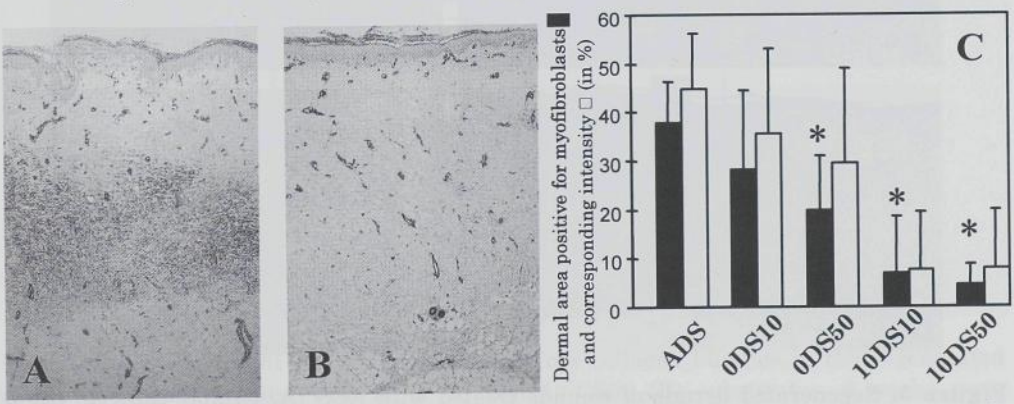

Figure 4. Alpha-smooth muscle actin staining for the detection of myofibroblasts in the granulation tissue, 3 weeks after wounding. In the granulation tissue of ADS-treated wounds (**A**) a clear area with myofibroblasts was detected, whereas in the 10-DS50 wounds only smooth muscle cells in vascular structures stained positive (**B**). (**C**) Percentage of dermal area with myofibroblasts determined with image analysis. Open bars indicate the intensity of the staining in the areas positive for myofibroblasts. The wounds treated with 0-DS50, 10-DS10, and 10-DS50 had significantly less dermal area containing myofibroblasts compared to the ADS treated wounds ($p<0.04$). SD is indicated by bars.

Histological observations after six weeks of healing

After 6 weeks of healing, tissue (1 cm in width) was dissected from the middle of the wound, to give a complete view of the regenerated tissue in between the wound edges. The observed acceleration of ECM deposition in the wounds treated with 10-DS10, 10-DS50 and 0-DS50 dermal substitutes at 3 weeks resulted in a regenerated tissue with thicker collagen bundles organised with a basket weave pattern as in normal skin at 6 weeks (Fig. 5B, D). This regenerated dermal tissue could only be distinguished from normal skin by a slightly increased cell density and the presence of more vascular structures. In the wounds treated with ADS and 0-DS10, a granulating area in the middle of the regenerated dermal tissue was still present. This area (Fig. 5A (in between arrowheads), C) was characterised as immature granulation tissue with a high cell density and with thin collagen bundles having a preferential orientation parallel with the epidermis. This is typical of scar tissue.

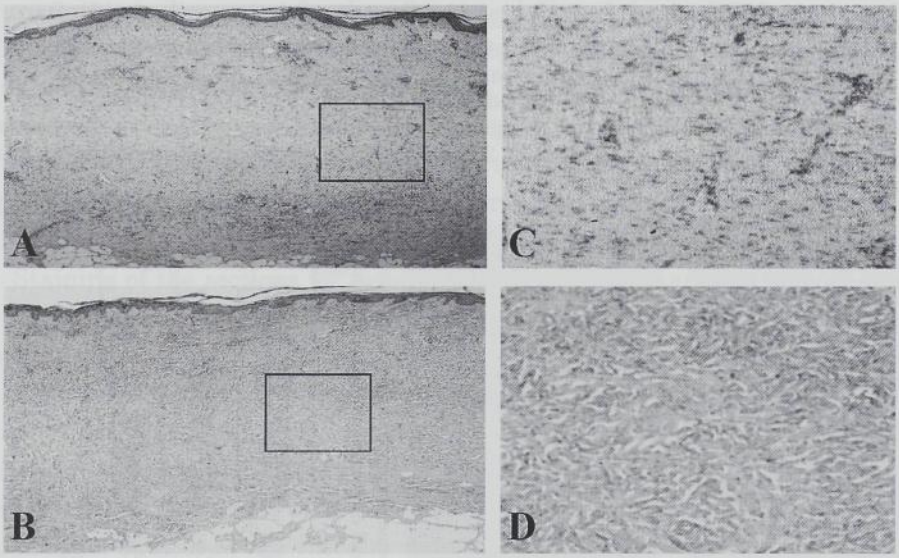

Figure 5. Regenerated dermis of wounds treated with ADS (**A, C**) and 10-DS50 (**B, D**) substitutes, 6 weeks post-wounding. Regenerated dermis of the ADS-treated wounds contained more cells and especially in the middle of the dermis (area between arrowheads) had less remodelled ECM than the 10-DS50-treated wounds. (C) and (D) 2.5-times higher magnification of insets in A and B, respectively, clearly demonstrating in the ADS treated wound preferential organisation of cells and ECM parallel with the epidermis. Original magnification of figure A and B: 35x

From investigations with polarized light on the maturation of the collagen bundles, it was evident that this area contained little to no mature collagen bundles (Fig. 6A, between arrowheads). The regenerated dermis of the wounds treated with 0-DS50, 10-DS10 and 10-DS50 (Fig. 6B) showed almost only mature collagen bundles.

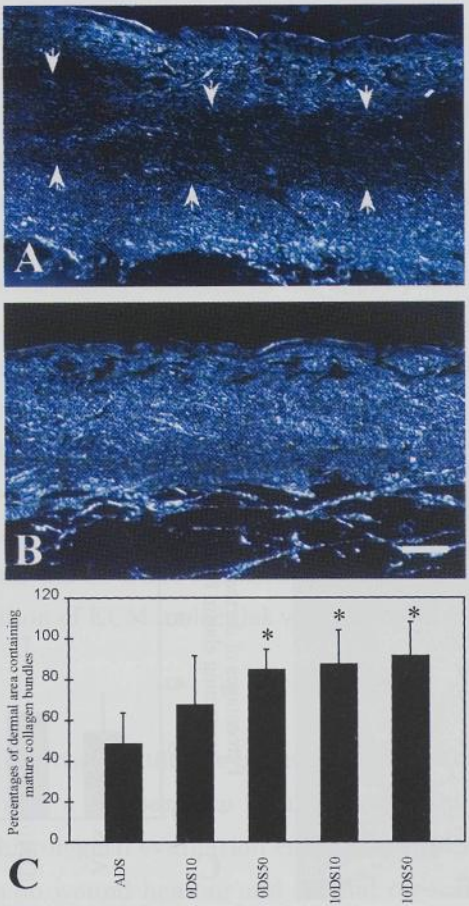

Figure 6. Polarized light image identifying mature collagen bundles in the regenerated dermis of wounds treated with ADS (**A**) and 10-DS50 (**B**) substitutes, 6 weeks post-wounding. In the middle of the regenerated dermis of the ADS-treated wounds, an area with immature collagen bundles was present. This area was absent in the dermis of the 10-DS50 treated wounds, indicating faster regeneration and remodelling of the ECM in these wounds (bar = 160 μm). (**C**) For each group, the average percentages of dermal area containing mature collagen bundles is shown. The wounds treated with 0-DS50, 10-DS10, and 10-DS50 had significantly more dermal area with mature collagen bundles than the ADS-treated wounds ($p<0.05$). SD is indicated by bars.

Elastin staining also identified an area in the middle of the regenerated tissue negative for elastin (Fig. 7A and 7B, area between arrowheads), which was larger for the ADS treatment (Fig. 6A) than for the 10-DS50 treatment (Fig. 6B). The treatments 0-DS50, 10-DS10 and 10-DS50 showed significantly higher percentages of dermal area with mature collagen bundles and elastin in the regenerated tissue than the ADS treatment (Fig. 6C and 7C).

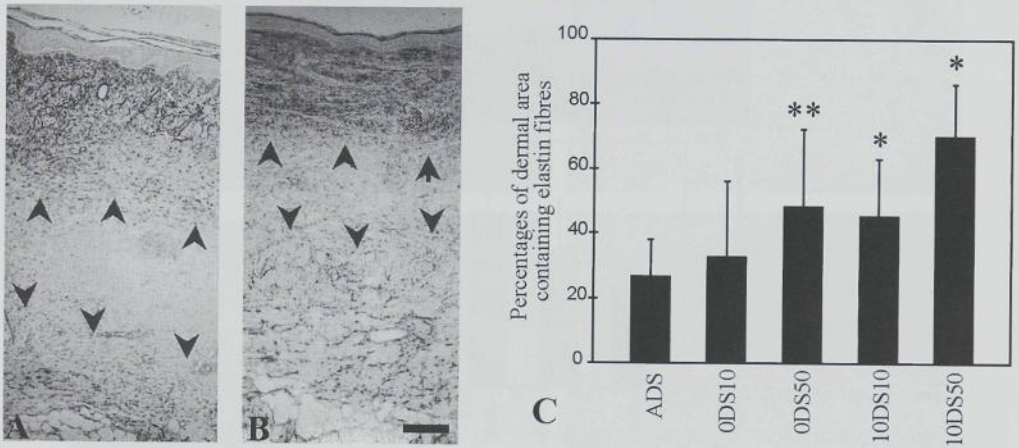

Figure 7. Elastin staining of the regenerated dermis of wounds treated with ADS (**A**) and 10-DS50 (**B**) substitutes, 6 weeks post-wounding. In the area between arrowheads elastin staining is absent. For the ADS treated wound this area was clearly larger than for the 10-DS50 treated wounds (bar = 260 μm). (**C**) For each group the average percentages of dermal area containing elastin staining is shown. The wounds treated with 0-DS50, 10-DS10, and 10-DS50 had significantly more dermal area with elastin staining than the ADS-treated wounds (**$p<0.05$, unpaired t-test; *$p<0.05$, paired t-test). SD is indicated by bars.

Fibroblast density measurements in cultured dermal substitutes

Additional prepared dermal substitutes with fibroblasts, which were fixed on the day of the operation (n=5 for each treatment group), were stained for nuclei with propidium iodine. The total fibroblast numbers were estimated with image analysis. The average number of fibroblasts found for the 0-DS10 substitute $1.05x10^6$ (SD±0.35) corresponded to the number of fibroblasts seeded in the substitute 4 to 16 h earlier. However, for the 0-DS50 substitutes a lower number $4.1x10^6$ (SD±0.75) was found than was seeded initially. Of the cultured substitutes, the 10-DS10 substitutes contained $4.9x10^6$ (SD±1.54) fibroblasts and the 10-DS50 substitutes $7.5x10^6$ (SD±1.26). Figure 8 illustrates the cellular distribution throughout the different dermal substitutes. The 0-DS10 and 0-DS50 showed fibroblasts attached to matrix fibres throughout the dermal substitute but with higher densities in the upper part. In the cultured dermal substitutes, fibroblasts were distributed more evenly indicating that they had migration into the dermal substitute. Staining of the dermal substitutes for α-smooth-muscle positive myofibroblasts showed variable percentages of positive fibroblasts which varied more between the different pigs than between the different dermal substitutes. The percentages of myofibroblasts were never higher than 30%. In addition, the cultured dermal substitute surface was somewhat reduced by contraction, i.e. 3% (SD±3%, n=12) for the 10-DS10 substitutes, and 6% (SD±4%, n=12) for the 10-DS50, and some deposition of ECM molecules was observed (not shown).

Discussion

Only a few studies investigated the influence of fibroblasts in skin substitutes on dermal regeneration in a comparative fashion taking into consideration wound contraction and other histological evaluation criteria. Murphy *et al.* (1990)[34] showed various positive effects on wound healing and dermal regeneration with a cultured collagen-glycosaminoglycan substitute containing autologous fibroblasts and keratinocytes in a guinea pig model, but the authors used open non-treated wounds as controls. Boyce *et al.* (1991)[35] used athymic nude mice to compare collagen-glycosaminoglycan substitutes with cultured human keratinocytes with and without human fibroblasts. For the wounds treated with substitutes with fibroblasts, they found positive effects on epidermal regeneration, but they did not see any improvement

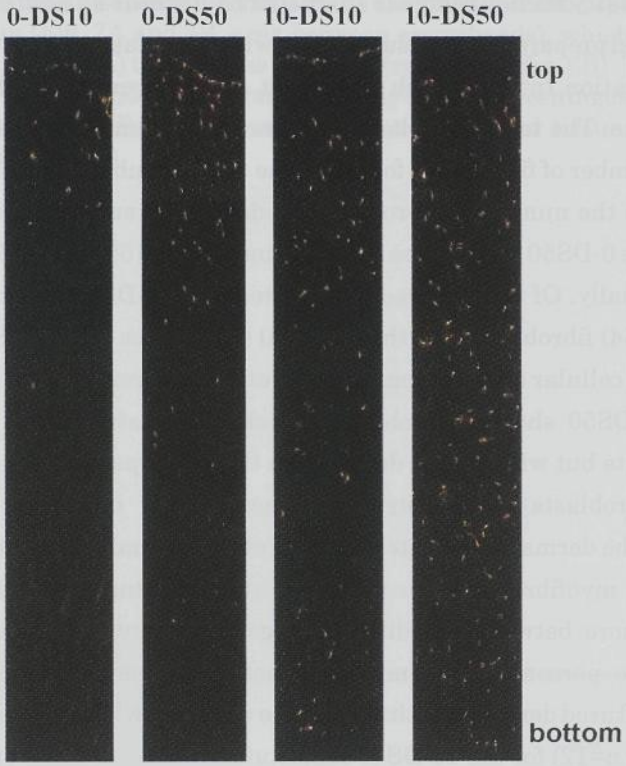

Figure 8. Distribution of fibroblasts on the day of operation within the seeded and pre-cultured dermal substitutes stained with propidium iodide to identify cell nuclei. Original magnification: 45x

on wound contraction and they did not investigate the quality of the regenerated tissue. In a guinea pig dermal wound model, Marks *et al.* (1991)[36] showed that the addition of fibroblasts to a collagen sponge ($2x10^4$ cells/cm^2, cultured for 1 week) accelerated the ultimate tensile strength of the wounds and the fibroblasts significantly retarded the collagen sponge biodegradation. However, up to the last time point of evaluation (30 days) they did not observe any positive effects on epithelialization or on collagen fiber diameter and orientation. In addition, the animal models used might not be very relevant to the human situation, since rodent skin does not have the same architecture as human skin. The porcine model we used is a more suitable model to study dermal regeneration.

