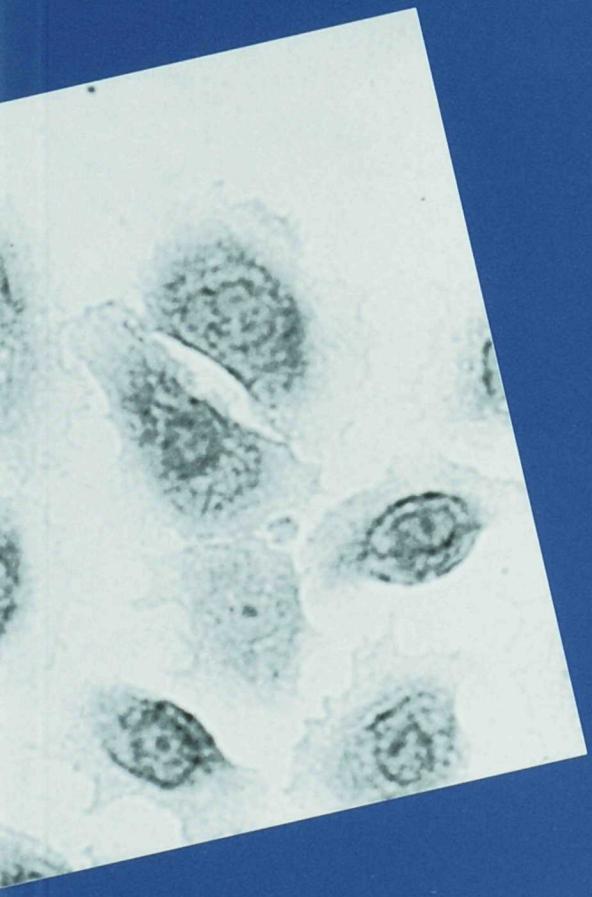
Regulation of expression of complement components, complement regulatory proteins, and chemokines in keratinocytes



Marcel Pasch

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Publication of this thesis was financially supported by:

Huidstichting Chanfleury van IJsselsteijn, European Immunodermatology Society, Stiefel Laboratories, Janssen-Cilag, Fujisawa, BIOGEN, Galderma, Yamanouchi Pharma, Bauerfeind, Bipharma Weesp, Glaxo Wellcome, Leo Pharma, Novartis Pharma, Roche Nederland, Schering Nederland, UCB Pharma.

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Regulation of expression of complement components, complement regulatory proteins, and chemokines in keratinocytes
Thesis University of Amsterdam

ISBN 90-9013570-7
NUGI 743

Printed by PrintPartners Ipskamp

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ACADEMISCH PROEFSCHRIFT

Ter verkrijging van de graad van doctor
aan de Universiteit van Amsterdam
op gezag van de Rector Magnificus
prof.dr. J.J.M. Franse
ten overstaan van een door het college van promoties ingestelde
commissie, in het openbaar te verdedigen in de Aula der Universiteit
op dinsdag 28 maart 2000, te 14.00 uur

door

Marcel Christian Pasch

geboren te Solingen

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The studies described in this thesis were conducted at the Department of Dermatology (Neurozintuigen Laboratory, Laboratory of Experimental Dermatology), Academic Medical Center, University of Amsterdam

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Chapter One

General Introduction

Part of this chapter has been published in: Asghar SS and Pasch MC, Complement as a promiscuous signal transduction device. Lab. Investigation 78: 1203-1225, 1998

At present, the mechanisms leading to inflammatory diseases of the skin are not fully understood. Recently, keratinocytes have been shown to be initiators of inflammation in the skin[1]. They comprise about 95% of the cells in epidermis and are known to produce cytokine (and chemokines) network. More recently, they were shown to be capable of expressing at least two components and several proteins belonging to another powerful system of inflammation, namely the complement system. Both the cytokine/chemokine and the complement systems are of great importance for initiation and maintenance of inflammation.

Several models to study skin inflammation have been proposed in recent years by many investigators. An overview of the potential events involved in keratinocyte mediated skin inflammation is summarized as a unified model in **Figure 1**. Several immunological factors, such as cytokines, released from infiltrating inflammatory cells and keratinocytes, can induce the release of cytokines/chemokines from keratinocytes. Some cytokines have been shown to induce the synthesis of at least two complement components from keratinocytes.

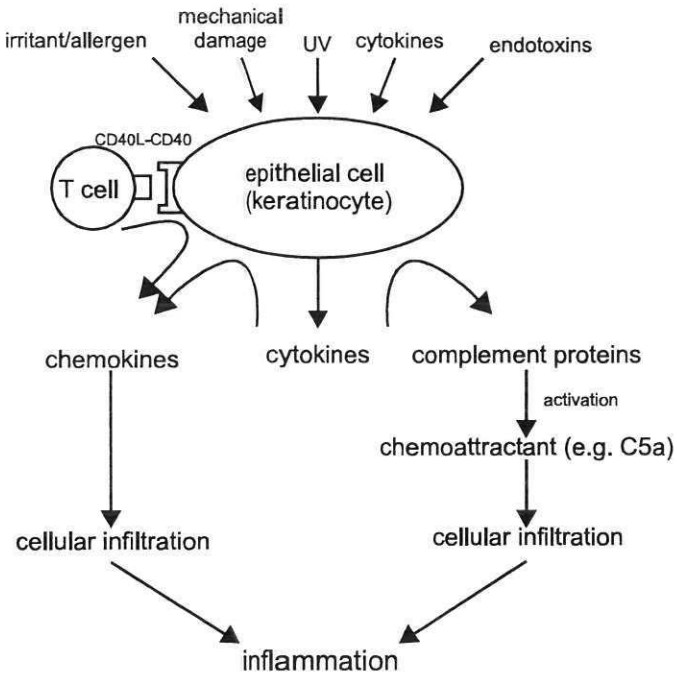

Figure 1. Overview of the potential processes involved in epithelial cell mediated skin inflammation

Activated T-cells are known to express CD40 ligand (CD40L) transiently and interact with CD40 expressing keratinocytes to release at least one cytokine from keratinocytes. Activated T cells may be postulated also to induce the release of some complement components from these cells and to express some other complement proteins on them. Some cytokines can up-regulate the expression of adhesion molecules on endothelial cells of cutaneous blood vessels and skin structures and cells. Chemokines and chemotactic complement fragments such as C5a finally make inflammatory cells to traverse across adhesion molecules expressing endothelium and infiltrate the skin, resulting in inflammation. Several external factors, such as mechanical damage, allergens, ultraviolet (UV) irradiation, and bacterial endotoxins also trigger keratinocytes to release cytokines (and chemokines). It may be postulated that at least some of the external factors can induce keratinocytes to enhance expression of complement proteins and thereby induce inflammation.

In the following sections the complement system and the cytokine/chemokine network, and their expression in keratinocytes will be briefly described. A short description of the role of these systems in inflammation of the skin will also be given.

THE COMPLEMENT SYSTEM

Complement is one of the most powerful effector systems involved in body's defense and inflammation. It consists of some 30 proteins which include soluble complement components, soluble regulators of complement activation, cell membrane embedded regulators of complement activation and complement receptors[2;3]. All these proteins together provide a system capable of destroying a large variety of pathogens without damaging autologous cells. Complement destroys pathogens either directly by causing their lysis or indirectly by recruiting phagocytic cells. Besides being a destructive entity for pathogens, the complement system has long been known to be a mediator of a variety of functions such as chemotaxis, phagocytosis, mast cell degranulation, and B and T cell activation. Recently, the complement system has been assigned new functions, many of them mediated by complement-receptors. These include enhancement of immunogenicity of antigens[4] and regulation of IgE synthesis[5;6]. Some complement-receptors also regulate activation of complement on body cells[2;3;7;8].

The primary site for the biosynthesis of plasma complement is the liver. However, cells of organs other than liver also produce complement components. The list of cells which have been shown to produce complement is growing rapidly and includes monocytes/ macrophages, fibroblasts, astrocytes, keratinocytes, endothelial cells, leukocytes, cells of renal glomerulus, and synovial lining cells[9-11]. Locally produced complement is believed to be involved in triggering and perpetuating inflammation at local tissue level under the conditions when recruitment of plasma complement does not occur[9-11]. It also appears to perform other functions assigned to complement in general, at tissue level.