In the past, the amplification of keratinocytes was considered to be the limiting step for a cultured skin graft.[22,37] Improved culture methods have made this more realizable. In addition, thin split-skin harvest methods have reduced the risk of creating cosmetically unattractive donor-sites. However, if for the creation of a cultured composite graft high numbers of fibroblasts are also required , the need for more donor-site tissue is likely to be associated with deeper donor-site defects. To overcome this problem, we investigated subcutaneous fat as a fibroblast source.[28] In a single-stage operation, subcutaneous fat was harvested, fibroblasts isolated, disposed of vascular fragments, and seeded at a density of $1x10^5$ cells/cm^2 in our dermal substitutes before they were implanted in full-thickness wounds. These dermal substitutes were able to improve tissue regeneration and healed with significantly less wound contraction (24.5% (SD±3.4%, n=13)) than to the wound contraction using acellular dermal substitutes (35.5% (SD±4.8%, n=15)). In spite of these promising results, augmenting the number of freshly isolated subcutaneous fibroblasts in the dermal substitute instead of cultured dermal fibroblasts reversed the positive effects observed. This was probably caused by impurities in the freshly isolated subcutaneous fibroblast population, e.g. inflammatory cells and collagen fragments (unpublished data).

The concept of a dermal equivalent seeded with fibroblasts seems to be a simple one. However, one has to consider that the fibroblast environment in a dermal equivalent may result in phenotypic changes[38], which might negatively influence the dermal regeneration process after transplantation. Fibroblasts can differentiate into a contractile phenotype, the myofibroblast, capable of rapid matrix contraction[39,40], or they might exhibit increased proteolytic activity once seeded in a dermal substitute.[41] In general, these processes are undesirable at the moment of skin grafting but may also be used to advantage. In the case of Dermagraft^R, the pre-culture of fibroblasts in the vicryl mesh caused degradation and replacement of the substitute material with fibroblast-synthesized ECM. This resulted in a reduction in the inflammatory reactions occurring in vivo with hydrolysis of the vicryl material.[16] Another point to consider is that prolonged culture periods could also result in a filling of the pores of the dermal substitute. This could eventually inhibit graft vascularisation and/or interfere with epidermal graft survival due to limited wound-fluid exchange and reduced diffusion

of nutrients to the keratinocytes. The acellular dermal substitute used in the present study allowed fast vascularisation[42] and the pre-culture of fibroblasts in the dermal substitute did not impede this. In comparison to the total amount of dermal substitute, the amounts of ECM deposited by the fibroblasts in the 10-DS10 and 10-DS50 substitutes were low and the contribution of these proteins to the observed accelerated dermal regeneration is likely to be small. Moreover, in vitro studies with substitutes cultured under similar circumstances but seeded with $1x10^6$ fibroblasts/cm^2 and cultured for 7 days showed a decrease of 9% in total hydroxyproline content, indicating some degradation of the substitute.[43] In addition, in vitro we observed some reduction in the areas of the 10-DS10 and 10 DS50 substitutes (3% and 6%, respectively). Staining for α-smooth-muscle actin positive myofibroblasts in the dermal substitute showed a variable percentage of positive cells which varied most between animals and not so much between instantaneously seeded or pre-cultured dermal substitutes. This was previously observed by Desmoulière *et al.* (1992)[44] in different fibroblast populations cultured on plastic (passages 4 and 5). Normally, the myofibroblast characteristics are lost during culture[45], but the number of passages used in our present study (p4-p6) is probably too low to achieve this.

The improved tissue regeneration with the cultured dermal substitutes seems to be correlated with the higher numbers of fibroblasts present in the dermal substitutes at the moment of transplantation. This is reinforced by similar fibroblast numbers found for the 0-DS50 and 10-DS10 treatments and their comparable wound-healing results. For the 10-DS50 treatment the fibroblast numbers only doubled during the 10-day culture period. Moreover, after the seeding of $5x10^5$ fibroblasts/cm^2 (0-DS50) almost 20% of the initial cell numbers was lost and during the culture period fibroblasts migrated out of the dermal substitute. Therefore, it is not advisable to culture such high cell numbers in a dermal substitute for 10 days. In order to minimize the loss of fibroblasts, one could seed lower cell numbers several times and culture them for a shorter period. In addition, it is also possible that higher cell numbers than found in the 10-DS50 substitutes improve dermal tissue regeneration even more and reduce wound contraction further.

The culture period could be important for the fibroblasts to adapt to their new environment and to firmly attach to the substrate. In this, the elastin coating of the

dermal substitute might be important. Fibroblasts are able to adhere strongly to elastin and receptor binding alters the cell metabolism[46]. Furthermore, elastin is capable of modulating proteolytic activity[47] and elastin peptides stimulate the growth of skin fibroblasts.[48] Moreover, we previously showed that the elastin component and fibroblast seeding retarded biodegradation of the substitute.[30,47]

Three weeks after healing, the wounds treated with 10-DS50 showed almost no staining for myofibroblasts positive for α-smooth-muscle actin. At this time point, the 10-DS50 wounds already had contracted by 20% and it can not be excluded that myofibroblasts were present during the first two weeks of healing. However, our definition of wound contraction is the percentage of original wound area after being corrected for local growth of animal. If the latter correction was not made, the 10-DS50 wounds showed no contraction of the original wound area and the 20% wound contraction could then also be considered to represent retarded growth of the wound tissue. The remarkable inhibition of fibroblast differentiation might be caused by several factors, e.g. reduced inflammatory signals and proteolytic wound activity. Nevertheless, apart from several growth factors[24,50], it is still not clear how components in the direct surroundings of fibroblasts induce myofibroblast differentiation. We believe that the early presence of fibroblasts in the dermal substitute might reduce the need of fibroblasts to migrate into the wounds from the subcutaneous tissue[28]. Concurrently, it might be that migrated fibroblasts encounter more activating signals which render these fibroblasts more susceptible to differentiation.

After three weeks, a typical area appeared in the middle of the granulation tissue of wounds which healed with more wound contraction. This area was characterized by high cell densities, preferential cell organisation parallel with the epidermis, and being positive for α-smooth-muscle actin. The existence of this area seemed to correlate with thicker layers of granulation tissue, which in part can be explained by the increased contraction of the wound tissue. This typical area remained present until six weeks after wounding, by which time most cells had disappeared and were replaced by thin immature collagen bundles, again organised parallel with the epidermis. This area remained negative for elastin and might evolve into scar tissue.[51] With the 10-DS50 treatment, this typical area was not observed, and after 6 weeks the regenerated dermis consisted almost completely of randomly organised mature

collagen bundles. Although significantly more elastin was present in the regenerated tissue of the 0-DS50, 10-DS10 and 10-DS50 wounds than in the ADS wounds, the elastin fibres were not yet completely regenerated. However, this observation may be due to the relatively short period of our evaluation.

In conclusion, we demonstrated that the cosmetic results of wound healing correlated with histological observations. Wounds with more myofibroblast formation healed with more contraction, showed more scar formation, were not supple, and remained more reddish in colour. The best healing was observed with a dermal substitute seeded with a high number of fibroblasts and cultured for 10 days prior to grafting. Furthermore, the accelerated and improved regeneration of dermal tissue was correlated to the higher number of fibroblasts present in the dermal substitute at the moment of grafting.

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Chapter 5

Allogeneic Fibroblasts in Dermal Substitution Induce Inflammatory Responses and Interfere with Dermal Tissue Regeneration

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Submitted for publication

Abstract

In the field of skin transplantation, dermal substitution with inclusion of connective-tissue synthesizing fibroblasts is a main objective. The use of allogeneic fibroblasts instead of autologous fibroblasts is more practical in the preparation of an "off-the shelf' skin substitute. In this study, we compared different allogeneic fibroblast populations with autologous fibroblasts in dermal skin substitution.

Three allogeneic fibroblast populations were isolated from a female pig bred under *spf* conditions (SPF fibroblasts), and from a female and a male pig (female and male fibroblasts) of the same breeding strain as two female acceptor pigs (autologous fibroblasts). The histocompatibility of the three donor pigs with the two acceptor pigs was tested with a Mixed Lymphocyte Reaction (MLR). Full-thickness wounds on 2 pigs were treated with dermal substitutes seeded with four different fibroblast populations (n=5) and transplanted in combination with split-skin mesh grafts. Six weeks after transplantation, wound contraction was measured by planimetry and wound cosmetic parameters were evaluated by two independent observers in a blinded fashion. At 2, 4 and 6 weeks biopsies were taken from the wounds. Histology was evaluated for the presence of inflammatory responses, the quality of dermal tissue regeneration, and the progress of ECM remodelling and collagen-bundle maturation.

The MLRs of both acceptor pigs showed the highest responses to peripheral blood mononuclear cells (PBMCs) of the SPF pig, and were low or negative to the PBMCs of the female and male donor pigs. Cosmetic appearance and wound contraction showed significantly better results for the wounds treated with autologous fibroblasts compared to wounds treated with SPF fibroblasts. The wound histology showed in all wounds treated with allogeneic fibroblasts an increased presence of inflammatory cells throughout the six weeks of the experiments. This inflammatory response was characterized by the presence of multiple foci with mixed lymphocytic and granulomatous inflammatory cells. In addition, the increased inflammatory responses retarded dermal tissue regeneration, remodelling of the ECM, and maturation of the newly formed collagen bundles. Six weeks after transplantation, the wounds treated with allogeneic fibroblast populations had dermal areas still granulating with thin collagen bundles organised parallel with the epidermis as in scar tissue. In contrast, the wounds treated with autologous fibroblasts showed a dermal tissue with mature collagen bundles organised randomly as in normal skin.

We concluded that for optimal restoration of dermal skin function without scar formation, the use of skin equivalents with autologous fibroblasts is to be preferred over skin equivalents with allogeneic fibroblasts.

Introduction

Ever since the first creation of a living-skin substitute by Bell et al. (1), much effort has been made to create an "off-the-shelf' skin replacement. The most practical choice would be the use of allogeneic cells. The advantages over the use of autologous cells are in eliminating patient donor sites, decreasing operating time, and avoiding a delay in treatment required for autologous cell culture and multiplication (2,3). During the last two decades, the attention was mainly focussed on cultured keratinocyte grafts and their application on burns and chronic ulcers (4). The results from these studies showed that keratinocyte allografts not only accelerate healing but also decrease the pain in both split-thickness skin graft donor sites and chronic ulcers (5,6). In addition, it became evident that allogeneic keratinocytes do not survive and are replaced by autologous keratinocytes (7,8). However, controversy still exists as to how long allogeneic keratinocytes survive (3). In immuno-competent animals, keratinocyte survival appeared to be relatively short (< 1 week) (9), whereas in patients with large burns a degree of immunosuppression exists which might favour prolongation of allogeneic cell survival (10,11). Furthermore, take and survival of allogeneic keratinocytes seem to be enhanced in the presence of dermis or a dermal equivalent (5,11,12). With respect to dermal substitution, it is commonly accepted that cultured keratinocyte grafts alone are insufficiently effective and that for adequate wound treatment both skin components, i.e. dermis and epidermis, are needed.

In developing a dermal equivalent, there are several reasons to include fibroblasts. The presence of fibroblasts in dermal equivalents not only stimulates keratinocyte outgrowth, differentiation and basement membrane regeneration (13-15), but is also likely to accelerate dermal tissue regeneration (16,17). An important question remained to be answered: 'can allogeneic fibroblasts be used and are they as beneficial for wound healing as autologous fibroblasts?' This is also clinically important, especially since commercial skin equivalents containing allogeneic fibroblasts are being developed and emerge onto the market.

In the literature, the data are not conclusive regarding the immunogenicity of human fibroblasts (18-22), and whether or not they are able to persist in newly regenerated skin or are replaced by host cells without adverse effects on tissue regeneration (2). In most clinical studies it is difficult to evaluate the effects of allogeneic fibroblasts due to the small numbers of patients evaluated, their multiple wound variables, and the lack of histological data and controls. The purpose of this

study is therefore to compare the use of allogeneic fibroblasts populations to the use of autologous fibroblasts in dermal substitution using a porcine full-thickness wound model. For the allogeneic fibroblast populations we chose two populations of female, and male origin genetically closely related to the acceptor animals and one population of female origin (SPF) that was less related. The genetical relationship between donors and acceptors was investigated with Mixed Lymphocyte Reactions. In the wounds treated with dermal substitutes seeded with the different groups of fibroblasts, we investigated inflammatory reactions in the granulation tissue and the quality of the dermal tissue which was regenerated after six weeks.

Materials and methods

Mixed Lymphocyte Reaction (MLR)

Heparinized peripheral blood from the pigs, from which the different fibroblasts populations were isolated, was layered on a density gradient (Lymphoprep, Nycomed, Oslo, Norway) and centrifuged to remove erythrocytes and granulocytes. The interface was washed 3 times with PBS solution containing 1% BSA to remove thrombocytes. Since the time-point of isolation was different for each pig, peripheral blood mononuclear cells (PBMCs) were cryopreserved in FCS containing 10% DMSO in liquid nitrogen until use. To measure the MLR response, $2x10^4$ allogeneic PMBCs were incubated with $2x10^4$ autologous PBMCs for 6 days in round-bottom 96-wells plates. Cells were cultured in 200 µl DMEM media containing 10% FCS and 50 µM ß-mercapto-ethanol. The numbers of proliferating cells were determined by adding 0.3 µCi ^{3}H-thymidine (Amersham, Aylesbury, UK) to each well sixteen hours before harvesting. Incorporation was measured by liquid scintillation counting in average disintegrations per min (DPM). As controls, $2x10^4$ PMBCs of each animal were incubated separately to measure background proliferation.

Preparation of dermal substitute grafts

The allogeneic and autologous fibroblasts were isolated from skin biopsies taken from the backs of the pigs. Two allogeneic fibroblast populations were from a female

and male pig (**female** and **male**) of the same breeding strain as the acceptor animals from which the **autologous** fibroblasts were isolated. The third allogeneic fibroblast population was isolated from a female pig of a different breeding strain which was bred under *spf* conditions (**SPF** fibroblasts). From all tissue biopsies epidermis was cutt off, dermal tissue was minced and digested for 1-2 hrs at 37°C in a phosphate buffer solution (PBS) containing 0.25% (w/w) collagenase A and dispase (Boehringer Mannheim, Mannheim, Germany) (1 ml/biopsy). The digest was sieved over a 70-μm cell strainer (Falcon, Becton Dickingson, Mountainview, AL), washed in culture media and subsequently cultured in DMEM supplemented with 10% (v/v) FCS, 1 mM L-glutamine and antibiotics (penicillin (100 IU/ml), streptomycin (100 mg/ml)) (all from Life Technologies, Breda, The Netherlands). Fibroblasts were passaged with PBS solution containing 0.25% trypsin and seeded in the dermal substitute the day before operation at an identical density ($5x10^5$ fibroblasts/cm^2) between passages 4 and 6. Dermal substitutes were seeded by inoculating the upper side of the pre-moistened matrices with culture medium containing the fibroblasts (1 ml/10 cm^2 of substitute). The seeding volume was absorbed by the substitute. The dermal substitute was a non-cross-linked native bovine collagen matrix (type I collagen from bovine skin) coated with a 3% (w/w) α-elastin hydrolysate (80% MW 60 kDa and 20% MW 200 kDa from bovine ligamentum nuchae) and was kindly provided by Dr. P. Tewes-Schwarzer (Dr Otto Suwelack Nachf. GmbH & Co., Billerbeck, Germany) (23). For each group extra substitutes were prepared which were fixed on the day of operation in 4% paraformaldehyde PBS solution for 16 h at RT.