The physiological and pathological importance of local synthesis of complement in different organs is beginning to be realized[10;11]. Here we focus our attention upon the complement system, its synthesis and regulation of its synthesis in keratinocytes and involvement of keratinocyte-derived complement in skin inflammation.

Mechanisms of complement activation

Several reviews on the complement system have recently been published[12-14]; therefore only a passing reference of the two pathways of complement will be made here (**Figure 2**). In general, complement activation on foreign target cells via the classical pathway is initiated after the target cells are sensitized with the antibody. After the binding of an antibody to a cell surface antigen, the first component of complement (C1) is activated as follows. One of the subunits of C1, C1q, recognizes and interacts with the Fc portion of the antibody. This results in the activation of other subunits, C1r and C1s, within C1. Activated C1s ($C1\bar{s}$) then split the fourth

component of complement (C4) into a smaller fragment, C4a, and a larger one, C4b. In freshly formed C4b, a thioester bond becomes exposed, which reacts rapidly with any nearby electron-donating group. Thus, some of the freshly formed C4b can react covalently with the amino or hydroxyl group on sensitized target cells. C4b also has a binding site for the second component of complement (C2), which leads to the generation of C4b2 complex on the target cell. In this complex, if C2 is appropriately oriented toward $C1\bar{s}$, it is cleaved by this enzyme. C4b2a complex, thus formed, is a protease with its active site in C2a. This enzyme, known as the C3-convertase of the classical pathway, can cleave native C3 to generate C3b.

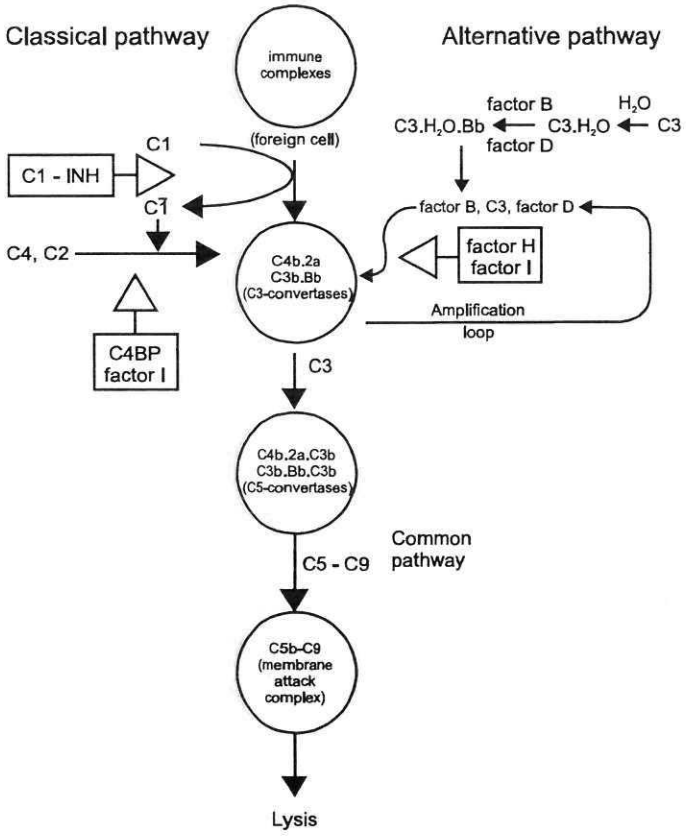

Figure 2. Diagrammatic representation of the mechanism of lysis of foreign cell by classical and alternative pathways of the complement system. Both pathways are described briefly in the text. To keep the diagram simple, smaller complement products have not been depicted and the role of properdin in stabilizing C3/C5-convertase of alternative pathway has not been illustrated. Fluid phase complement regulatory proteins have been boxed with attached large empty arrow heads indicating the steps at which they regulate complement activation.

The activation of the alternative pathway is initially dependent on the continuous exposure of a thioester bond otherwise buried inside the C3 molecule[15]. This exposure takes place likely as a result of normal thermal unfolding. The exposed thioester bond is highly reactive and reacts with nucleophilic groups such as amino and hydroxyl groups. In fluid phase, most of the C3 with an exposed thioester bond reacts with water to form $C3.H_2O$. This intermediate can combine with factor B present in body fluids to generate $C3.H_2O.B$ complex which, in the presence of an enzyme factor D, is converted to $C3.H_2O.Bb$ complex. This complex is an enzyme known to be the initial C3-convertase of the alternative pathway. It can cleave C3 to generate C3b fragments. At this point, freshly formed C3b, which like C4b also has an exposed thioester bond, can rapidly bind covalently to hydroxyl or amino groups of molecules present in the membranes of foreign target cells or in the self cells present in the immediate vicinity. Membrane-bound C3b combines with factor B. In the presence of factor D, the C3b.Bb complex is generated. This complex is known as the C3-convertase of the alternative pathway. On foreign target cells, this enzyme performs its function of cleaving C3 to C3b, whereas on self cells, it is destroyed as we will see below (" *Control of complement activation* "). Fluid phase C3bBb is produced when C3b and factor B bind to each other through their mutual binding sites, followed by the action of factor D. Fluid-phase as well as membrane-bound C3bBb is stabilized by properdin. Both C3-convertases generate large amounts of C3b and thus cause amplification of C3b generation and C3bBb formation in fluid phase and on cell surfaces.

C3b generated by the classical or alternative pathway C3-convertases combines with the respective dimolecular C3-convertases (C4b2a and C3bBb, respectively). This results in the generation of C5-convertases of the classical ($C4bC2aC3b_n$; n represents the number of C3b molecules) and alternative ($C3bBbC3b_n$) pathways. C5-convertases are formed mainly on the cell surface but not significantly in fluid phase because of the low degree of quick availability of an appropriate orientation for attachment of C3b to soluble C3bBb. C5 then combines with C4b and C3b subunits of C5-convertase of the classical pathway or C3b and Bb subunits of C5-convertase of the alternative pathway in an orientation suitable for cleavage by C2a or Bb, respectively, within these C5-convertases. C5-convertases cleave C5 to C5a and C5b. Metastable C5b remains loosely bound to C5-convertases; C6 then combines with it. The binding of C7 to C5b6 complex causes the formation and release of C5b-7 from the C5-convertases and exposure of its metastable membrane binding site. At this point, the C5b-7 complex can bind to a foreign invading target cell or host cell present in the immediate vicinity. Incorporation of C8 and then C9 into the C5b-7 complex causes self-assembly of the cytolytic membrane attack complex (C5b-9; MAC) on a foreign cell and non-cytolytic MAC on a self cell, as will be described in more detail in the following sections. Cytolytic MAC can form pores in the membrane of the foreign target cell, resulting in its death.

Control of complement activation

As described above, C3b generated by classical and alternative pathway C3-convertases can cause subsequent activation of complement on a foreign invading cell such as a bacterium and eliminate it. Similarly, C3b formed by C3-convertases of both pathways can get fixed on a self cell, cause subsequent activation of complement on it, and can damage it. Although elimination of the foreign cell by the complement system is highly desirable, damage of self cells must be prevented. Damage of self cells is prevented by the following mechanisms, which control activation of complement in fluid phase and on the surface of autologous cells.

Mechanisms that control activation of complement in fluid phase

The mechanisms that protect self cells from classical or alternative pathway-mediated lysis by keeping complement dormant in fluid phase involve several plasma inhibitors and inactivators that act at virtually every step of the complement cascade. These regulators minimize the formation of complement fragments, such as C4b and C3b, and complement complexes, such as C5b-7, so that a minimum of them in active form collide with self cells and a minimum of complement activation on self cells occurs. These include C1-inhibitor (C1-INH), factor H, C4-binding protein (C4BP), factor I, vitronectin and clusterin (**Figure 2, Table 1**).

C1-INH has recently been reviewed[16], and its structure is known. C1-INH keeps the activation of C1 checked in circulation and body fluids. It efficiently interacts with $C1\bar{r}$ and $C1\bar{s}$ to form C1r-C1s-$(C1\text{-}INH)_2$ complex and causes the dissociation C1r and C1s from C1q. Inhibition of $C1\bar{s}$ results in the inhibition of both the cleavage of C4 and C2 and the formation of C4b and C2a. C1-INH inhibits C1 100-times more strongly in fluid phase than on cell surface[17].