Operation, grafting, and bandaging procedures

The protocol was approved by the University of Amsterdam Committee of Animal Welfare. Two female Yorkshire pigs (15 kg at arrival) were included in the study. Animal anaesthetics: before each surgical procedure, wound evaluation and bandage changing, the pigs were sedated by intramuscular injection with azaperon 4 mg/kg (Stressnill, Janssen-Cilag, Gent, Belgium). Complete anesthesia was induced with a face mask with 5% isoflurane and a 50:50 mixture of nitrous oxygen and oxygen (3-5 L/min). Anesthesia was maintained with 1%-2% isoflurane and the same air mixture. If necessary, post-operative pain was treated with a subcutaneous injection containing flunixine 50 mg/50 kg/day (Finadyne^R, Schering-Plough, Segré, France).

Before the operation, in which the full-thickness wounds were created, 0.001 mg/kg atropine was included as additional sedative. After complete anesthesia was induced, animals were intubated and artificial respiration was applied. During the operation, vital functions were monitored and fluid loss was compensated by an intravenous infusion with Ringers solution. This anaesthesia was antagonised with 0.005 mg/kg sufentanil (Sufenta^{R}, Janssen-Cilag, Gent, Belgium) and post-operative analgesia was provided with 0.05 mg/kg buprenorphine (Temgesic^{R}, Reckitt & Colman Products Ltd, Hull, UK), both administrered intravenously.

One week before the operation, the hair was clipped from both dorsal flanks and the skin was disinfected with i.e. hibiscrub, 70% alcohol solution containing 0.6% chlorohexidin, and with 2% iodine solution. A grid was tattooed by cutting the skin with a scalpel till subepidermal depth and applying tattoo paste. On the day of the operation, the skin was disinfected as described above and full-thickness wounds (2.5 x 2.5 cm) were created on the back of the pigs using a dermatome. First split-skin mesh grafts (0.2 mm thick) were harvested from the wound sites, moistened in PBS, and expanded at a ratio of 1:3. The wounds were re-excised to the subcutaneous fat layer. The wounds were grafted with the fibroblast-seeded dermal substitutes and covered with the split-skin mesh grafts. Each treatment group of grafts was applied 5 times on both animals in a randomised fashion. The wounds were protected against dehydration and bacteria with a polyether urethane top layer (Exkin^{R}, X-Flow, Twente, The Netherlands). Protection against mechanical trauma was achieved by wound coverage with one layer of hydrophilic gauzes (5x5 cm) fixed with adhesive tape, two layers of hydrophilic gauzes (20x20 cm) fixed with elastic adhesive tape from the back to the midriff/abdomen, and elastic stockings (Tubigrip). The pigs were housed individually and fed twice a day. At the moment of the operation the pigs weighed 30-35 kg and after 6 weeks 70-75 kg.

Macroscopical evaluations of wound healing

Wounds were evaluated weekly for 6 weeks. Wound contraction was followed by tracing the wound edges and the tattooed grid on transparent film. Wound contraction was measured by planimetry, expressed as percentage of reduction of original wound area, and was corrected for the locoregional growth of the animal. The final wound evaluation included a blinded score for three cosmetic wound parameters

by two independent experienced observers. They scored on a 1-5 scale for wound colour (pink - purple/red), smoothness and skin level of epidermis, and wound suppleness or stiffness. The overall score ranged from 3 (normal skin) to 15 (excessive scarring) and was averaged for each group in both animals.

Histological analysis of wound healing

After 2 and 4 weeks, a 4 mm punch biopsy was taken from each wound and after 6 weeks a cross biopsy from the middle of the wounds was taken (0.5 x 3-4 cm). The biopsies were fixed in 4% formaldehyde PBS solution for at least 16 h at RT, and subsequently embedded in paraffin according to standard procedures. Hematoxylin and eosine (H&E) stains were used to visualize cell infiltration and dermal architecture. In each biopsy, the degree of inflammatory response was scored on a 0 - 5 scale (0 a few lymphocytes/granulocytes - 5 high numbers of inflammatory cells and presence of localisd foci of mixed lymphocytic and granulomatous responses).

Six weeks post-wounding, the dermal architecture was analysed for the presence of non-remodelled amorphous ECM, cellularity, and collagen-bundle orientation (random, as in normal skin or parallel with the epidermis as in scar tissue). The maturation of collagen fibers was investigated with polarized light in H&E stained wound sections.

Statistical analysis

In each animal, five wound per treatment group were used (randomly assigned to different wound locations). The results were analysed for significant differences with a paired Student's *t*-test. A p-value below 0.05 (two-sided) was considered to be statistical significant. The cosmetic appearance scores were evaluated using the non-parametric Wilcoxon signed rank test.

Results

Mixed Lymphocyte Reactions (MLR)

In Figure 1, the MLR responses are shown of the PBMCs of the acceptor pigs (A and B) to the different allogeneic PBMC populations. The SPF pig, which was genetically the least related to the acceptor pigs, evoked the highest response, i.e. 4-6

times higher than background values. The MLR responses to the female and male PBMCs were relatively low: only the MLR with PBMCs of pig A and the male pig induced some response, almost 3 times higher than background values. The figure shows a representative example of 3 independent experiments and the mean values (n=4) were corrected for the sum of the background counts of the two corresponding single PBMC populations (<1500 DPM).

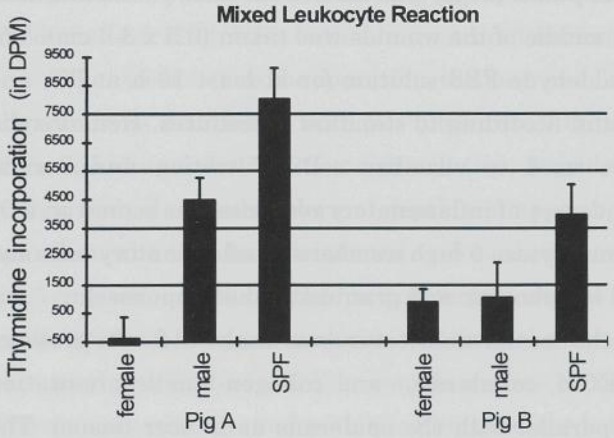


Figure 1. Mixed Lymphocyte Reaction responses of PBMCs of pigs A and B to PMBCs of the allogeneic pigs, female, male and SPF in DPM. The data shown are a representative example of 3 independent experiments and the mean values (n=4) were corrected for the sum of the background counts of the two corresponding single PBMC populations which was never higher than 1500 DPM

Wound cosmetic parameters and contraction six weeks post-wounding

The three cosmetic parameters scored were wound colour, epidermal skin level and smoothness, and elasticity/pliability of the regenerated skin. In Figure 2 the cosmetic appearance of the wound is shown for each treatment group 6 weeks post-wounding. The wounds treated with the dermal substitute seeded with autologous fibroblasts (A) showed the strongest resemblance to normal skin, whereas the wounds treated with the dermal substitutes seeded with allogeneic fibroblasts populations of female, male and SPF origins (B-D) were of mixed red/pink colour and had an uneven skin level with inflexible fibrotic wound areas. The wound treated with the substitute seeded with SPF fibroblasts had contracted the most. The overall scores for the cosmetic appearance for each treatment group in both pigs is shown in Figure 3A. In both animals, the substitutes seeded with SPF fibroblasts significantly worsened the cosmetic outcome of wound healing. In Figure 3B, the average wound contraction of each treatment group on both pigs is shown. In pig A the substitutes seeded with male and SPF fibroblasts, and in pig B the substitutes seeded with SPF fibroblasts induced significantly more wound contraction than did treatment with the dermal substitute seeded with autologous fibroblasts.

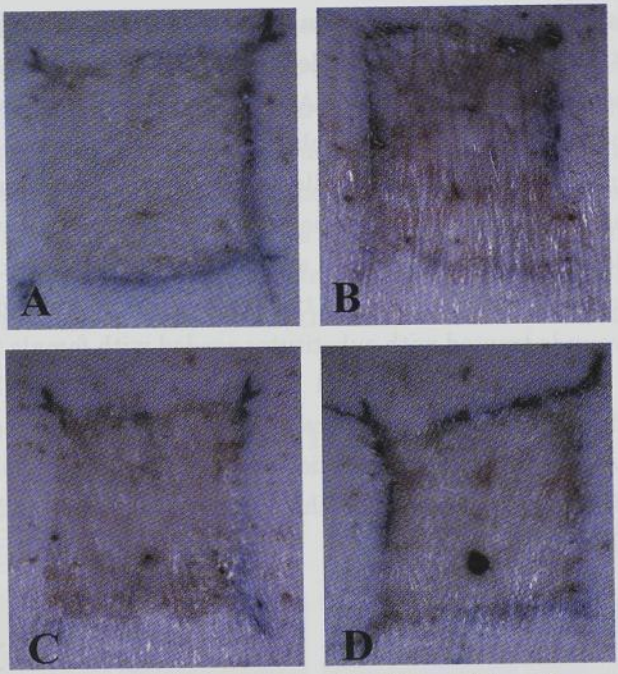


Figure 2. Cosmetic appearance of wounds 6 weeks after grafting. The wound treated with a substitute seeded with autologous fibroblasts (A) resembled normal skin the most. The wounds of the other groups, female (B), male (C) and SPF fibroblasts (D), still had red and fibrotic wound areas. The wound treated with the substitute seeded with SPF fibroblasts had contracted the most.

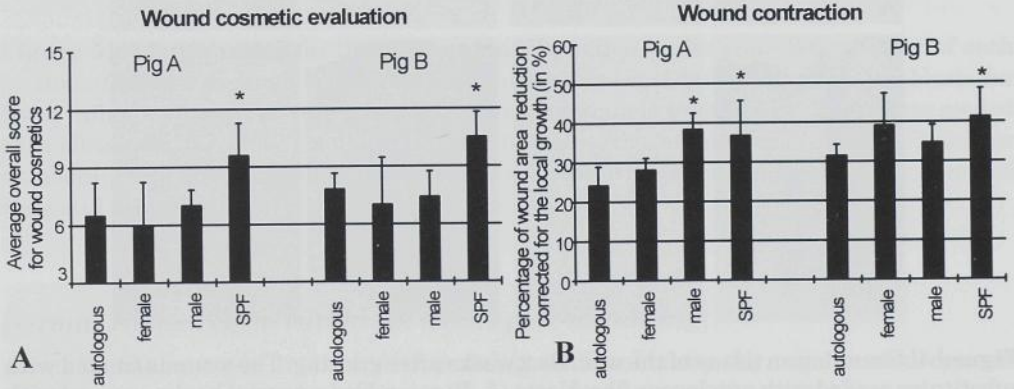


Figure 3. Overall cosmetic scores (A) and average wound contraction (B) of the different treatments six weeks after grafting. The X-as indicated the seeded fibroblast populations. The SPF fibroblasts significantly worsened the cosmetic outcome of wound healing compared to autologous fibroblasts (* $p<0.05$, Wilcoxon signed rank test). The SPF fibroblasts induced in both pigs significantly more wound contraction compared to treatment with autologous fibroblasts. The male fibroblasts did the same in pig A (* $p<0.05$, paired *t*-test).

Histological observations and inflammatory responses during healing

In Figure 4, a representative example is shown of the granulation tissue of each group 2 weeks post-wounding. In Figure 4A, C, E, and G an overview is given of the dermal area (in between epidermis and subcutaneous fat), whereas Figure 4B, D, F and H are enlargements of insets to illustrate the cellular composition of the granulation tissue. The granulation tissue of the wounds treated with substitutes seeded with autologous fibroblasts (Fig. 4A and B) show the presence of some diffusely distributed inflammatory cells, which is a typical image for normal healing wounds. In contrast, the wounds treated with substitutes seeded with female fibroblasts (Fig. 4C and D), male fibroblasts (Fig. 4E and F) and SPF fibroblasts (Fig. 4G and H) showed a marked increase in the numbers of inflammatory cells together with the presence of localised mixed lymphocytic and granulomatous responses. These inflammatory foci were never observed in the wounds treated with substitutes seeded with autologous fibroblasts.

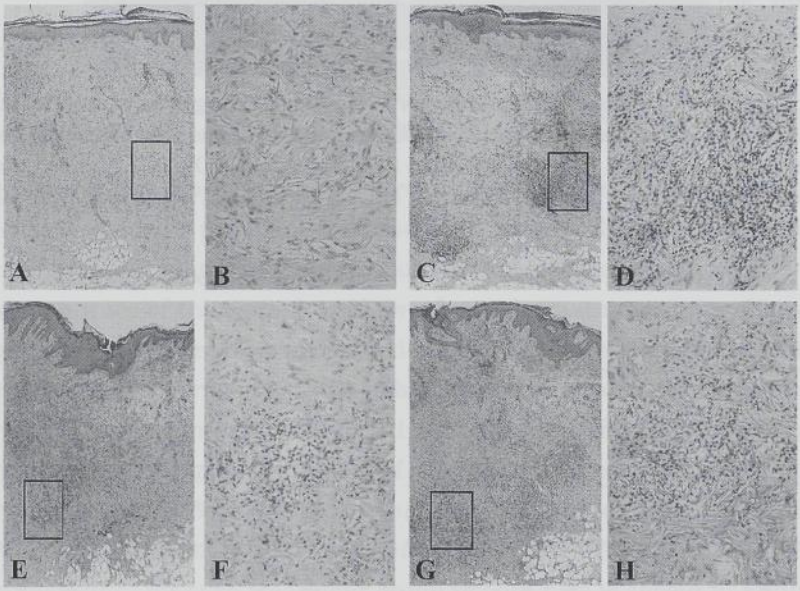


Figure 4. Granulation tissue of the wounds 2 weeks after grafting. The wounds treated with substitutes seeded with autologous fibroblasts (A, B) resembled a normal healing wound with the presence of few granulocytes and lymphocytes. In contrast, the 'allogeneic' wounds with female (C, D), male (E, F), and SPF fibroblasts (G, H), showed an increased numbers of inflammatory cells and the presence of mixed granulomatous and lymphocytic inflammatory foci. The increased inflammation also appeared to over-activate the keratinocytes in the epidermis, as evidenced by increased thickness of epidermis and large rete ridges. A, C, E and G: original magnification 20x, B, D, F and H: 5x enlargements of insets.

Furthermore, in the wounds treated with substitutes seeded with allogeneic fibroblasts the epidermis appeared to be overstimulated, was thicker, and had larger rete-ridges when compared to wounds treated with autologous fibroblasts seeded substitutes. In time, the granulation tissue matured progressively and the numbers of lymphocytes and granulocytes diminished. In Figure 5, the scores for the intensity of the inflammatory responses in time are shown for both pigs A and B. The number of inflammatory cells in the wounds treated with substitutes seeded with allogeneic fibroblasts never subsided to the level of the wounds treated with substitutes seeded with autologous fibroblasts.

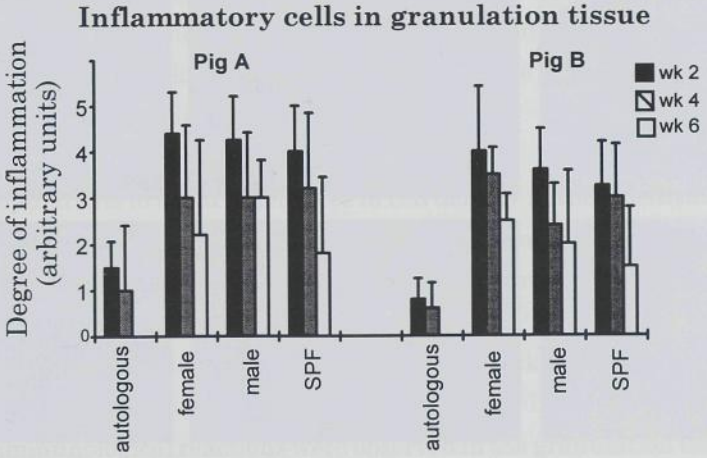
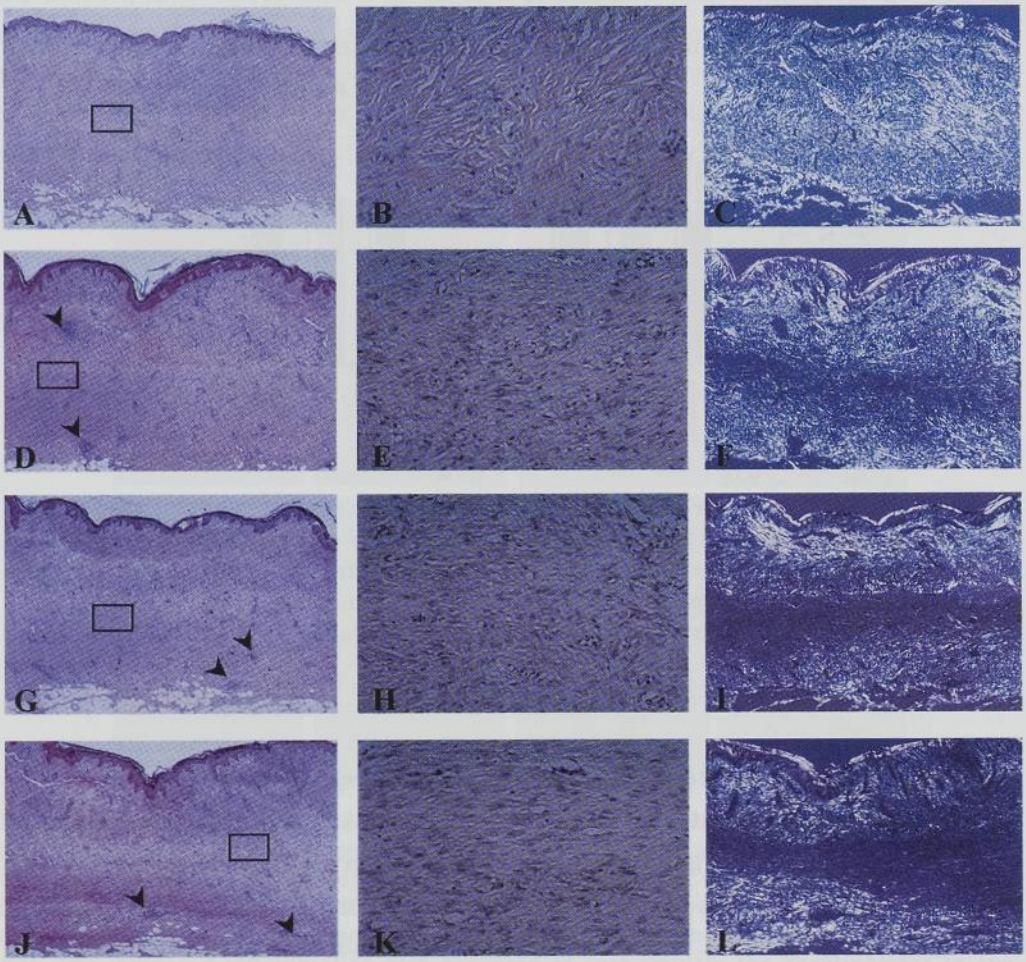


Figure 5. Relative scores for the degree of inflammation in the granulation tissue of each treatment in time. In wounds treated with substitutes seeded with autologous fibroblasts far fewer inflammatory cells were observed than in the wounds treated with substitutes seeded with allogeneic fibroblast populations. After 6 weeks, the 'autologous' wounds had similar numbers of inflammatory cells as normal skin, whereas in the 'allogeneic' wounds the numbers were still increased.

Dermal tissue regeneration six weeks post-wounding

In the wounds treated with autologous fibroblasts, the regenerated dermal tissue showed randomly organized collagen bundles as in normal skin after six weeks (Fig. 6A and B). Moreover, all collagen bundles turned out to be of a mature nature under polarized light (Fig. 6C). In contrast, in the wounds treated with allogeneic fibroblasts the scar tissue contained areas which were still granulating (fibroblasts

origins: female Fig. 6D-F, male Fig. 6G-I, and SPF fibroblasts Fig. 6J-L). These areas contained higher cell numbers than the wounds treated with substitutes seeded with autologous fibroblasts which are illustrated in Fig. 6B, E, H and K (higher magnification of insets). The collagen bundles in these areas were still of an immature nature (Fig. 6F, I and L) and had a tendency to be organised parallel with the epidermis, which is typical for scar-tissue formation. Furthermore, wounds treated with allogeneic fibroblasts still contained inflammatory cell foci (arrowheads in Fig. 6D, G and J).



Discussion

Previously, we demonstrated that a dermal substitute in combination with split-skin mesh grafts is capable of improving dermal tissue regeneration compared to split-skin mesh-graft treatment alone (23,24). Since this dermal substitute consisted of native non-cross-linked collagen and elastin, the bio-absorption of the material occurred without significant inflammatory reactions (24,25). Furthermore, the addition of autologous fibroblasts to the dermal substitute not only showed survival and proliferation of these fibroblasts after implantation, but also an additional improvement in dermal tissue regeneration (17,26). The use of autologous fibroblasts in the clinic implies a delay of treatment, for which the use of allogeneic fibroblasts could be advantageous. The present study was designed to establish whether or not allogeneic fibroblast populations were able to stimulate dermal tissue regeneration without inducing adverse inflammatory reactions.

Dermal substitutes were implanted within 24 hours after seeding of the fibroblast populations to avoid differences in cell density in the substitute which could occur with culture of substitutes in vitro. The SPF allogeneic fibroblast population was the least related to the acceptor pigs, which was also reflected in the highest MLR response in both acceptor pigs. The response with PBMCs from the other two allogeneic female and male pigs was comparable to background levels; only in pig A the PBMCs of the male pig did induce some response. Despite these negative MLR responses, immunological reactions were observed in the granulation tissue of wounds treated with these fibroblasts. This is not surprising since it is known that even after HLA matching and negative MLR responses, allogeneic skin grafts were ultimately rejected (27). In our study, allograft rejection was never observed. However, the observed inflammatory and immunological responses retarded dermal tissue regeneration and appeared to induce more scar tissue. In addition, SPF fibroblasts

< Figure 6. Regeneration of dermal tissue of the wounds treated with substitutes seeded with autologous (A-C), female (D-F), male (G-I), and SPF fibroblasts (J-L) six weeks after grafting. Figures A, D, G, and J give an overview of the dermal tissue; B, E, H, and K are enlargements of insets illustrated in A, D, G, and J, respectively; and C, F, I and L are images of the regenerated tissue under polarized light which highlights matured collagen. The regenerated tissue of the 'autologous' wounds resembles normal skin, whereas the 'allogeneic' wounds still had granulating areas in the middle of the regenerated tissue with increased cell numbers and non-remodelled ECM with immature collagen bundles. In addition, the 'allogeneic' wounds still contained mixed granulomatous and lymphocytic inflammatory foci (arrow heads). A, D, G and J, original magnification 13x; B, E, H, and K 120x, and C, F, I and L 15x.

induced significantly more wound contraction and significantly impaired the wounds' cosmetic appearance compared to autologous fibroblasts. For the female and male fibroblast populations the average contraction was higher in both pigs compared to that of the autologous fibroblasts, but only the contraction induced by male fibroblasts in pig A proved to be significantly higher. In addition, the contraction data correlated well with the levels of the MLR responses found in both pigs.

In a similar porcine wound model, Reagan *et al.* (18) compared the use of acellular to cellular allogeneic dermal grafts. They found that the cellular dermal grafts elicited a drastic inflammatory response which seemed to be directed against epithelial follicular elements but not against fibroblasts or extracellular matrix elements. This inflammatory response also increased wound contraction and worsened wound cosmetic outcomes and, as was found in our study, these grafts did not show signs of rejections. In the literature, rejection reactions after renal transplantation have been reported to increase the presence of myofibroblasts and tissue fibrosis (28). Since myofibroblasts mediate wound contraction (29), it is feasible that the observed inflammatory reactions induced fibroblast differentiation into myofibroblasts or prolonged the presence of myofibroblasts. This could explain the increased wound contraction and scar formation we found in the wounds treated with the 'allogeneic' substitutes.

In most studies fibroblasts were included in a dermal equivalent for their positive effects on epidermal regeneration. In the present study, differences in outgrowth or closure of the meshed split-skin graft interstices could not be observed, since the wounds completely epithelialized in between the evaluation time points 1 and 2 weeks post-wounding. Hansbrough *et al.* (30) showed that allogeneic fibroblasts allowed rapid epidermal regeneration of meshed epidermal interstices xenografted to nude mice in a vicryl mesh dermal substitute. Treating burn patients with this 'allogeneic' dermal substitute (Dermagraft^{R}), they did not observe evidence of immunological rejection towards the allogeneic fibroblasts or to the vicryl fibres 14 days after grafting (31). However, in their histological analysis the authors mentioned that there were minimal signs of inflammatory reactions: giant cells were observed and vicryl fibres were extruded from the wound surface. Unfortunately, the study design allowed only comparison to treatment with split-skin mesh grafts and not to a dermal substitute with autologous fibroblasts. The skin equivalent, Graftskin^{R}, contains both male allogeneic fibroblasts and keratinocytes and is nowadays called Apligraf^{R}. This

substitute has been applied on patients with surgical wounds and chronic ulcers without clinical signs of rejection (21,32,33). The immunocompatibilty is claimed to be based on several factors: the lack of antigen-presenting cells bearing MHC class-II molecules, the cytokine environment which down-regulates unprimed T-cells, and the limited access of host immune cells to graft cells. Apligraf® is a modification of the organotypic collagen gel construct originally described by Bell et al.(1). Bell also reported that these grafts populated with allogeneic fibroblasts were accepted across the barriers of histocompatibility (19,20), but persistence of the allogeneic fibroblasts was only investigated by karyotyping of allogeneic cells in isogeneic transplanted rats. Furthermore, this added confusion to this research field, since the majority of the evidence seemed to be in favour of non-survival of cultured allografted cells, especially for keratinocytes (3). More recently, Otto *et al.* (11) also detected male fibroblasts and keratinocytes in a female patient up to 2.5 years after grafting with a more sophisticated molecular detection technique. Although the quality of the regenerated dermis was not investigated, the long survival of male fibroblasts and keratinocytes seems to indicate that in these types of skin equivalent allografted cells may survive longer than expected.

The basis for transplantation rejection is the expression of MHC-I and MHC-II molecules. If CD8+ T cells interact with MHC-II molecules, this immediately elicits a cytotoxic response (34). On resting fibroblasts, the expression of MHC-I is low and MHC-II not detectable. After fibroblast activation, the MHC-I levels are up-regulated and in chronic inflammatory loci the expression of MHC-II on fibroblasts has been reported (35,36), especially in the presence of IFN-g (37). In vitro, IFN-g induced MHC expression on fibroblasts did not seem to be able to stimulate or only moderately stimulate unprimed allogeneic T-cell or lymphocyte responses (38,39). However, when mixed with primed T cells, the allogeneic fibroblasts did induce T cell proliferation (40). In addition, T cell-fibroblast interactions (both autologous and allogeneic) increased the secretion of inflammatory cytokines IL-1, IL-2 and TNF-a (41). In wound healing the latter are able to contribute to increased inflammation and tissue fibrosis (42). The culture conditions of cells also play a role in the expression of MHC molecules. The coculture of keratinocytes with 3T3-fibroblasts induced expression of both MHC-I and II on these fibroblasts (43) and the 3T3 fibroblasts were able to sensitize a graft recipient for accelerated second-set rejection (44). The data presented in our present study also seem to support the fact that allogeneic fibroblasts are

recognized by lymphocytes and provoke inflammatory responses. We believe, therefore, that for true skin substitution the use of autologous fibroblasts in a dermal equivalent is to be favoured rather than that of allogeneic fibroblasts. Nevertheless, when they are used as a temporary cover to stimulate wound healing, allogeneic fibroblasts might play a role in triggering the immune system. This can be especially interesting in chronic wounds in which the immune status is often impaired.

In conclusion, for optimal restoration of dermal skin function without scar formation, the use of skin equivalents with autologous fibroblasts is to be preferred to the use of skin equivalents with allogeneic fibroblasts.

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Chapter 6

Cadexomer-iodine Ointment Shows Stimulation of Epidermal Regeneration in Experimental Full-thickness Wounds

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Abstract

The use of iodine in wound healing is still controversial. Both wound healing stimulating effects and toxic effects leading to impaired wound healing have been reported. In order to study the direct effects of iodine on wound healing without interference of infectious pathogens, we investigated wound-healing parameters in noninfected experimental full-thickness wounds in the pig. Topical iodine treatment with an ointment consisting of a combination of iodine and cadexomer (modified starch), was compared with cadexomer ointment, the vehicle without iodine, and with treatment with saline. Treatment lasted for 30 days, followed by 30 days of wound assessment. The rate of epithelialization, wound contraction, systemic iodine absorption and several immunohistochemical markers were evaluated.

All 36 wounds healed without macroscopic signs of wound infection and re-epithelialized within 21 days. During the first 9 days of treatment, wounds treated with cadexomer-iodine ointment showed significantly more epithelialization than the wounds treated with either cadexomer or saline. In addition, the epidermis of wounds treated with cadexomer-iodine ointment had significantly more epithelial cell layers from day 12 to day 30 and these wounds stained for chondroitin sulphate proteoglycans in the newly formed basement membrane zone, which was not observed with the other treatments. No negative effects of cadexomer-iodine ointment on the formation of granulation tissue, neovascularization or wound contraction were observed. During the treatment systemic iodine absorption was physiologically acceptable. These results showed that treatment with cadexomer-iodine containing ointment had positive effects on epidermal regeneration during the healing of full-thickness wounds in the pig compared with ointment alone or saline treatment.