Factor H[18], C4BP[19] and factor I[20] have recently been reviewed. They prevent the assembly of C3/C5-convertases of the classical and alternative pathways in fluid phase. Factor H and C4BP combine with freshly formed C3b and C4b, respectively. They then develop cofactor activities for the enzyme factor I, which cleaves the α-chains of C3b and C4b to inactivate them before they collide with self cells. In this way, they protect self cells. In the context of this thesis description of factor H is important. Models of domain structures of factor H are depicted in **Figure 3.** C4BP controls the activation of the classical pathway and factor H controls the activity of both pathways more efficiently in fluid phase than on cell surface.

Two structurally distinct serum proteins, vitronectin[21] and clusterin[22], whose structures and functions have recently been reviewed, inhibit MAC formation by identical mechanisms. Both bind to C5b-7 as it is assembled in fluid phase. C5b-7 complex in which vitronectin or clusterin has been incorporated is unable to fix on cell membranes but can take up C8 and C9 molecules to form nonlytic C5b-8 and C5b-9 complexes, respectively. Thus, vitronectin and

clusterin render the MAC nonlytic and offer protection to the self cell.

Humoral mechanisms minimize the formation and maximize the inactivation of complement fragments and complexes. In this way, these mechanisms prevent complement fixation and activation on body cells, thereby preventing their damage.

Table 1. *Fluid-Phase and Cell-Membrane Regulators of Complement Activation**

Regulator	Ligand specificity	Functional activity
Fluid phase regulators		
C1 stage		
C1-INH	C1r/C1s	Inhibits C1r and C1s mainly in fluid phase
C3/C5 convertase formation stage		
C4BP	C4b	Cofactor for factor I in cleavage of C4b
Factor H	C3b	Cofactor for factor I in cleavage of C3b
Factor I	Factor H/C4BP MCP/CR1	Cleaves C3b and C4b using cofactors
MAC formation stage		
Vitronectin	C5b-8/C9	Prevents the assembly of cytolytic MAC
Clusterin	C5b-8/C8	Prevents the assembly of cytolytic MAC
Membrane embedded regulators		
C3/C5-convertase formation stage		
DAF	C4b/C3b	Dissociates C2a and Bb from C4b and C3b
MCP	C3b/C3b	Cofactor for factor I in cleaving C4b and C3b
MAC formation stage		
CD59	C5b-8/C9	Prevents assembly of cytolytic MAC on self cell
HRF	C5b-8/C9	Prevents assembly of cytolytic MAC on self cell

* Abbreviations used: C4BP, C4-binding protein; CR1, complement receptor-1; DAF, decay-accelerating factor; HRF, homologous restriction factor; INH, inhibitor; MAC, membrane attack complex; MCP, membrane cofactor protein.

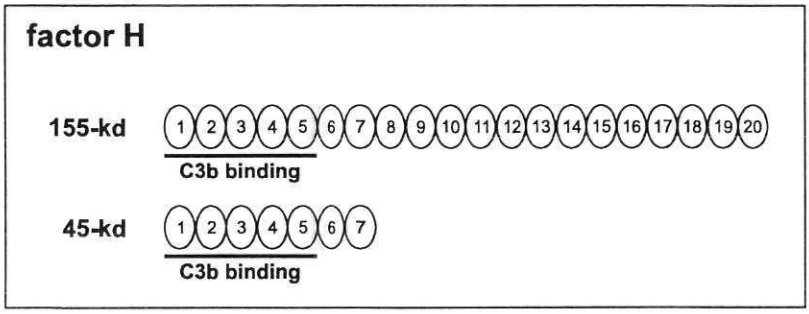

Figure 3. Schematic representation of the structural organization of the large and the small forms of factor H protein. The 155-kD factor H protein consists of 20 short consensus repeats (SCRs) (open circles) and the 45-kD factor H protein of 7 SCRs. The first 5 SCRs are involved in binding of C3b.

Mechanisms that control activation of complement on autologous cell surface

Despite efficient control of activation of the classical and alternative pathways by plasma regulators, some complement fragments and complement complexes can escape regulation, get deposited on self cells, and activate the rest of the complement cascade on their surfaces[2;13;14]. Activation of complement by either pathway can potentially damage self cells. Activation of complement on self cells is, however, prevented by multiple membrane-embedded regulators of complement. These include decay-accelerating factor (DAF), membrane cofactor protein (MCP), complement receptor-1 (CR1), CD59 and homologous restriction factor (HRF). The ligand specificities and functional activities of these regulators are summarized in **Table 1**.

DAF[13], MCP[13], and CR1[8] restrict complement activation on self cells at the C3/C5-convertase formation stage. Models of the domain structures of DAF, MCP, and CR1 are shown in **Figure 4**. The cell type distribution of DAF is very wide. When C4b and C3b are fixed on a self cell, DAF present in the membrane of the same cell binds to the deposited C4b and C3b and inhibits the interaction of C2 with C4b and of factor B with C3b. It also dissociates C2a and Bb from preformed C4b2a and C3bBb, respectively. Thus, DAF inhibits activation of complement on the surface of a self cell at the C3-convertase formation stage and protects the cell from both pathways of autologous complement. MCP is also expressed on a wide variety of cell types. When C4b and C3b formed during complement activation are fixed on the membrane of a self cell, MCP present in the membrane of the same cell combines with these fragments and develops cofactor activity for factor I, which then proteolytically degrades C4b and C3b to inactive products iC4b and iC3b, respectively. Thus, MCP interferes with ongoing classical and

alternative pathways on an autologous cell by intercepting the formation of C3/C5-convertases and mediating their destruction. CR1 is present on a limited number of cell types (**Table 1**). When C4b and C3b formed during complement activation are fixed on the membrane of a self cell, CR1 present in the membranes of CR1 bearing cells combines with these fragments and acts as a cofactor for the enzyme factor I, which cleaves C3b to iC3b; iC3b to C3c and C3dg; C4b to iC4b; and iC4b to C4c and C4d. Thus, CR1 can regulate the formation and activities of C3/C5-convertases of the classical and alternative pathways on cells on which it is expressed.

CD59[23] and HRF[2;24] control complement activation on the surface of a self cell at the MAC formation stage. Both have a wide tissue distribution. The amino acid structure of CD59 has been determined; a schematic model is presented in **Figure 4**. The amino acid structure of HRF is not yet known, but it has been shown to be a distinct 65-kD single chain protein that during storage is cleaved into a fully active 38-kD product. HRF, like DAF and CD59, is anchored to the cell membrane through its glycosylphosphatidylinositol (GPI)-anchor. CD59 inhibits complement lysis by preventing the assembly of cytolytic MAC. It accomplishes this by binding to an epitope on the α-chain of C8 that is exposed when C8 interacts with the C5b-7 complex on the cell membrane, on the one hand, and to an exposed site of C9, on the other. HRF inhibits the assembly of MAC in a manner reminiscent of CD59.

DAF, MCP, HRF, and CD59 (and CR1 on CR1 bearing cells) act synergistically to control different steps of complement activation on self cells and protect them from both complement pathways.

Control of cell lysis by elimination of membrane attack complex (MAC)

The above-described humoral and membrane-associated mechanisms, in most instances, do not allow cytolytic MAC formation on autologous cells. However, if activation of complement is so extensive that a limited number of cytolytic MAC complexes have been formed on the body cells, these complexes are eliminated by vesicular shedding and endocytosis[26;27].

Expression of functional receptors for complement components and their fragments on keratinocytes

Local complement activation can have profound non-lethal effects on keratinocytes through interaction with specific complement receptors or through membrane attack complex-induced cell activation[3].