Introduction

Cadexomer-iodine ointment is an hydrophilic modified-starch polymer with 0.9% iodine immobilized within the matrix, in an ointment base of polyethylene glycol and poloxamer. When applied on a wound, cadexomer-iodine ointment becomes moist by absorbing the wound exudate and gradually releases the incorporated iodine [13]. Several multicentre studies have found that in combination with compression therapy cadexomer-iodine ointment is an effective debrider and antiseptic agent for chronic wounds such as venous leg ulcers [17,18,25,31,35,42,43]. Used as a dressing it removes pus, debris and wound exudate from wounds and reduces bacterial counts. As a result,

wound healing is stimulated, which is indicated by a reduction in the healing time of chronic wounds.

In the treatment of open infected wounds the use of antiseptics is preferred to antibiotics because of the risk of development of bacterial resistance. Topical treatment with iodine has proven to be effective against a wide range of bacterial and fungal infections [3,5,14,15,28,29,37,46] and to our knowledge bacterial resistance against iodine has never been reported. Nevertheless, its use in wound care is still somewhat controversial owing to reports suggesting that iodine may retard wound healing [16,30]. In one study povidone-iodine used on experimental burn wounds did delay wound healing [21] and on large burns it has been seen as the cause of metabolic acidoses and renal insufficiency resulting from systemic iodine uptake [7,41]. Further studies on animals and on cells in vitro have confirmed that iodine indeed affects cellular viability in a dose-dependent fashion and delays wound healing, which reinforces the opinion that iodine should not be used in the treatment of open wounds [2,4,22,26,32].

However, results from other studies suggest that iodine applied at lower concentrations is nontoxic to wounds, and may even accelerate wound healing. For example, two clinical studies have shown that topical iodine treatment significantly stimulates the epithelialization of small burns [12,36] and similar findings have been described for cadexomer-iodine ointment treatment of chronic wounds [25,31,35] and of partial-thickness wounds in the pig [29]. Furthermore, animal studies using the rabbit ear chamber model have shown that povidone-iodine does not adversely influence epithelialization and granulation [6,8,34].

The contradictions in the previous findings are probably a result of differences in application to the wounds and local concentration of iodine in tissue. Furthermore, wound healing studies in patients are often difficult to evaluate. Standardization is hardly possible owing to the multiple variables related to wound aetiology and the absence of internal controls. To overcome these problems, we investigated whether cadexomer-iodine ointment or the cadexomer vehicle itself were able to stimulate healing of acute noninfected wounds in a pig wound model. Both treatments were compared with a treatment with saline-moistened gauzes. During the evaluation, biopsies were taken from each wound and several parameters of the healing process were assessed: cosmetic appearance by photography, wound contraction and epithelialization by planimetry, and basement membrane regeneration, vascularization and granulation tissue formation by immunohistochemistry.

Materials and methods

Materials and antibodies

The cadexomer-iodine ointment, Iodosorb^{R}, consisted of a formulation of cadexomer, a hydrophilic starch polymer cross-linked with epichlorohydrin in which 0.9% (w/w) iodine was incorporated, polyethylene glycol and poloxamer. The cadexomer ointment used had an identical formulation to the cadexomer-iodine ointment but without iodine; both ointments were from Perstorp Pharma, Lund, Sweden. Opsite^{R} (Smith & Nephew, Hull, U.K.) is an oxygen-permeable polyurethane adhesive transparent film. From DAKO A/S (Copenhagen, Denmark) we purchased monoclonal mouse antibodies anti-α smooth muscle actin (dilution 1:200), polyclonal rabbit-antibodies anti-human Von Willebrand Factor (1:1500), biotinylated swine-antibodies anti-rabbit immunoglobulins, biotinylated rabbit-antibodies anti-mouse immunoglobulins and streptavidin biotinylated horseradish peroxidase complex (StreptABcomplex/HRP). Monoclonal mouse antibodies anti-chondroitin-4- and -6-sulphate (1:400) and the peroxidase substrate diaminobenzidine (DAB) were obtained from Sigma Chemical Co. (St. Louis, MO). All primary antibodies were shown to cross-react with porcine antigens.

Surgical procedures and skin biopsies

The operation procedures were performed as described previously [9,10] and the protocol was approved by the Ethical Committee of Animal Welfare of the University of Amsterdam. In brief, female Yorkshire pigs, weighing approximately 20 kg at the start of the study, were used. A grid was tattooed on the back of the animals to facilitate the measurement of wound surfaces by planimetry. Six identical full-thickness wounds (3.0 cm x 3.0 cm and 0.24 cm deep) were created with an electro-dermatome. Two wounds were dressed with approximately 5 ml of cadexomer-iodine ointment, two with 5 ml of cadexomer ointment and two with 5 ml of saline on 5 x 5 cm gauzes. These dressings were fixed with Opsite, covered with another layer of hydrophilic gauzes which were fixed with adhesive tape (Curafix, Lohmann, Almere, the Netherlands) and elastic stockings (Tubigrip, Seton Health Care, Oldham, UK). This procedure protected the wounds from mechanical trauma. During the next 30 days, the wounds were cleaned every 3 day with saline and the treatments were repeated. All wounds were treated with occlusion to create an optimal wound environment for cell migration and granulation tissue formation and to prevent rapid desiccation of the saline gauzes and the ointments.

Treatment application and evaluation were not blinded and evaluation of

epithelialization was based on colour differences of the wound surface (Fig. 1). Wound edges, epithelialized area and the tattooed grid were traced on transparencies every third day for 30 days and after 36, 44 and 58 days. Reepithelialization was calculated as the percentage of epithelialized wound area divided by the total wound area. Wound contraction was calculated as the reduction in original wound area and corrected for growth of the animal. Punch biopsies and photographs were taken of each wound every sixth day for 36 days and on days 44 and 58. The biopsies were fixed in a 4% formalin PBS solution for 8-12 h at room temperature, processed by routine histological procedures, and embedded in paraffin. Biopsies were used to visualize wound histology, e.g. epidermal regeneration (rete-ridges, number of epithelial cell layers, presence of chondroitin sulphate basement membrane proteoglycans), angiogenesis (staining for von Willebrand Factor), and granulation tissue formation and thickness (staining for α-smooth muscle actin).

Immunohistochemistry

Sections of 5-6 μm thickness were mounted on lysine-coated glass slides. The sections were deparaffinized in xylol and hydrated through a graded series of ethanol. All incubations were performed at room temperature unless stated otherwise. To remove endogenous peroxidase activity the slides were incubated for 30 min in a 0.3% H_2O_2/methanol solution, and washed with water and PBS. Non-specific binding of antibodies was avoided by 15 min preincubation with a PBS solution with 10% normal human AB serum. The sections were incubated for 1 hour with primary antibodies (diluted as indicated above) and washed three times in PBS before the appropriate biotinylated secondary antibody was applied (diluted 1:400 in PBS 10% AB serum). After an incubation of 30 min, sections were washed with PBS twice and incubated for another 30 min with the streptABcomplex/Hrp (diluted 1:200 in PBS). After extensive washing, the colour reaction was performed for 7 min with 50 mM Tris/HCl buffer (pH 7.8) containing 0.05% DAB substrate and 0.03% H_2O_2. Finally, the sections were counterstained with haematoxylin, mounted in glycergel and examined microscopically.

For the Von Willebrand staining, the sections were predigested with a 0.25% pepsin solution for 30 min at 37°C, in order to unmask antigens. Sections of normal human and pig skin served as positive controls. As negative controls, adjacent sections of the wound biopsies were stained with nonimmune IgG from the same animal as the primary antibodies at the same dilution. No staining was observed in negative controls. Microscopic examinations were carried out by three experienced researchers. Photographs of representative histological stainings were taken with an Olympus SC35 camera with 64T (EPY-135) Ektachrome film (Kodak, Netherlands).

Measurement of plasma iodine levels

From each of the six animals studied, 10 ml blood was collected on heparin on every third day from day 0 until day 30, and on days 36, 44 and 58. After centrifugation, plasma samples were stored deep frozen until assay. After digestion of organic matter with nitric acid, the total iodine content was assessed using inductive coupled plasma analysis with mass spectrometric detection. The analysis was performed by Biospectron AB, Tågarp, Sweden.

Statistical analysis

A total of twelve wounds per treatment, i.e. two on each pig were evaluated. The location of treatments was varied in a balanced design. A significant difference between two treatments was defined as $p<0.05$, using one-tailed Student's t-test.

Results

Cosmetic appearance and epithelialization

During the first 12 days of treatment, all three treatments yielded red granulating wounds which closed by epithelialization and wound contraction. Wound closure was fastest for wounds treated with cadexomer-iodine ointment. Epithelialization measured by planimetry as the percentage of closed wound areashowed that after 6 and 9 days wounds treated with cadexomer-iodine ointment had significantly more epithelialized wound area ($p<0.05$) compared with the wounds treated with both cadexomer and saline (Fig. 1). Figure 2 shows a representative example of the wounds 12 days post-wounding. At this time point, the cadexomer-iodine ointment treated wounds still showed significantly more reepithelialization ($p<0.05$) than saline treated wounds. At later time points wound closure was almost complete and the differences between the treatment were no longer significant.

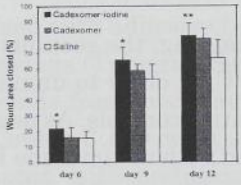

Figure 1. Rate of epithelialization. Results are expressed as mean percentage (±SD, n=12) of wound area covered after contraction. * After 6 and 9 days, cadexomer-iodine ointment treated wounds showed significantly more wound closure compared those treated with cadexomer or saline ($p<0.05$). ** After 12 days, cadexomer-iodine ointment treated wounds were significantly more epithelialized than the saline treated wounds ($p<0.05$)(per treatment n=12, ±SD).

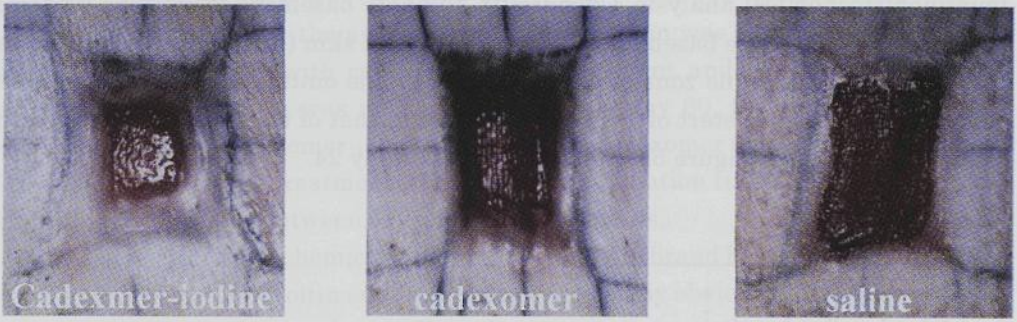

Figure 2. Cosmetic appearance of the wounds after 12 days. **A.** Cadexomer-iodine ointment treated wound showing red granulation tissue and epithelialization. **B.** The cadexomer treated wound only shows epithelialization at the wound-edges. **C.** The saline treated wound showed little epithelialization.

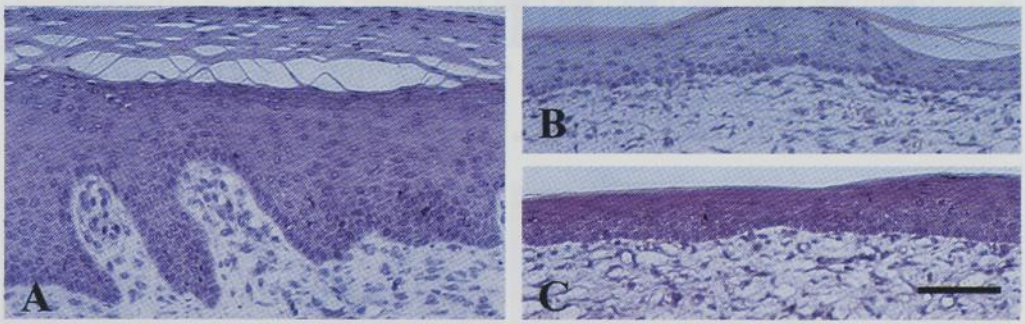

Figure 3. Haematoxylin and eosine stainings of wound sections 18 days post-wounding. **A.** Cadexomer-iodine ointment treated wound showing a thick epithelium with the presence of rete ridges and a thick stratum corneum. **B, C.** Cadexomer and saline treated wounds, respectively, showing a flat epidermis without rete ridges. Scale bar = 40 μm.

Epidermal regeneration

Histological analysis of sections routinely stained with Haematoxylin/Eosin showed that the epidermis was thicker for the cadexomer-iodine ointment treated wounds than for the other treated wounds (Fig. 3). Significant differences were found in the number of cell layers for cadexomer-iodine ointment treated wounds compared with the other wounds starting 18 days post wounding and lasting until the end of treatment ($p<0.01$)(Fig. 4). In addition to the higher number of cell layers of the cadexomer-iodine ointment treated wounds, they also showed more rete ridges and a thicker stratum corneum, both markers for epidermal differentiation and keratinocyte differentiation. The advanced epidermal differentiation was confirmed by

immunohistochemical analysis. Chondroitin sulphate basement membrane protein, normally present in the basement membrane of intact skin (Fig. 5a), was detected in the basement membrane zone of the cadexomer-iodine ointment treated wounds as early as 12 days after start of treatment, but not in that of the wounds treated with cadexomer or saline. Figure 5b-d illustrates this at day 24.

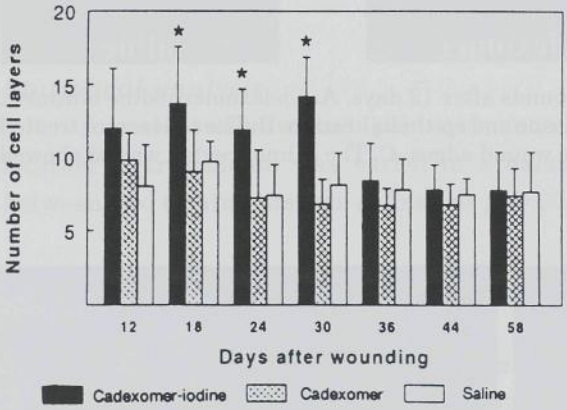

Figure 4. The mean number (n=12, ±SD) of epidermal cell layers at different time points after wounding. * After 18, 24 and 30 days, cadexomer-iodine ointment treatment induced significantly more epidermal cell layers than the other treatments ($p<0.05$). Wound biopsies 6 days after wounding showed no reepithelialization.