Most of the activities of complement are mediated through interaction of complement fragments with specific receptors on effector-cell membranes. Key players in this are CR1, CR2,CR3 and CR4, which bind to large, opsonized fragments of C3 and C4, and C3aR and C5aR

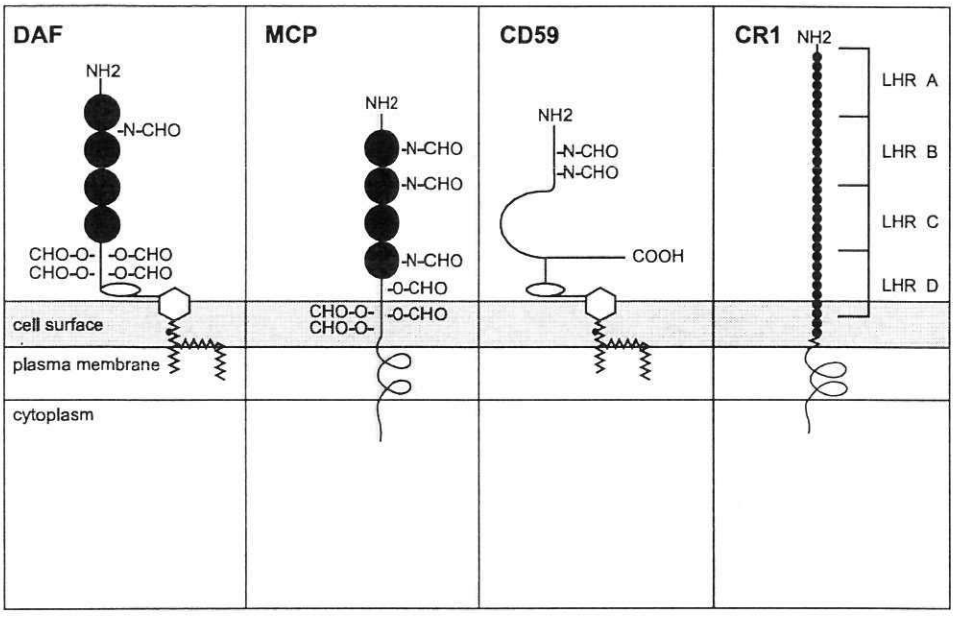

Figure 4. Diagrammatic representation of structural organization of membrane embedded complement regulatory proteins. DAF: A single chain protein containing four short consensus repeats (SCRs) (closed circles) at amino terminus. Short consensus repeats are referred to repeating domains of approximately 60 amino acids which are believed to be involved in protein-protein interactions[25]. Locations of a single N-linked carbohydrate side chain (-N-CHO) and of multiple O-linked carbohydrate chains (-O-CHO-) present in DAF are shown. A glycosylphosphatidylinositol (GPI)-moeity at the carboxy terminal is involved in anchoring DAF into the cell membrane. MCP: A single chain protein which contains four SCRs (closed circles) at amino terminus. Locations of three -N-CHO- side chains are indicated. Next is an STP-rich heavily O-glycosylated region followed by a hydrophobic transmembrane spanning domain and basic amino acid-region typical of cytoplasmic anchor and the rest of the cytoplasmic tail. CD59: Made up of 103 amino acid residues. Amino terminus 70 of these are rich in cysteine and constitute the main body and the remaining 33 a hydrophobic tail. Residue 71 appears to be the site of GPI-anchor attachment through which the molecule is embedded in cell membranes. CR1: The extracellular domain of a most common allotype (allotype A; frequency 0.82) of CR1 consists of 30 SCRs (closed circles) 28 of which are organized into four homologous repeats (LHR) each comprising of 7 SCRs. The extracellular domain is followed by a transmembrane spanning segment and a cytoplasmic tail. SCRs 1-2 (most N-terminal) of LHR-A have a binding site for C4b and SCRs 8-9 of LHR-B and SCRs 15-16 of LHR-C have binding sites for C3b. Collagen region of C1q binds to CR1 at two points: SCRs 22-28 of LHR-D, a region most homologous to SCR of C1r and C1s, and the last two SCR 29 and 30.

which bind to small chemotactic fragments of C3 and C5. Data indicate that CR1, the receptor for C3b, and CR2, the receptor for C3d, are distributed among cells of the epidermis as well as on cultured keratinocytes[28]. *In situ* hybridization experiments in this study also suggested the expression of CR1 and CR2 mRNA in human epidermis. Expression of CR2 has also been shown at protein and mRNA level[29] on keratinocytes and keratinocyte cell lines RHEK-1 and HeLa. There are no reports on expression of CR3, CR4, and C1qR on keratinocytes.

Recently, Werfel *et al.*[30] have studied the expression of C5aR on keratinocytes. Among the evidences which favored the conclusion that C5aR is not expressed by keratinocytes were the fact that C5a did not cause transient Ca^{2+} fluxes in keratinocytes and C5aR mRNA could not be detected in keratinocytes or in HaCat cells. C3aR on keratinocytes remains to be studied.

Expression and regulation of complement proteins in keratinocytes

In the skin, epidermis is separated from the dermis by a basement membrane. The epidermal compartment is avascular. In the dermal compartment, plasma in the blood vessels is separated from the dermal tissue by vascular endothelial cells and underlying basement membrane which act as a molecular sieve and restrict the passage of large molecules, including complement components, into the dermis[31]. Thus, plasma complement will not reach the dermis and the epidermis in the presence of an intact microvascular system. The absence of plasma complement within dermal-epidermal tissue is perhaps an advantage in that it prevents the damage to self cells but is disadvantageous in that an important component of immune defense is missing at this vital site. Therefore, there should exist a mechanism(s) which switches on the local synthesis of complement in the skin when it is needed and shuts it off when it is not needed. One major cell type which can synthesize complement locally could be the keratinocyte, and signals which could switch on the synthesis of complement by keratinocytes could be some cytokines. Keratinocytes respond to a variety of cytokines[32], resulting in the expression of several molecules including adhesion molecules and MHC antigens. Therefore, it is highly likely that keratinocytes also synthesize many other proteins, including complement, in response to cytokines. However, complement synthesizing capacity of keratinocytes has not yet been thoroughly explored.

The possibility that keratinocytes may be capable of synthesizing complement was raised by Basset-Seguin *et al.*[33] who showed that C3dg, a 41-kD fragment of α chain of C3, is present in normal human epidermal basement membrane but absent in dermal vascular basal membrane. They hypothesized that epidermal keratinocytes synthesize C3 whose breakdown product, C3dg, is passively incorporated into the adjacent epidermal basement membrane and becomes its integral part. Later studies by these workers showed that human keratinocytes and

a human keratinocyte cell line, A431, in culture produce C3[34] and factor B[35]. This was demonstrated at mRNA level from harvested cells and at protein level from products released in culture by Dovezenski *et al.*[28]. More recently, Terui *et al.* showed that constitutive secretion of C3 by keratinocytes was very low but synthesis of C3 was greatly enhanced by IFN-γ and TNF-α[36].

Synthesis of C3 and factor B raised the question as to whether keratinocytes are able to synthesize other components of complement as well, particularly the late components. Perhaps keratinocytes will be found to produce a complete functional complement system. Keratinocytes themselves can synthesize inflammatory cytokines, raising the prospect that some cytokines can switch on complement biosynthesis in an autocrine manner.

Keratinocyte-derived complement can provide immune defense against pathogens but it may also damage cells in the epidermis. Activation of this complement must, therefore, be tightly regulated likely by the same mechanisms which regulate complement activation in the plasma. In the plasma, activation is restricted by fluid phase regulatory proteins (*e.g.*, C1-inhibitor, C4 binding protein, factor H, factor I) that inhibit at several stages in the complement pathways (**Figure 2, Table 1**). Human keratinocytes have not yet been studied for their ability to synthesize these proteins. The synthesis and the regulation of synthesis in keratinocytes of even factor H which plays a pivotal role have not yet been studied. By virtue of being able to inactivate C3b, factor H is a restrictive factor in the assembly of C3/C5 convertase of both the classical and alternative pathways. Production of C3 and factor B suggests that keratinocytes may also be able to produce factor H to regulate the activation of these components in fluid phase as a part of the mechanism of prevention of epidermal cell damage by autologous complement. The role of factor H in suppression of complement at C3/C5-convertase stage demands that, if produced by keratinocytes, its production be up-regulated when there is up-regulation of C3 and factor B production. Therefore, the mechanisms for the regulation of the synthesis of factor H may also involve some inflammatory mediators.