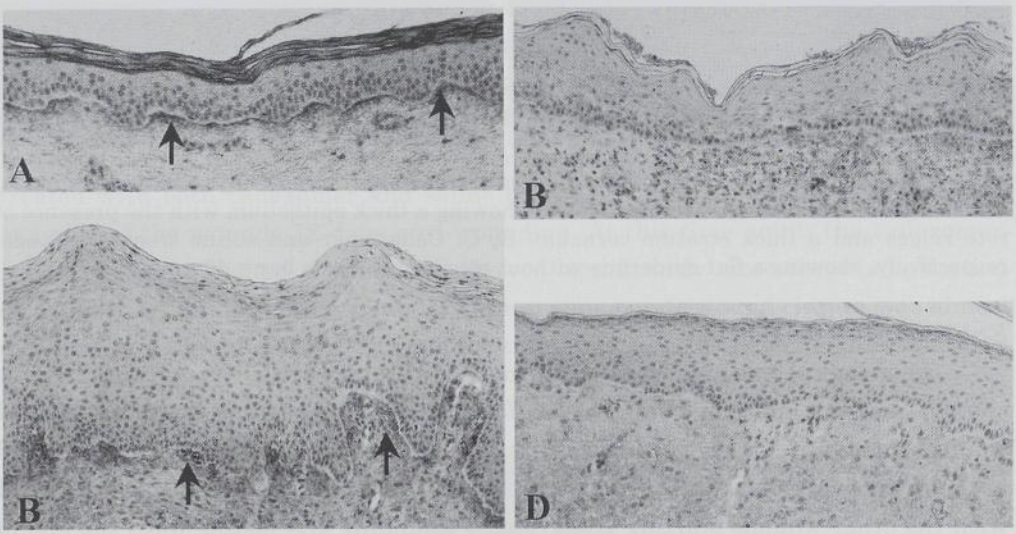

Figure 5. Chondroitin sulphate stainings of normal pig skin and the different wounds 24 days after treatment. **A.** Normal skin positive for chondroitin sulphate in the basement membrane (arrows). **B.** Cadexomer iodine ointment treated wound clearly showing rete ridges and chondroitin sulphate staining in the basement membrane zone (arrows). **C, D.** Cadexomer and saline treated wounds, respectively, are negative for chondroitin sulphate in the basement membrane. Scale bar= 80 μm.

Dermal regeneration

During the study, the average wound contraction was somewhat higher with saline treatment than with cadexomer-iodine ointment and cadexomer treatment although this difference was never significant. On day 60, the final average area reduction with the cadexomer-iodine ointment and cadexomer treatments was around 70% and for the saline treatment 75% (Fig. 6). Granulation tissue thickness was not significantly different between the different treatments.

The immunohistochemical stainings for von Willebrand Factor, α-smooth muscle actin and dermal chondroitin sulphate did not reveal any obvious differences between the treatments (results not shown). Vascular structures were already abundant 6 days after the start of the treatment and at the same time myofibroblasts positive for α-smooth muscle actin appeared in the lower dermis. They were abundant in the granulation tissue after 12 and 18 days and started to disappear after 24 days when the rate of wound contraction decreased. Chondroitin sulphate proteoglycans were abundant in the granulation tissue.

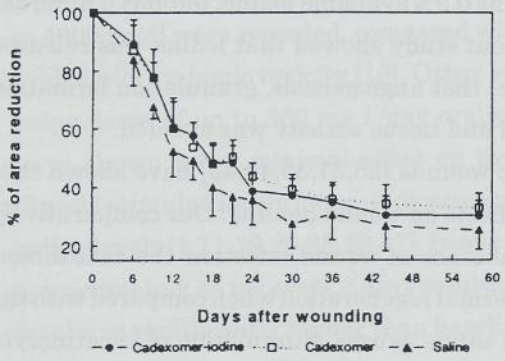

Figure 6. The average wound contraction (n=12, ±SD) measured by planimetry during the evaluation period. Results are expressed as percentage reduction of original wound area and were corrected for growth of the animal ($p>0.05$).

Systemic iodine absorption

After the start of cadexomer-iodine ointment treatment, the total iodide concentrations in plasma were significantly raised to approximately three times baseline values ($p<0.05$)(Fig. 7). After the end of the treatment, the levels returned to normal within one week.

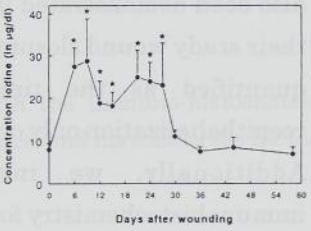

Figure 7. Mean total protein-bound iodine plasma levels of the 6 pigs during the evaluation period (±SD). * $p<0.05$ vs baseline values.

Discussion

The reason for the use of iodine in wounds is its antimicrobial activity. One likely mechanism which enhances the antimicrobial capacity of granulocytes and macrophages is the stimulation of the oxygen-dependent myeloperoxidase-H_2O_2-halide system in phagosomes by iodine [23]. Nevertheless, iodine has been shown to be cytotoxic in vitro, and its use in wound therapy has been discouraged. However, the observed cell toxicity in vitro has been shown to be dose-dependent [26] and in consequence tissue toxicity is likely to be dependent on the frequency and formulation of the application used [12,19]. Two reviews on the effects of iodine on wound healing have indicated that povidone-iodine does not retard wound healing when it is applied in small quantities [12,27]. The povidone-iodine preparations used nowadays are a solution and an ointment containing 10% povidone-iodine with 1% available iodine, and a povidone-iodine cream containing 5% povidone-iodine with 0.5% available iodine. Cadexomer-iodine ointment contains 0.9% available iodine, but has a different carrier and release mechanism. The present study showed that iodine was released from its ointment at a sufficiently low level that angiogenesis, granulation formation and wound contraction were not inhibited and tissue toxicity was avoided.

Previous clinical studies on chronic wounds [25,31,35,42,43] have shown that cadexomer-iodine ointment has positive effects on wound healing. Our comparatively acute wound model showed that in the absence of wound infection the cadexomer-iodine ointment treatment stimulated epidermal regeneration when compared with the treatments with cadexomer ointment and saline gauzes. Stimulation of keratinocyte proliferation, migration and differentiation was demonstrated by significantly faster epithelialization, a significantly greater number of epidermal cell layers, the presence of rete ridges, and early deposition of chondroitin sulphate basement membrane proteins. These effects were most likely caused by the release of iodine from its ointment. Faster epithelialization of cadexomer-iodine ointment treated wounds has also been demonstrated in partial thickness wounds by Mertz et al.[29]. However, in their study wound closure was achieved by re-epithelialization from hair follicles and quantified as the time needed to achieve wound closure. In our study reepithelialization only occurred from wound edges and was quantified by planimetry. Additionally, we investigated basement membrane regeneration by immunohistochemistry for which we have shown previously that chondroitin sulphate

staining is an appropriate marker [24].

It is not clear whether the observed stimulation of keratinocytes was direct or not. In cell culture it has been demonstrated [33] that iodine is able to modulate cytokine production of macrophages towards a pro-inflammatory profile. The IL-6 production is decreased while the TNF-α production is increased. In addition, low concentrations of cadexomer-iodine ointment, but not of cadexomer ointment, have also been shown to stimulate proliferation of fibroblasts [40]. Whether iodine also directly modulates keratinocyte proliferation or exerts its effects by acting on cytokine production of macrophages or other cell types remains to be established.

Wound therapy with products containing iodine results in systemic iodine uptake which is related to the size and depth of the wound [19] and which may cause side effects related to changes in thyroid function [44,45]. However, normal subjects without pre-existing thyroid disease are able to tolerate systemic iodine uptake well without any physiological disturbance. In a study on burn patients, serum levels of up to 4900 μg/dl were recorded, compared with normal serum levels of 4-8 μg/dl, without evidence of systemic toxicity [19]. Other studies of increased iodine intake in humans, using doses of up to 350 mg I^-/day orally as well as different topical dose regimens, have shown no or minimal effect on thyroid function except an increased level of thyroid stimulating hormone, indicating physiological adaptation to increased plasma iodine levels [1,11,19,20,38,39,47]. In our study, the iodine dose applied to wounds was approximately 45 mg every 3 days resulting in iodine plasma levels of 25-30 μg/dl. This level was significantly higher than baseline values (7-9 μg/dl), but is unlikely to affect thyroid function or will lead to organ toxicity. After cessation of iodine therapy iodine plasma levels returned to normal as a result of rapid renal clearance [1,47].

In conclusion, the cadexomer-iodine ointment stimulated epidermal regeneration of acute full-thickness wounds in pigs. The combination of antimicrobial activity and stimulation of the wound healing process may be clinically useful.

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Chapter 7

Summary and Concluding Remarks

Tissue engineering and artificial skin substitution is a rapidly progressing field in the centre of the attention of dermatologists, plastic surgeons, burn wound specialists, and pharmaceutical companies and many research groups. The objective of most skin substitutes is stimulation of wound healing, especially in chronic wounds, and diminishment of the time needed for wounds to close (1-3). Surprisingly, little attention was given to effects of artificial skin substitutes on dermal tissue regeneration and to the role of fibroblasts. The experiments described in this thesis focus on these aspects in the healing of full thickness skin defects.

New treatments can only be tested in patients after efficacy and safety have been proven in appropriate animal models. It is unrealistic to think that wound healing studies in animal models can be completely replaced by in vitro models. In wound healing, a multitude of factors is involved, forming a complex interplay of signals that wound cells are receiving. The complexity of signals during early wound healing is reviewed in **chapter 1** combining the scientific fields of molecular biology, biochemistry and tissue physiology.

The architecture and complexity of human skin is unique. In non-primates, the skin of the pig seems to be most appropriate model to study skin tissue regeneration, wound contraction and scar formation (4). Important is that new treatments which are analysed in an animal or clinical setting should be evaluated with objective methods which not only allow measurement of the degree of scarring, but preferably also visualize the scar tissue formed. Wound healing can be easily analysed from the exterior, but wound histology seems to be even more important (5).

Scarring not only becomes more severe when the wound depth increases, but also becomes permanent (6,7). Scarring is a problem of dermal molecular architecture and not so much of epidermal regeneration or epidermal-dermal attachment. The latter often occurs in scars from wounds which were treated with cultured epidermal sheets

(8). The lack or excess of specific proteins and architecture of collagen bundles will determine the quality or severity of the scar.

The Department of Dermatology, Wound Healing Research Group, Academic Medical Centre, Amsterdam, Burns Centre, Red Cross Hospital and Dutch Burns Foundation, Beverwijk have developed a dermal substitute, which consists of a native collagen 3D scaffold coated with 3% elastin hydrolysate (9,10). It provides a suitable substrate for the ingrowth of cells and the deposition of extracellular cellular molecules (ECM). In a pig model, full thickness wounds treated with these substitutes showed a stimulation of dermal tissue regeneration, and a reduction of wound contraction and scar formation (9).

The objectives of the experiments in this thesis were to optimize the healing of full thickness skin defects so that they would heal without scar formation. This was performed by the application of the artificial dermal substitute containing fibroblasts populations and topical dressings. In **chapter 2**, the healing of wounds treated with the collagen/elastin dermal substitutes combined with split-skin mesh grafts were compared to wounds treated with split-skin mesh grafts without dermal substitutes. Healing processes were investigated and followed by immuno-histochemistry. Results indicated that the dermal substitute accelerated the deposition of chondroitin sulphate basement membrane proteins in the basement membrane zone, induced a faster remodelling of the ECM in the sub-dermal area, and stimulated the regeneration of elastin in this area. This reduction in scar formation was furthermore evidenced by the reduced presence of thin collagen bundles organised parallel with the epidermis.

The presence and localisation of chondroitin sulphate basement membrane protein (probably bamacan) in the basement membrane zone proved to be a good marker to study basement membrane regeneration. Other basement membrane proteins studied in relation to basement membrane regeneration, e.g. laminin, collagen IV and VII, are present within the basement membrane zone already within 14 days after wounding (5,11). However, the presence of these proteins or the presence of hemidesmosomes is related to the formation of anchoring fibrils. Since the regenerated epidermis has to resist to shearing forces, the presence of rete ridges and fibrils extending much deeper in the dermis is more vital (12). Elastin fibrils and the chondroitin sulphate basement membrane protein, bamacan, are likely to have an

important function in the firm attachment of the epidermis to the dermis (13-15). Apparently, the induced stimulation of dermal tissue by the dermal substitute positively influenced the regeneration of a functional basement membrane. In addition, the immunohistochemical parameters described in this chapter proved to be very useful for the evaluation of consecutive wound healing studies. In addition, patient studies investigating scar formation by means of wound histology were often limited to the dermal tissue directly underlying the epidermis (16-19). This study also demonstrated that proper scar evaluations should include the entire reticular dermal layer.

In full thickness wounds, there is a delay of at least 3 days before fibroblasts start to migrate into the wound (20,21). The addition of fibroblasts to a dermal substitute is likely to stimulate dermal tissue regeneration, since fibroblasts are the cells responsible for the synthesis and remodelling of the newly deposited ECM. Indeed, the addition of fibroblasts to the collagen/elastin dermal substitute has been found to stimulate dermal tissue regeneration and reduce wound contraction of full thickness wounds more than the acellular substitute did (22). In **chapter 3**, the survival of fibroblasts seeded in the dermal substitute was investigated. A relatively low number of fluorescently labelled fibroblasts was seeded and was shown to proliferate after being transplanted in the wound. After 5 days, their cell number had almost tripled. This study proved that fibroblasts seeded in a dermal substitute and transplanted in wounds within a few hours after seeding had survived. This is important, because vascular structures are absent in the dermal substitute (20) and the supply of nutrients and removal of waste products are impaired during the first days after transplantation. In addition, the presence of fibroblasts in the dermal substitute also seems to inhibit the migration of other mesenchymal cells and significantly retards substitute degradation. Cell migration is correlated to proteolytic activity necessary for tissue invasion (reviewed in **chapter 1)**. It is likely that the apparent reduction of mesenchymal cell migration also correlates with the retarded degradation of the dermal substitute. Moreover, this also supports the idea that the control of fibroblast cell migration from the subcutis into the wound bed and their role in fibroplasia could be crucial for the control of scar formation (6). How fibroblasts present in the dermal substitute influenced tissue regeneration and scar formation at

later time points after wounding was investigated in **chapter 4**.

In **chapter 4**, living dermal substitutes were created in different ways and compared in the pig wound model. The seeding density of the fibroblasts was varied and instantaneous seeding was compared to a preculture period of 10 days. The length of the preculture period was chosen as the longest period still feasible in a clinical setting (23). Fibroblasts were precultured in the substitute to increase fibroblast deposition of the ECM molecules in the substitute. The best wound healing was observed with the precultured substitutes seeded with the highest fibroblast densities. Three weeks after wounding, this treatment almost completely abolished the presence of myofibroblast positive for alpha-smooth muscle actin. The wounds with the presence of more myofibroblast in the granulation tissue healed with more contraction and scar formation, were not supple, and remained more reddish in colour. Six weeks after wounding, the precultured substitutes seeded with the highest fibroblast densities showed a dermal tissue which consisted of only mature collagen bundles oriented in a basket weave pattern. This was never observed in previous studies.

The improved healing observed with the precultured substitutes seeded with the highest fibroblast density could be related to three factors: the increased deposition of ECM molecules, the numbers of fibroblasts in the substitute or alterations in the phenotypes of the fibroblasts in the cultured substitutes. The increased deposition of ECM molecules is unlikely to be responsible for the observed improvements in tissue regeneration. Histologically, a substantial deposition of ECM molecules was not observed in the substitutes. Furthermore, the substitutes seeded with the highest fibroblast density transplanted instantaneously and the precultured substitute seeded with the lower fibroblast density showed comparable wound healing results. The number of fibroblasts present in the substitute at the moment of grafting did correlate with the improved regeneration of dermal tissue, whereas the percentages of α-smooth muscle actin positive myofibroblasts did not correlate. Fibroblast populations in cell culture always seem to contain a variable percentage of α-smooth muscle actin positive myofibroblasts (24).

These results look very promising for treatment of patients. However, autologous fibroblasts were used in this study and this means that for the treatment of large wound surfaces substantial numbers of autologous fibroblasts would be

required. In this respect, it is also important to note that the seeding of fibroblasts in the dermal substitute at high density resulted in the loss of about 20% of the fibroblasts. Apparently, they leaked out of the substitute. Furthermore, fibroblasts also migrated out of the substitutes during the 10 days of preculture. Future studies should investigate which method is most optimal to obtain the highest fibroblasts densities in the dermal substitute in the shortest culture period. In addition, new developments to improve fibroblast isolation methods and culture conditions would help in the generation of more fibroblasts in the same or shorter period.

The generation of sufficient autologous fibroblasts for dermal substitution implies a delay in wound treatment, which could be solved by the use of allogeneic fibroblasts (25). In **chapter 5**, the use of allogeneic fibroblast populations for dermal substitution was investigated and compared to the use of autologous fibroblasts. Studies investigating the use of allogeneic fibroblasts for skin substitution in relation to dermal tissue regeneration are presently not published. Most studies only included autologous or allogeneic fibroblasts for their stimulating activity on epidermal regeneration and keratinocyte differentiation (26,27).

The results of this study clearly showed that allogeneic fibroblasts induced specific inflammatory reactions in the granulation tissue, which adversely affected tissue regeneration. After 6 weeks of healing the regenerated dermal tissue of the wounds treated with substitutes seeded with allogeneic fibroblasts showed more scar formation when compared to the wounds treated with autologous fibroblasts. Furthermore, wound contraction was significantly increased in the wounds treated with allogeneic fibroblast populations of which the mixed lymphocyte reactions induced the strongest responses. This study shows that the use of skin equivalents with autologous fibroblasts is preferred to skin equivalents with allogeneic fibroblasts, when the objective is the restoration of dermal skin function without scar formation. For the treatment of chronic wounds, products are already on the market which contain allogeneic fibroblasts (28,29). In those wounds, faster healing rates are considered of more importance than scar formation. Their future marketing strategy is going in the direction of skin substitution and the results of this study is highly relevant for these developments.

In chronic wounds and burns, skin substitution is also accompanied with a high

risk of failure due to bacterial infection (30) and the presence of excess of proteolytic activity (31-33). This could result in the loss of expensive autografts and even reverse the healing process resulting in increased scar formation. In the past, the infection control with antimicrobials has been shown to impair wound healing, which was explained by their toxicity for cells (34,35). The use of antibiotics is less favoured because of the danger of bacterial resistence. In **chapter 6**, a wound dressing, cadexomer-iodine ointment, containing the strong antimicrobial iodine was applied to full thickness wounds and wound healing was investigated in time. The cadexomer-iodine ointment was compared to two treatments being cadexomer without iodine and saline gauzes. In the past iodine containing wound products proved to be toxic for cells in a dose dependent fashion (36). The cadexomer-iodine dressing contains a slow iodine release mechanism. This mechanism proved to control the iodine concentration in the wound at sufficiently low levels so that adverse effects on wound healing were not observed. In fact, the released iodine of the ointment stimulated epidermal regeneration when compared to both other treatments. The stimulation of epidermal regeneration was concluded after observations showing the presence of a thicker epidermis, more and larger rete-ridges and the deposition of chondroitin-sulphate basement membrane protein. Systemic iodine absorption was observed but remained far below systemic toxic levels and was cleared from the blood stream within one week after treatment cessation.

The development of the artificial dermal skin substitutes has learned us more about wound healing and tissue regeneration. The fibroblasts play a central role in tissue regeneration. The understanding of fibroblast behaviour and differentiation will not only help in development of engineered tissues, but could also result in the discovery of new anti-fibrotic agents. The latter has important implications for the treatment of fibrotic diseases, e.g. scleroderma, and fibrosis associated with inflammatory reactions in organs.

In the next years, the artificial skin will find its way into clinical practice. This clinical implementation will raise new problems. These problems will concern: nutrition of the cells in living skin substitutes after the first days of transplantation; the control of bacterial contamination and proteolytic activity; the harvesting and culture of autologous cells. In the clinic, wound aetiology differs so much that the ideal

wound treatment should be able to interact with the wound environment and respond to it in an adequate manner. This can only be achieved by the use of skin cells in combination with a dressing providing nutrients for the cells, and allowing the control of bacterial contaminations and excess inflammation.

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Chapter 8

Samenvatting en Conclusies

Het onderzoek naar weefsel engineering en naar artificiële huidvervangende producten is een onderzoeksveld met veel progressie en staat in het middelpunt van de aandacht van dermatologen, plastische chirurgen, brandwond specialisten en de farmaceutische industrie. Het doel van de meeste huidvervangende materialen, met name voor chronische wonden, is een stimulatie van het genezingsproces en het verkorten van de tijd die nodig is voor het dichtgaan van wonden (1-3). Tot onzer verbazing is er tot nu toe weinig aandacht geschonken aan de kwaliteit van de genezing van diepe huiddefecten en de invloed van een artificieel huidsubstituut op het geregeneerde onderhuidse bindweefsel, de dermis. Vooral de rol van fibroblasten in dit proces werd weinig onderzocht. De experimenten beschreven in dit proefschrift richten zich met name op deze punten.

Nieuwe huidvervangende materialen kunnen alleen in patiënten getest worden nadat de werkzaamheid en veiligheid is bewezen in adequate diermodellen. Het is niet realistisch te denken dat wondgenezing studies in diermodellen volledig vervangen kunnen worden door kweekmodellen in het laboratorium. Tijdens de wondgenezing spelen er enorme aantallen factoren en moleculen een rol die een complex netwerk van signalen vormen. De cellen in de wond ontvangen uiteindelijk de signalen en reageren dus op veranderingen in hun directe omgeving. Om te begrijpen hoe cellen zullen of kunnen reageren is kennis nodig uit vele wetenschappelijke richtingen zoals de moleculaire biologie, biochemie en weefsel fysiologie. In de introductie van dit proefschrift is geprobeerd een uitgebreid overzicht te geven van de complexiteit van de signalen tijdens de vroege wondgenezing.

De architectuur en complexiteit van de humane huid is uniek. Buiten de primaten lijkt de huid van het varken het meest op die van de mens en de Yorkshire varkens bleken een zeer geschikt type proefdieren om de wondgenezing, wondcontractie en littekenvorming te bestuderen (4). Een geschikt diermodel is echter niet voldoende. Het is evenzo belangrijk om nieuwe wondbehandelingen te evalueren

met methodes waarmee de mate van littekenvorming zo objectief mogelijk is te bepalen. Alhoewel van de buitenkant een litteken makkelijk lijkt te beoordelen is de waarde en objectiviteit van deze beoordeling vele malen groter als dit wordt onderbouwd met histopathologie van het litteken (5).

Als de wond dieper is wordt er meer littekenweefsel gevormd, dat dan ook niet meer zal verdwijnen (6,7). In het verleden werden diepe wonden al behandeld met gekweekte opperhuid oftewel gekweekte epidermis. Bij deze behandeling bleek dat er vaak een slechte huidregeneratie optrad. De nieuw gevormde epidermis was snel beschadigd en er ontstond vaak spontane blaarvorming (8). Verlittekening daarentegen is echter niet een probleem van de epidermis of van de aanhechting van de epidermis aan de dermis, maar eerder een probleem van de moleculaire architectuur van de nieuwgevormde dermis. De aan- of afwezigheid van specifieke eiwitten en de architectuur van de collageen bundels zijn bepalend voor de ernst van het litteken.

Binnen de afdeling dermatologie in het AMC, in samenwerking met het brandwondencentrum en de Brandwonden Stichting in Beverwijk, heeft de Wound Healing Research Group een artificieel huidsubstituut ontwikkeld. Dit huidsubstituut dient in eerste instantie als vervanging van de dermis en heeft een sponsachtige matrix structuur. De matrix is gemaakt van natief collageen gecoat met een 3% elastine hydrolysaat suspensie (9,10). Dit substituut is een goed substraat voor de ingroei van huidcellen en vormt een 3-dimensionale basis voor de aanmaak van nieuwe huid. In het diermodel bleek dat dit huidsubstituut getransplanteerd in diepe wonden leidde tot een genezing waarbij meer dermaal weefsel werd aangemaakt. Wondcontractie en verlittekening waren beduidend verminderd (9). Het doel van experimenten beschreven in dit proefschrift was om te proberen de genezing van diepe huiddefecten nog verder te optimaliseren zodat er helemaal geen verlittekening meer plaats zou vinden. Dit is gedaan door huidcellen, fibroblasten, aan het artificiële substituut toe te voegen en te kijken naar het effect van een wonddressing met een sterke anti-microbiële werking op de genezing van diepe wonden.

In hoofdstuk 2 is de genezing van wonden die waren behandeld met het collageen/elastine dermale substituut in combinatie met een epidermaal transplantaat

(split-skin mesh graft) vergeleken met wonden die waren behandeld met alleen het epidermale huidtransplantaat. De genezing van de wonden werd onderzocht door te kijken naar de distributie van verschillende eiwitten in het wondweefsel m.b.v. immunohistochemische technieken. De resultaten lieten duidelijk zien dat in de aanwezigheid van het artificiële dermale substituut de aanmaak van chondroitine sulfaat proteoglycanen werd gestimuleerd in de basaal membraan zone en er een versnelde 'remodeling' van extracellulaire matrix eiwitten in de onderlaag van de dermis plaats vond. Op deze plaats in de dermis werd ook nieuwvorming van elastine vezels waargenomen, waar dit niet geval was wanneer het dermale substituut afwezig was. De stimulatie van dermale weefselregeneratie ging gepaard met een verminderde aanwezigheid van dunne collageen bundels parallel georiënteerd aan de epidermis, een fenomeen dat karakteristiek is voor littekenweefsel. Deze studie laat ook zien dat goede histologische karakterisatie van littekenweefsel betekent dat de hele reticulaire dermis moet worden beoordeeld en niet alleen de bovenkant van de reticulaire dermis (11-14).

Bij genezing van diepe wonden duurt het minimaal drie dagen voordat huidcellen naar het wondgebied toe gaan migreren (15,16). Door fibroblasten toe te voegen aan het artificiële dermale substituut is het misschien mogelijk het wondgenezing proces te versnellen en/of de aanmaak van nieuwe huid verder te stimuleren. Voor de aanmaak en 'remodeling' van nieuwe huid zijn voornamelijk de fibroblasten verantwoordelijk. Deze hypothese bleek inderdaad waar. Bij de behandeling van wonden in het diermodel bleek dat de toevoeging van fibroblasten aan het dermale substituut niet alleen de aanmaak van nieuwe huid stimuleerde, maar ook nog eens de wondcontractie verminderde in vergelijking met het dermale substituut zonder cellen (17). Het is relevant voor klinische studies om te kijken hoeveel van de getransplanteerde fibroblasten overleven omdat met name gedurende de eerste dagen na de transplantatie er nog geen vaatstructuren in het substituut aanwezig zijn en de aanvoer van voedingstoffen en afvoer van afvalstoffen verstoord is (15).

In hoofdstuk 3 is dit onderzocht en hiervoor werden fluorescerend gelabelde fibroblasten gebruikt. Na vijf dagen werden de cellen uit het wondweefsel geïsoleerd en bleek dat het aantal fluorescerende cellen bijna was verdrievoudigd. Dit geeft niet

alleen aan dat de gezaaide fibroblasten inderdaad de zaai en transplantatie procedures overleven, maar ook dat ze snel na de transplantatie gaan proliferen. Tevens werd er gekeken naar de invloed van gezaaide fibroblasten op de vroege wondgenezingsprocessen. Hierbij bleek dat de vroege aanwezigheid van fibroblasten in het dermale substituut de ingroei van mesenchymale cellen uit de wondranden verminderde en dat de afbraak van het dermale substituut significant vertraagd werd. Omdat voor celmigratie en weefselinvasie proteolytische enzymactiviteit nodig is (samengevat in de introductie) lijkt het voor de hand te liggen dat de verminderde celmigratie mogelijk een rol speelt bij de vertraagde afbraak van het dermale substituut. Hoe deze vroege effecten van fibroblasten in het dermale huidsubstituut uiteindelijk de huidregeneratie en littekenvorming beïnvloeden werd onderzocht in hoofdstuk 4.

In hoofdstuk 4 zijn op verschillende manieren dermale substituten gecreëerd die fibroblasten bevatten. Er werden 2 variabelen getest: de hoeveelheid fibroblasten gezaaid in het huidsubstituut en het tijdstip van transplanteren van de substituten na het zaaien van de fibroblasten. Substituten werden direct getransplanteerd na het zaaien of eerst 10 dagen gekweekt alvorens ze werden getransplanteerd. De verschillende substituten werden onderling vergeleken in het varkens proefdiermodel. Een kweekperiode van 10 dagen is de langste periode die nog realiseerbaar lijkt in een klinische setting (18). Het idee achter het kweken van fibroblasten in het dermale huidsubstituut is dat gedurende deze periode de fibroblasten extracellulaire matrix in het substituut kunnen afzetten, waardoor de aanmaak van nieuwe huid versneld wordt. De meest optimale wondgenezing werd gevonden in wonden behandeld met dermale substituten gezaaid met de hoogste aantallen fibroblasten en gekweekt in het substituut gedurende 10 dagen. De genezing van deze wonden leidde tot enkele opmerkelijke resultaten; drie weken na de operatie bleek dat er in het wondweefsel geen myofibroblasten positief voor de α-isovorm van gladde spiercel-actine aanwezig waren. Normaal gesproken zijn op dit tijdstip altijd nog veel van deze myofibroblasten aanwezig die de extracellulaire matrix actief samentrekken en verantwoordelijk worden geacht voor het optreden van wondcontractie. Verder bleek na zes weken dat deze wonden een geregeneerde huid hadden, die alleen bestond uit rijpe collageen

bundels met een oriëntatie net zoals in de normale huid. Deze wonden hadden geen tekenen van verlittekening meer, een observatie die nog niet eerder was waargenomen in eerdere studies. Verdere analyses leidden tot de conclusie dat de genezing van de wonden in deze studie gerelateerd kon worden aan het aantal fibroblasten dat aanwezig was in het dermale substituut op het moment van transplanteren en niet zo zeer aan de verhoogde aanwezigheid van ECM in de gekweekte dermale substituten. Deze resultaten lijken veelbelovend voor de behandeling van patiënten. Hierbij moet men echter wel voor ogen houden dat voor de behandeling van grote wondoppervlaktes er grote aantallen fibroblasten nodig zijn. In een klinische setting zou dit nu nog betekenen dat er veel donor-site weefsel nodig is en/of de kweek periode van 10 dagen te lang is. In principe zou de periode tussen het tijdstip van isoleren van de cellen en de behandeling van de patiënt zo kort mogelijk moeten zijn. Dit om wondcontractie processen voor te zijn en wondinfecties te voorkomen. Deze problemen zouden echter opgelost kunnen worden wanneer er lichaamsvreemde (allogene) fibroblasten gebruikt kunnen worden in plaats van de fibroblasten van de patiënt zelf (autologe fibroblasten)(19).