More important for the survival in the face of complement attack is the fact that human keratinocytes express membrane regulators, decay accelerating factor (DAF), membrane cofactor protein (MCP), and in particular abundance, CD59[28;37]. Owing to the high level of expression of membrane regulators, human keratinocytes and human keratinocyte cell line SCC-12F are extremely resistant to complement lysis[38;39]. In human diseases aberrant expression of complement regulatory proteins on some cell types has been observed. These include paroxysmal nocturnal hemoglobulinuria, in which increased susceptibility of eryhrocytes to complement mediated lysis is caused by genetic deficiencies in expression of DAF and myocardial infarction in which infarcted areas of the myocardium is selectively deficient in CD59[2]. Decreased expression of complement regulatory proteins has also been observed on keratinocytes (and also on melanocytes and other cells) in lesional in comparison to non-lesional epidermis in vitiligo[40].

The mechanisms and the factors which regulate the expression of complement regulatory proteins on different cell types including keratinocytes have not been studied.

Complement, keratinocytes and skin inflammation

The support for the above concept that complement biosynthesis and its regulation by cytokines occur within the epidermis could be gathered from several observations in inflammatory diseases such as psoriasis, and in bullous diseases such as pemphigus. In psoriasis which is characterized by epidermal acanthosis, elongation of the rete ridges, and a mononuclear cell infiltrate in the upper dermis, complement synthesis and activation seems to take place in the epidermis; not in the circulation. This is evidenced by (1) the presence of C5b-9 complexes in the lesion and in the circulation without an associated presence of C1r-C1s-C1-INH and C3b.Bb.P complexes[41], and (2) high levels of complement activation products, particularly C5a-des arg in the lesional epidermis but not in the circulation[42;43]. These findings, particularly the latter one, suggest that C5a in the lesion could not have originated from the plasma. They strongly suggest that both complement production and activation take place in the lesional epidermis in psoriasis. If complement production takes place in the lesional epidermis, the most likely source of production of this complement is the keratinocyte.

In some bullous diseases, the source of complement, which attacks keratinocytes and adjacent structures, has not been determined but appears to be keratinocytes. For example, in pemphigus, the blood vessels remain intact excluding the possibility that it is the plasma complement which reaches the epidermis and attacks keratinocytes. Most likely, complement initially synthesized by keratinocytes constitutively may be attacking keratinocytes and adjacent structures in the presence of autoantibodies. This may release cytokines from keratinocytes, which in turn may enhance the synthesis of complement; blister fluid from patients with pemphigus has been shown to contain cytokines[44]. More and more keratinocyte derived complement can then attack keratinocytes and activate them. Because of their high resistance to complement attack[38], cell lysis is not a predominant phenomenon in pemphigus.

CYTOKINES AND CHEMOKINES

Immunocompetent cells are able to secrete various soluble proteinaceous mediators which are required for growth, differentiation, and communication between different cells. These mediators are collectively called cytokines and can be divided into interleukins, interferons, tumor necrosis factors, growth factors, chemokines, and hematopoietic growth factors. Cytokines

and chemokines (chemotactic cytokines) exert their activities via inducible specific cell surface receptors present both on primarily immunological cells as well as many other cell types. Binding of cytokines/chemokines to their receptors activates signazling pathways which result in cellular responses. Some cytokines/chemokines have pleiotrophic biological properties and overlapping effects, whereas others have comparatively more restricted biological activities and less overlapping effects. Cytokines/chemokines do not function in isolation. They strongly influence the biologic consequences of each other.

In the epidermis, keratinocytes, Langerhans' cells, melanocytes, Merkel cells, and infiltrating inflammatory cells, have been demonstrated to release cytokines. In this overview attention is focused on the role of some well known cytokines/chemokines in cutaneous biology.

Cytokines and skin inflammation

Keratinocytes synthesize a broad array of cytokines[32], which is summarized in **Table 2**. Cytokines released by keratinocytes have profound effects on a variety of cell types. The reverse is also true; cytokines released from several cell types, including keratinocytes themselves, also have profound effects on keratinocytes. Some of the cytokines cause expression of several adhesion molecules on keratinocytes and endothelial cells, resulting in attraction of inflammatory cells. In the skin, inflammatory cells also produce cytokines. The net result of cytokine production by keratinocytes and inflammatory cells is the recruitment of more inflammatory cells into the dermal and epidermal compartments of the skin and progression of inflammation.

Those cytokines which are important within the context of this thesis will be discussed here briefly. A passing reference of their involvement in some inflammatory diseases of skin will also be made.

Interleukin-1 alpha

Interleukin-1 alpha (IL-1α) is a proinflammatory cytokine that belongs to the IL-1 family and has similar biologic activities as IL-1ß, despite a minimal sequence similarity (~ 25% amino acid homology). Another member of the IL-1 family is IL-1 receptor antagonist (IL-1ra) which binds to the IL-1 receptor without inducing intracellular signaling. IL-1α is synthesized as a 31-kD precursor protein which is processed intracellularly to a mature extracellular form with a molecular weight of 17-kD. The mature form is a potent modulator of immune responses. It is also a very potent inducer of acute phase responses and inflammation. IL-1α is primarily produced by cells of the mononuclear phagocytic lineage, but it is also produced by keratinocytes, endothelial cells, fibroblasts, melanocytes, and numerous other cells.

Table 2. *Keratinocyte derived cytokines*[32]

Resting keratinocytes	
Interleukins	IL-1α, IL-6, IL-7, IL-11, IL-15
Tumor necrosis factors	TNF-α
Growth factors	TGF-ß
Hematopoietic growth factors	GM-CSF, G-CSF
Activated keratinocytes	
Interleukins	IL-1α, IL-1ß, IL-6, IL-7, IL-10 (?), IL-11, IL-12, IL-13, IL-15, IL-1RA
Interferons	IFN-α, IFN-ß
Tumor necrosis factors	TNF-α
Growth factors	TGF-α, TGF-ß, PDGF, bFGF
Chemokines	IL-8, Gro-α, Gro-ß, Gro-γ, ENA-78, IP-10, MIP-2, MCP-1, RANTES
Hematopoietic growth factors	IL-3, G-CSF, M-CSF, GM-CSF, SCF

IL-1 production by keratinocytes is stimulated by a variety of agents, including other cytokines, endotoxins, and microorganisms. In human keratinocytes, synthesis of IL-1α was shown to be up-regulated by TNF-α[45], IL-1 itself[46], GM-CSF[47], UVB[48], LPS[49], heat-shock proteins of E.coli[49], and scabies mites[50]. Binding of group A streptococcus via MCP to keratinocytes[51] induces IL-1α synthesis. IL-1α appears to be induced in herpes simplex virus (HSV) and human papiloma virus (HPV) infected keratinocytes[52;53].

IL-1α has a wide range of effects on a wide variety of cell types. As regard its inflammatory effects on cells in the skin, it can release various inflammatory mediators from several cell types belonging to the dermis and epidermis and can induce expression of cell surface proinflammatory molecules. In dermal endothelial cells, IL-1α induces release of cytokines[54;55]. Activation of endothelium by IL-1α *in vitro* results in a major increase in C3 and factor B secretion and generation of C3a in the vicinity of endothelial cells[56]. IL-1α causes major phenotypic changes in human skin microvasular endothelial cells[57] and induces cell proliferation,

vasodilatation and up-regulation of intercellular adhesion molecules[58]. In dermal fibroblasts, IL-1α induces the release of several cytokines[59;60] and complement proteins[61;62]. IL-1α induces up-regulation of the synthesis of IL-8[59] and RANTES[63;64] which leads to attraction of inflammatory cells to the skin. Human melanocytes are strongly influenced by IL-1α resulting in production of chemotactic cytokines IL-8 and MCAF[65], induction of ICAM-1 expression and decreased proliferation and pigmentation[66;67]. IL-1α is involved in physiological differentiation[68] and desquamation[69] of keratinocytes. It is likely to have a role in wound healing because it stimulates keratinocyte migration on collagen[70]. In keratinocytes, IL-1α induces the releases of several cytokines, including TNF-α[71], IL-6[48;72], IL-8[68;72], and IL-1α itself[46]. The effects of IL-1α on the synthesis of complement proteins by keratinocytes have not been thoroughly investigated. So far one report has appeared which shows some up-regulation of C3 release by IL-1α[36].

Because of its multiple biologic effects, the aberrant production of IL-1 during the immune response produces a spectrum of changes associated with various illnesses. In atopic dermatitis IL-1 was shown to be a part of both the early- and late-phase response within the skin. In psoriasis, contrasting data exist on the amount of IL-1α in the epidermis[73-75]. Regardless of these data, recent studies indicate that enhancement in the expression of IL-1α in inflammatory skin diseases like atopic dermatitis and psoriasis may be compensated by increases in expression of IL-1ra[74;76]. Enhancement in the synthesis of collagenases[77;78], and metalloproteinases[79], and inhibition of the synthesis of collagen by skin fibroblasts strongly suggest that IL-1α is involved in the breakdown of extracellular matrix and reduced rebuilding of this matrix. These data plus the fact that IL-1α is expressed in fibroblasts in systemic sclerosis[80] suggest that IL-1α may play a beneficial role in systemic sclerosis[81].

Interleukin 6

Interleukin 6 (IL-6) is a 26-kD molecule made up of 212 amino acids. Originally, IL-6 was identified as a factor that induced immunoglobulin production in activated B cells. This factor has now been found to exhibit a wide range of biological functions involving cells other than B lymphocytes. Considered a proinflammatory cytokine for several years, increasing amount of evidence suggests a predominantly anti-inflammatory and immunosuppresive role for IL-6. IL-6 directly inhibits several key inflammatory responses and it may play a role in shutting off the *in vivo* synthesis of IL-1 and TNF-α in several cell types[82]. IL-6 might also have indirect effects on inflammatory responses through the induction of various acute phase proteins in several cells, which in turn induce IL-1ra synthesis[83]. Unlike IL-1 and TNF-α which are difficult to detect in peripheral circulation, IL-6 appears in high concentrations in the circulation. IL-6 may thus be viewed as not only a locally acting cytokine but also the one which may play a role in orchestrating the systemic response of the host to local injury. IL-6 is a cytokine which affects the proliferation, differentiation, or specialized functions of numerous cell types; it affects not

only primary immunological cells like B cells, T cells, monocytes or NK cells but also many other cell types such as fibroblasts, hepatocytes, keratinocytes, melanocytes, and endothelial cells.

Human keratinocytes in culture spontaneously synthesize only small amounts of IL-6. However, synthesis is greatly enhanced by IL-4[45;84;85], IL-1[47;84], TNF-α[45;84], TGF-α[86], IL-13[85], IL-17[87], and IFN-γ[45;72;87]. HSV infection[52] and bacterial products like heat shock proteins and LPS of E.coli[49;85] have been shown to up-regulate IL-6 production by keratinocytes. Physical factors like UVB[88] and immunological factors like ligation of IgE with its low affinity receptor (CD23) on keratinocytes[89], interaction of CD40 on keratinocytes with CD40L (CD154) on T cells[90], and binding of group A streptococcus to the keratinocytes via MCP[51] up-regulate the synthesis of IL-6 in keratinocytes. So far, the only factor that has been demonstrated to down-regulate the synthesis of IL-6 in keratinocytes is IL-10[91].

IL-6 exerts diverse effects on a wide variety of cell types, including several resident cells of the skin. In contrast to IL-1α and TNF-α, IL-6 appears to have little effects on dermal endothelial cells; it probably does not change their expression of adhesion molecules nor their proliferation[92]. Dermal fibroblasts, which produce significant amounts of IL-6 themselves, are induced by IL-6 to release complement proteins[93;94]. IL-6 appears to play a role in repair of the skin; it not only induced the fibroblast synthesis of keratinocyte growth factor[95], but also the production of collagen, glycosaminoglycans, hyaluronic acid, and chondroitin sulfate[96]. Melanocytes respond to IL-6 with a decrease in proliferation and a decrease in tyrosinase activity[66;67]. The main effect of IL-6 on keratinocytes appears to be the induction of proliferation[84;97], probably via the induction of the receptor for epidermal growth factor[98;99].

The ability of IL-6 to enhance proliferation of keratinocytes suggests that this cytokine may play a crucial role in cutaneous lesions characterized by hyperkeratosis. Indeed, in psoriasis elevated levels of IL-6 are found, not only in the lesion but also in the peripheral circulation[84]. In this disease both keratinocytes[84;100-102] and fibroblasts[103;104] have been shown to produce increased amounts of IL-6. In granulomatous skin conditions a pathogenic role for IL-6 is presumed by the occurrence of IL-6 (and IL-1 and TNF-α) among the cellular infiltrates[105]. In systemic sclerosis aberrant fibroblast function is associated with fibrosis of the skin. The release of IL-6 by fibroblasts is significantly increased in this disease and may play an important role in the perpetuation of the fibroblast activation in the sclerotic lesion[106;107].

Tumor necrosis factor alpha

Another proinflammatory cytokine is tumor necrosis factor alpha (TNF-α). TNF-α is a 17-kD molecule which is secreted by a broad array of cells, including fibroblasts, vascular endothelial cells, keratinocytes, and melanocytes. It has many biological functions. It induces fever and plays a role in septic shock. TNF-α activates B and T cells, leucocytes, neutrophils, and

macrophages, and induces the expression of MHC class I and class II antigens on various cells.

Human keratinocytes in culture spontaneously synthesize small amounts of TNF-α but synthesis is stimulated by IFN-γ[71], IL-1α[71], skin irritants[108], and nickel[108]. Bacterial products like heat shock proteins and LPS of E.coli[49;109], and some proteins and superantigens from Staphylococcus aureus[110] also stimulate synthesis of TNF-α in keratinocytes. Physical factors like UVB[111], and immunological factors like ligation of IgE with its low affinity receptor (CD23) up-regulates the synthesis of TNF-α in keratinocytes[89].

Like IL-1α, TNF-α has numerous effects on many cell types. It exhibits proinflammatory action on most cell types of the skin by inducing the release of inflammatory mediators and inducing the expression of proinflammatory molecules on cell surfaces. In dermal endothelial cells, TNF-α induces the release of cytokines[55] and chemokines[112]. TNF-α causes major phenotypic changes in human skin microvasular endothelial cells, including the up-regulation of the expression of intercellular adhesion molecules and MHC class II molecules but does not influence cell proliferation[58;92;113;114]. In dermal fibroblasts, TNF-α induces expression of cytokines[62;95], chemokines[64], and complement proteins[62]. TNF-α alters melanocyte function; It decreases tyrosinase activity, pigmentation and proliferation of melanocytes but increases the matrix protein production[66;67]. TNF-α induces chemokine production and cell differentiation in melanocytes[66]. In keratinocytes, TNF-α induces the release of several cytokines, including RANTES[115], IL-1[45], and IL-6[45]. Recently, Terui *et al.* have demonstrated that TNF-α drastically up-regulates the release of complement protein C3 by human keratinocytes[36]. Effects of TNF-α on the synthesis of other complement proteins by keratinocytes have not been investigated. Proinflammatory effects of TNF-α on keratinocytes also include induction of expression of cell adhesion molecules, thereby enabling inflammatory cells to enter the epidermis[116]. TNF-α appears to play a role in induction of apoptosis in normal[117] and HPV transformed keratinocytes[118]. It has been suggested that synthesis of TNF-α by keratinocytes plays a role in antigen presentation in the skin, and in the elicitation phase of type IV allergic reactions[119].