In hoofdstuk 5 is het gebruik van verschillende lichaamsvreemde fibroblast populaties vergeleken met de lichaamseigen fibroblasten. Tot nu toe waren er geen studies gepubliceerd die het gebruik van allogene fibroblasten voor dermale huidsubstitutie hebben onderzocht in relatie tot dermale huidregeneratie. De resultaten van onze studie laten duidelijk zien dat alle geteste lichaamsvreemde fibroblast populaties ontstekingsreacties in het granulatieweefsel opwekten en dat na 6 weken meer verlittekening was opgetreden wanneer de genezing werd vergeleken met de wonden behandeld met lichaamseigen fibroblasten. Bovendien waren er wondgroepen, die waren genezen met significant meer wondcontractie. Dit waren wonden behandeld met dermale substituten gezaaid met lichaamsvreemde fibroblasten, die waren geïsoleerd uit varkens waarvan de gemixte lymfocyten reactie het hoogst was. Als conclusie kan ik stellen dat de toevoeging van autologe fibroblasten aan het dermale huidsubstituut is te preferen boven de toevoeging van lichaamsvreemde fibroblasten wanneer het doel van wondbehandeling is optimale huidregeneratie zonder littekenvorming. Voor de behandeling van chronische wonden zijn er

tegenwoordig 2 artificiële huid producten op de markt die allogene huidcellen bevatten (20,21). Ondanks dat het doel van deze producten de bevordering is van de genezing van chronische wonden, is nu al duidelijk dat de toekomstige marketing-strategie in de richting van volledige huidsubstitutie gaat. De resultaten van deze studie geven aan dat immunologische reacties tegen lichaamsvreemde cellen kunnen leiden tot meer littekenvorming.

In de praktijk komen er nog andere belangrijke problemen bij huidsubstitutie om de hoek kijken. Chronische wonden en brandwonden zijn bijna altijd gecontamineerd met bacteriën (22) en bevatten veel proteolytische enzymen (23-25). Dit betekent dat er een groot gevaar is dat kostbare huidsubstituten verloren gaan of te snel worden afgebroken en huidsubstitutie kan dan zelfs leiden tot een verslechtering van de genezing. In het verleden is meerdere malen aangetoond dat de meeste desinfecterende middelen (ook jodium houdende producten) slecht waren voor de wondgenezing, met name door hun toxiciteit voor huidcellen (26-28). Daarnaast is het gebruik van antibiotica minder wenselijk vanwege het gevaar van bacteriële resistentie.

In hoofdstuk 6 is de invloed van een wonddressing getest op de genezing van "schone" normaal genezende wonden. Deze dressing bestaat uit een cadexomer-jodium ointment en heeft een sterke antimicrobiele werking. De genezing van wonden behandeld met deze dressing werd vergeleken met de genezing van wonden behandeld met dezelfde dressing maar zonder jodium en wonden behandeld met gazen gedrenkt in fysiologisch zout. Deze laatste behandeling is een standaard behandeling in het AMC voor het schoonmaken van chronische wonden. De wonden behandeld met de cadexomer-jodium dressing genazen met een snellere regeneratie van de epidermis. Dit werd geconcludeerd uit observaties dat wonden sneller dicht gingen, een dikkere epidermis hadden die meer tekenen van differentiatie vertoonde in een vroeg stadium van de genezing. De cadexomer-jodium dressing laat langzaam de jodium vrij en klaarblijkelijk in zulke lage concentraties dat weefsel- of cel-toxiciteit niet werden waargenomen en de wondgenezing zelfs werd gestimuleerd. Systemische jodium opname werd ook waargenomen, maar bleef ver beneden toxische niveaus. Eén week na het stoppen van de behandeling was het jodium niveau in het bloedplasma weer

teruggekeerd op zijn normale niveau.

Het onderzoek naar artificiële huidvervangers heeft een belangrijke rol gespeeld bij het begrijpen van wondgenezingsprocessen en met name van de huidregeneratie. Bij de huidregeneratie spelen fibroblasten een centrale rol, maar er is echter nog maar weinig bekend over het gedrag en de differentiatie van fibroblasten, en of er verschillende fenotypes bestaan. Een beter begrip hierover is nodig en kan veel bijdragen in de ontwikkeling van artificiële huidproducten. Deze kennis kan tevens leiden tot de ontdekking van nieuwe anti-fibrotische stoffen, iets wat zeer relevant is voor andere fibrotische huidziektes zoals Scleroderma en keloid, en orgaan fibrose die ontstaat na ontstekingsreacties in het orgaan.

In de nabije toekomst zal artificiële huid zijn weg vinden naar de klinische praktijk. Deze klinische implementatie zal gepaard gaan met mogelijke nieuwe problemen: voeding van de huidcellen in het substituut gedurende de eerste dagen na transplantatie; de controle op bacteriegroei en de activiteit van proteolytische enzymen; en het oogsten en de vermeerdering van de autologe huidcellen. In de praktijk blijkt het dat de aetiologie van wonden zo verschillend is dat de meest ideale wondbehandeling er eentje moet zijn die interactief met de wondomgeving communiceert en daarop reageert. Dit kan gerealiseerd worden met behulp van een wondverband of huidsubstituut met daarin levende cellen in combinatie met een dressing die voedingstoffen voor de cellen bevat die in staat moet zijn om bacteriegroei en ontstekingsprocessen te remmen.

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Curriculum vitae

De auteur werd geboren in Bunschoten in het jaar 1967 november de negentiende als oudste in een gezin met drie kinderen. Vanaf zijn vijfde levensjaar bracht hij zijn jeugd door in Alkmaar waar hij lager en middelbaar onderwijs ontving. Na zijn diploma Atheneum B behaald te hebben, begon hij in 1986 met zijn studie scheikunde, waar al snel de liefde voor het levende organisme de boventoon begon te voeren en de richting biochemie werd gekozen. Er werden tijdens zijn studie meerdere stages gelopen met immunologische en moleculair biologische onderwerpen: op het CLB olv Rene van Lier en Rolien de Jong, bij de afdeling Moleculaire Biologie olv L.A..Grivell en in Parijs bij het bedrijf Roussel Uclaf op de afdeling Immunologie. Na zijn terugkeer uit Parijs studeerde hij af in Augustus 1992, waarna een carrière in het onderzoek werd nagestreefd maar niet als zijnde AIO. Dit bleek een bijna onmogelijke opgave en na de hoop al opgegeven te hebben, vond hij bij toeval een baan als analist bij de wondgenezingsgroep van Dr. Wiete Westerhof. Al snel werd de leiding van het basale onderzoek door Esther Middelkoop overgenomen en de interesse voor het wondgenezingsonderzoek verder aangewakkerd. Het arbeidscontract werd meerdere keren verlengd doordat er meerdere projecten voor industriële sponsors werden uitgevoerd. Tijdens het onderzoek werd zijn leven verrijkt met 2 lieve maar ondeugende kinderen. In de loop der tijd werd ook het idee opgevat dat het mogelijk moest zijn om op het onderzoek te promoveren en wat met dit boekje een feit lijkt te zijn geworden. Per 1 maart 1999 is hij gedurende 4 dagen werkzaam bij het bedrijf IsoTis in Bilthoven, waar hij in teamverband meewerkt aan de verdere ontwikkeling en klinische implantatie van een artificieel huidsubstituut, Biskin. Gedurende 1 dag per week is hij verbonden aan de Brandwonden Stichting, waar hij op de Research Afdeling meehelpt in de begeleiding van verschillende projecten binnen de onderzoekslijn "Huidequivalenten en Kwaliteit van Wondgenezing.

List of scientific publications

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4. Lamme EN, van Leeuwen RTJ, Jonker A, van Marle J, Middelkoop E. ***Living skin substitutes : survival and function of fibroblasts seeded in a dermal substitute in experimental wounds***. J.Invest.Dermatol. 111: 989-995, 1998

5. Loots MAM, Lamme EN, Zeegelaar JE, Mekkes JR, Bos JD, Middelkoop E. ***Differences in cellular infiltrate and extracellular matrix of chronic diabetic and venous ulcers versus acute wounds.*** J.Invest.Dermatol. In press.

6. Lamme EN, van Leeuwen RTJ, Brandsma K, Jonker A, van Marle J, Middelkoop E. ***Higher numbers of autologous fibroblasts in an artificial dermal substitute improve tissue regeneration and modulate scar-tissue formation in experimental full thickness wounds.*** Submitted to J.Pathol. 10-1998

7. Loots MAM, Lamme EN, Mekkes JR, Bos JD, Middelkoop E. ***Cultured fibroblasts from diabetic ulcers (diabetes mellitus type 2) show disturbed proliferation.*** In press Arch Dermatol Research 1998

8. Van Leeuwen RTJ, Lamme EN, Everts V, Verheijen JH, Middelkoop E. ***The proteolytic enzymes urokinase and MMP-1 and -9 are increased during early healing of full-thickness porcine skin wounds treated with artificial dermal substitutes.*** Submitted to J.Invest.Dermatol. 9-1998.

9. Lamme EN, van Leeuwen RTJ, Mekkes JR, Middelkoop E. ***The use of allogeneic fibroblasts for dermal substitution induces inflammatory responses and interferes with dermal tissue regeneration.*** Submitted to Transplantation 11-1998.

List of abbreviations

AA	arachidonic acid
AC	adenylate cyclase
ADAM	adamalysin-related proteinases
AJ	adherense junctions
AKAP	PKA-kinase anchoring proteins
Akt	Protein kinase B isoform
ATP	adenosine trisphosphate
BMP	bone morphogenetic protein
C1-inhibitor	complement-type1-inhibitor
cAMP	cyclic adenosine monophosphate
CAPK	ceramide activated protein kinase
CBP and P300	CREB binding proteins
CER	ceramide
CERase	ceramidase
CP	carboxypeptidase
CRE/AP1	cyclic-AMP response element
CREB	cyclic-AMP response element binding protein
CTAP-III	connective tissue-activating protein-III
DAG	diacylglycerol
ECM	extracellular matrix molecules
EGF	epithelial growth factor
ENA-78	epithelial-derived neutrophils chemoattractant-78
ERK	extracellular signal-regulated kinase
ERM	ezerin, radixin, moessin protein family
ET	endothelin
FAK	focal adhesion kinase
FAS	focal adhesion sites
FGF-1, -2	acidic- and basic-fibroblast growth factor
fMLP	N-formylmethionyl peptide
GAS	interferon-γ activated sequences
GCP-2	granulocyte chemotactic protein-2
GDP	guanidine di-phosphate
GM-CSF	granulocyte monocyte-colony stimulating factor
GRO	growth related oncogene
GTP	guanidine tri-phosphate
HA	hyaluronic acid
HB-EGF	heparin-binding EGF-like growth factor
HCC	hemofiltrate CC-chemokines
HETE	hydroxyeicosatetraenoic acid
HGF	hepatocyte growth factor
HPETE	hydroperoxyeicosatetraenoic acid
ICAM	intracellular adhesion molecule
IFN	Interferon
IGF	insulin-like growth factor
IL	interleukin
IP-10	interferon-inducible protein-10
IP3	inositol 1,4,5-trisphosphate
IRS	insulin receptor substrate protein
ISRE	interferon stimulated response element
JAK/TYK	Janus kinases
KGF	keratinocyte growth factor
LPA	lysophosphatidate
LPS	lipopolysaccharides
LT	leukotriene
LTBP	latent TGF-β-binding protein

MAPK	mitogen activated protein kinase
MCP	monocyte chemoactractant protein
MEKK	MAPK/ERK kinase kinase
Mig	monokine induced by IFN-γ
MIP	macrophage inflammatory proteins
MKK	MAP kinase kinase
MMP	metallo-proteinase
MT-MMP	membrane-bound MMP
MW	molecular weight
NAP-2	neutrophil activating protein-2
NO	nitric oxide
nSMase	neutral sphingomyelinase
p70-S6K	protein kinase (70 kDA) specific for ribosomal protein S6
PA	phosphatidate
PAF	platelet activating factor
PAI	plasminogen activator inhibitor
PAK	p21 activated kinase
PBP	platelet basic protein
PC	phosphatidyl choline
PDGF	platelet derived growth factor
PE	phosphatidyl ethanolamine
PECAM	platelet-endothelial cell adhesion molecule
PF4	platelet factor 4
PG	prostaglandin
PI	phosphatidylphosphate
PI-3-K	phophatidylinositol-3-OH kinase
PI-4P-5K	phosphatidylinositol-4-monophosphokinase
PIP2	phosphatidylinositol 4,5 bisphosphate
PIP3	phosphatidylinositol 3,4,5 trisphosphate
PKA and PKC	protein kinase A and C
PKC	protein kinase C
PLA2, PLC,PLD	phospholipase A2, C and D
PTP	protein tyrosine phosphatase
RANTES	regulated on activation, normal T cell expressed and secreted
SAPK	stress-activated protein kinase
SDF	stromal cell derived factor
SEK	SAPK/ERK kinase
SH	Src-kinase homology domain
SKALP	skin-derived anti-leukoproteinase
SM	sphingomyelinase
SMAD	stands for Sma and Mad gene proteins in drosophila and C.elegans
SMase	sphingomyelinase
SPARC	secreted protein acidic and rich in cysteine
SPH	sphingosine
STAT	signal transducers and activators of transcription
TAK1	TGF-beta activated kinase
TARC	thymus and activation-regulated protein
TGF-ß	Transforming growth factor ß
TIMP	tissue-inhibitor of MMP
TM	transmembrane spanning
TNF-α	tumor necrose factor-alpha
tPA	tissue-type plasminogen activator
Tx	tromboxane
uPA	urokinase-type plasminogen activator
V-ATPase	vacuolar H^+-ATPase
VEGF	vascular endothelial growth factor