Because of the pleiotrophic effects of TNF-α on several cell types in the human skin, increased production during immune responses of the skin may induce an array of effects associated with several skin diseases. A study on chronic granulomatous skin conditions, such as cutaneous leishmaniasis, granuloma annulare, leprosy, and hidradenitis demonstrated a contribution of TNF-α (and IL-6) to the granulomatous reaction[105]. Like IL-1α, TNF-α inhibits synthesis of collagen types I and III by dermal fibroblasts[120]. This modulation of extracellular matrix deposition may play an inhibitory role in the pathogenesis of sclerotic skin diseases. In transformed epidermal keratinocytes, TNF-α has been shown to induce the synthesis of a collagenase, suggesting a role for TNF-α in the invasive capacity of malignant human epidermal cells[121]. In the dermis of lesional skin in atopic dermatitis increased TNF-α activity can be found, which is released by keratinocytes[122] and/or dermal mast cells[123]. TNF-α is considered responsible

for the up-regulation of adhesion molecules on endothelial cells in atopic dermatitis[124]. These adhesion molecules may promote the influx of leukocytes, such as lymphocytes and eosinophils, to the dermis, which will induce the clinical signs of the disease. TNF-α appears to have an important role in the induction-phase of allergic contact dermatitis. Exposure of the skin to skin-sensitizing chemicals induces epidermal TNF-α synthesis and this is thought to provide the stimulus for the migration of Langerhans cells from the epidermis and their accumulation as immunocompetent dendritic cells in draining lymph nodes[125;126]. In psoriasis strongly increased amounts of TNF-α were found in lesional keratinocytes by Terajima *et al.*[127]. These authors suggest that in psoriasis, TNF-α might play an important role by the induction of vascular adhesion molecules hereby enabling inflammatory cells to enter the skin. Thus, TNF-α in concert with other skin derived inflammatory proteins may mediate inflammatory reactions during many pathologies of the skin.

Interferon gamma

Interferon gamma (IFN-γ) is a 34-kD homodimer proinflammatory cytokine, which is primarily produced by activated T cells and natural killer (NK) cells. Together with IFN-α and IFN-ß it belongs to the interferon family of cytokines. IFN-γ differs from other members of the family with regard to its primary sequence and cell surface receptor. It was first identified as an antiviral substance but was later found to be capable of orchestrating a remarkable range of distinct cellular programs, including the regulation of antigen presentation and leucocyte-endothelium interaction[128]. Furthermore, it plays an important role in the induction of synthesis of cytokines and components of the complement system in various cell types. The IFN-γ synthesis itself is regulated by cytokines including IFN-α/β, TNF-α, and IL-4[128].

As mentioned above, IFN-γ exerts a wide range of effects on a wide variety of cell types. Some effects appear to be cell-type specific but others are more general. Examples of the general effects of IFN-γ on cells of the skin are induction of expression of cell adhesion as well as MHC class I and II molecules on keratinocytes[87;129-131], endothelial cells[132-135], fibroblasts[136-138] and melanocytes[139-141]. Induction of cell adhesion molecules implicates IFN-γ in the modulation of leucocyte trafficking into the skin and up-regulation of MHC molecules implicates it in facilitation of some skin cells to act as antigen-presenting cells. IFN-γ is known to increase skin inflammation by induction of cytokine production by epidermal and dermal cells[71]. In keratinocytes, it augments the production of several cytokines, chemokines, and complement protein C3[36;71;87;142-145]. It also induces the expression of matrix metalloproteases[146] and of CD40[90;147] by these cells. Other important effects of IFN-γ on keratinocytes are induction of growth arrest and differentiation[148] and mediation in apoptosis by induction of FAS[149]. Also in dermal endothelial cells IFN-γ inhibits the proliferation[150] and induces the expression of CD40[151]. There are no reports showing induction of production of cytokines and complement in these cells

by IFN-γ. IFN-γ has major effects on dermal fibroblasts, which are frequently opposite to that of TGF-ß[152]. IFN-γ is often considered to be an anti-fibrotic cytokine because it inhibits fibroblast synthesis of extracellular matrix proteins[153], including collagens[154;155]. It also regulates production of collagenases[156;157] and other metalloproteinases[158]. IFN-γ has many stimulatory and inhibitory effects on the fibroblast release of some cytokines[159] and several complement proteins[160-162].

Because of its major effects on most cell types in dermis and epidermis, efforts have been made to illustrate the role of this cytokine in the pathogenesis of several skin diseases. In psoriasis, contrary to normal or non-lesional skin, active lesions have been shown to contain IFN-γ[163], which appears to be produced by infiltrated T cells[164]. This may play a role in trafficking inflammatory cells towards the skin through the involvement of adhesion molecules. Numerous reports on the role of IFN-γ in atopic dermatitis have appeared. Peripheral blood mononuclear cells in atopic dermatitis patients secrete less than normal amounts of IFN-γ[165;166]. Since IFN-γ inhibits the process of IgE class switch in B cells, the resulting relative deficiency of IFN-γ, due to decreased synthesis by T cells, may contribute to the increased IgE synthesis[167]. The intrinsic defect in IFN-γ secretion by T cells in atopic dermatitis may reflect a defect in general T cell activation, not only contributing to overproduction of IgE, but also explaining the high incidence of cutaneous infections[168]. Systemic treatment of atopic dermatitis patients with recombinant IFN-γ results in clinical improvement[169]. Production of immunoreactive IFN-γ by human keratinocytes has been shown in an early phase of allergic contact dermatitis[170].

Transforming growth factor beta

Transforming growth factor beta (TGF-ß) is a 25-kD homodimer of 12.5-kD subunits made up of 112 amino acids. Three structurally and functionally related members of the TGF-ß family have been identified in humans, TGF-ß1, 2, and 3[171]. TGF-ß isoforms are produced by cells of every leucocyte lineage. They are also produced by keratinocytes[172], melanocytes[173], and fibroblasts[174]. All of these cells do not constitutively produce all three isoforms of TGF-ß but most cell types appear to be inducible to synthesize all isoforms. Keratinocytes constitutively express TGF-ß3, but also synthesize other TGF-ß isoforms following appropriate stimulation, like UV exposure, thermal wounding, or stripping[172;175;176]. Cytokine regulation of TGF-ß synthesis in keratinocytes has not yet been shown.

TGF-ß isoforms act on a wide variety of cell types to regulate a variety of functions such as cell proliferation and differentiation, and induction of apoptosis. TGF-ß has profound, mainly inhibitory, effects on immune responses in inflammatory cells[177]. As regard effects on cells in the skin, TGF-ß has been studied most thoroughly on dermal fibroblasts. TGF-ß induces contractility of fibroblasts[178] and inhibits their proliferation[179]. It promotes extra-cellular matrix deposition through several distinct mechanisms, which include increased production of matrix macromolecules[180;181], proteoglycans[182;183] and collagens[184;185], and increased synthesis of protease

inhibitors[186;187]. TGF-ß also induces the synthesis of the chemokine MCP-1 in fibroblasts[188]. Little is known on the effects of TGF-ß on endothelial cells in the skin. It inhibits their proliferation and is known to down-regulate the expression of adhesion molecules on these cells. This may be important for inhibiting inflammatory responses by reducing adhesiveness of endothelial cells for inflammatory cells[189;190]. TGF-ß inhibits proliferation of melanocytes[191]. Other effects of TGF-ß on melanocytes have not been studied. In keratinocytes, TGF-ß inhibits the proliferation[192], increases the release of cytokines (IL-1 and IL-6)[193;194], and like in fibroblasts, changes extra-cellular matrix deposition through increased production of matrix macromolecules[195;196]. It also induces the synthesis of extracellular matrix proteases[197] and collagenases[198]. High levels of TGF-ß induce apoptosis in human keratinocytes[199]. Effects of TGF-ß on synthesis of complement proteins by dermal and epidermal cells have not yet been studied. Both stimulatory and inhibitory effects of TGF-ß on complement production have been reported in other cell types[200;201].

TGF-β appears to be involved in regulation of cell proliferation *in vivo*. Several neoplastic cells, including human melanoma[202] and HPV transformed keratinocytes showed profound resistance to growth-inhibitory effects of TGF-ß[203;204]. During wound repair TGF-ß promotes extra-cellular matrix deposition and induces angiogenesis[205] and strong TGF-ß immunoreactivity in migrational epidermis is seen[206]. TGF-ß appears to play a major role in hypertrophic scars and keloid[207;208]. In psoriasis, TGF-ß showed reduced effects on psoriatic dermal endothelial cells. In other words, endothelial cells had reduced susceptibility to TGF-ß induced down-regulation of adhesion molecules. As a consequence, these endothelial cells maintain their expression of cell adhesion molecules and allow infiltration of lymphocytes into the psoriatic plaque[189]. There are no reports on the role of TGF-ß in the pathogenesis of atopic dermatitis and allergic contact dermatitis.

Chemokines and skin inflammation

Chemokines have emerged as critical mediators of inflammation. They frequently act as effector pathway for other cytokines and directly activate effector cells of inflammation. Chemokines have additional functions but chemotactic activity is their hallmark. Their molecular weights are in the range of 8- to 10-kD. The classification of chemokines (**Table 3**) is based on the position of the first two N-terminal-conserved cysteine residues. One family has two N-terminal cysteine residues separated by one nonconserved residue. This family is called the CXC (X = any amino acid) family. The second family has two N-terminal-conserved cysteine residues in juxtaposition and is referred to as the CC family. The third family has a single cysteine residue in the conserved position and is known as the C family[209].

In virtually all inflammatory conditions studied so far, increased systemic or lesional

Table 3. *Classification and members of the chemokine family*[*209]

Chemokine family		
CXC chemokines	**CC chemokines**	**C chemokines**
Ckα-1	C-10	ATAC
CTAP-III	CCF-18	Lymphotactin
ENA-78	Eotaxin	SCM-1
GCP-2	HCC-1	
Gro-α, Gro-β, Gro-γ	I309	
IL-8	MCP-1, MCP-2, MCP-3, MCP-4	
IP-10	MIP-1α, MIP-1β, MIP-1γ	
LIX	MRP-2	
Mig	RANTES	
NAP-2		
PBP		
PF4		
SDF-1		
β-thromboglobulin		

* Abbreviations used: Ckα-1, chemokine alpha 1; CTAP-III, connective tissue-activating peptide; ENA-78, epidermal cell-derived neutrophil chemotactic activity; GCP-2, granulocyte chemotactic protein; Gro, growth factor-related oncogene; IL-8, interleukin-8; IP-10, inducible protein-10; LIX, lipopolysaccharide-induced CXC chemokine; Mig, monokine induced with γ-interferon; NAP, neutrophil-activating peptide; PBP, platelet basic protein; PF, platelet factor ; SDF, stromal cell-derived factor; CCF-18, CC chemokine F-18; HCC-1, hemofiltrate-derived CC chemokine; MCP, monocyte chemotactic protein; MIP, macrophage inflamatory protein; MRP, MIP-related protein; RANTES, Regulated upon Activation, Normal T Expressed, and presumably Secreted; SCM, single C motif.

levels of chemokines have been seen. In psoriasis, IL-8 appears to be responsible for the attraction of neutrophils[210-212] and MCP-1 may be responsible for the presence of macrophages and dendritic cells in the lesion[213]. RANTES may be the chemokine responsible for attracting eosinophils toward the lesional skin[115]. In atopic dermatitis, scales show high levels of RANTES,

explaining the dominance of mononuclear cell infiltrates and eosinophils in the lesion. High levels of IL-8 can be found in the epidermis and plasma of most atopic patients[214;215]. The origin of chemokines in these inflammatory skin diseases has not been established but appears to be keratinocytes[115;213;216]. However, the mechanisms by which their production is regulated in the skin have not been studied extensively. T cells may play a role in inducing epidermal keratinocytes to release chemokines via their cytokines or co-stimulatory molecules. This is supported by the fact that the T cell inhibiting drug cyclosporin A did not affect the IL-8 mRNA expression in cultured keratinocytes but reduced its expression in psoriatic lesions where there is infiltration of T cells[217]. Some studies have shown the role of cytokines in keratinocyte synthesis of chemokines but the influence of direct interaction between keratinocytes and lymphocytes via pairs of co-stimulatory molecules, like CD40 - CD40L, needs to be investigated. The following paragraphs describe only those chemokines which are relevant within the context of this thesis. Chemokines shown to be produced by keratinocytes are listed in **Table 2**. Other chemokines described so far are listed in **Table 3**.

Interleukin 8

Interleukin 8 (IL-8) belongs to the CXC family of chemokines. It is the most potent chemotactic factor for neutrophils and binds to chemokine receptors CXCR-1 and CXCR-2[218]. It also attracts basophils and CD8+CD56+ T cells. In order to attract cells from circulation IL-8 preferentially binds to the endothelium of the postcapillary venule, where it regulates leukocyte adherence and diapedesis. In addition to chemotaxis, the primary effects of IL-8 on neutrophils are secretion of granular enzymes, induction of respiratory burst, stimulation of phagocytosis, and up-regulation of surface expression of CR1 and CR2. IL-8 exhibits weak histamine releasing activity in IL-3 primed basophils, suppresses myeloid colony formation, and exhibits angiogenic activity.

IL-8 is produced by almost all types of human cells. In the skin, keratinocytes, endothelial cells, fibroblasts, Langerhans' cells, and melanocytes produce IL-8[54]. Production of IL-8 by these cells is stimulated by a variety of stimuli and leads to influx of inflammatory cells into the skin resulting in an inflammatory infiltrate and to activation of dermal and epidermal cells.

In human keratinocytes, constitutive synthesis of IL-8 was shown to be up-regulated by IL-17 (in synergy with IFN-γ)[87], TNF-α[68], ligation of CD40[147], exposure to UVB[219] or to relatively high temperature[220]. In melanocytes and dermal fibroblasts, production of IL-8 is stimulated by proinflammatory cytokines IL-1α[59;65] and TNF-α[65;221]. In latter cell type, production is also stimulated by IL-1ß[59] and leukoregulin[222], but inhibited by IFN-γ[159]. In skin derived endothelial cells expression of IL-8 was induced by IL-1ß and TNF-α[112]. Exposure of transformed human microvascular endothelial cells to IL-10, alpha-melanocyte stimulating hormone, and UVB

increased release of IL-8[223;224]. IL-8 exerts a variety of effects on keratinocytes. It induces chemotaxis of cultured keratinocytes *in vitro*[225]. In these cells, IL-8 down-regulates the expression of the IL-10 receptor gene[226], induces expression of HLA-DR antigen[227], and promotes cell proliferation[228]. Endothelium of postcapillary venules and small veins in the skin have been demonstrated to express binding sites for IL-8 (and RANTES), which may be internalized, transported transcellularly, and released onto the luminal surface[229].

Because of its chemoattractive properties, IL-8 is considered an important factor in many inflammatory diseases of the skin, like psoriasis, atopic dermatitis, and allergic contact dermatitis. It has been demonstrated that psoriatic fibroblasts[230] and psoriatic suprabasal keratinocytes[231] produce significantly more IL-8 than fibroblasts and keratinocytes from healthy skin. This IL-8 may play a role in induction of angiogenesis in the psoriatic dermis[232] and in hyperproliferation of keratinocytes in the psoriatic epidermis[97], which may be facilitated by the increased keratinocyte expression of IL-8 receptors in psoriasis[233]. In concert with C5a/C5a des arg, IL-8 may also play a role in attraction of inflammatory cells towards the psoriatic skin, although circulating T cells in patients with severe psoriasis appear to show a decreased response to IL-8[234]. Decreased response to IL-8 was demonstrated to be even more pronounced for circulating T cells in atopic dermatitis[234], another inflammatory skin disease in which epidermal IL-8 immunoreactivity has been shown to be up-regulated[214]. Some studies on the role of IL-8 in contact eczema have shown increased keratinocyte synthesis of IL-8 in allergogenic, tolerogenic, and irritant contact skin reactions[235;236]. This suggests that non-specific stimuli can cause the induction of IL-8 production by epidermal keratinocytes in contact eczema and that IL-8 may play a critical role in the early response to immunogenic or inflammatory signals in this disease.

RANTES

RANTES is a small molecule comprising 68 amino acid residues. It belongs to the CC family of chemokines. It binds to chemokine receptors CCR1, CCR3, CCR4 and CCR5, which are expressed on memory T cells, activated naive T cells, eosinophils, monocytes, dendritic cells, and basophils[218;237]. Like other chemokines, RANTES also has non-chemotactic activities including activation of T cells[209].

Synthesis of RANTES has been detected in cells of many organs, including several cell-types of the skin. Keratinocytes[143], fibroblasts[238], and dermal endothelial cells[112] synthesize RANTES but nothing is known about its production by melanocytes. Very limited number of studies have been carried out on cytokine regulation of synthesis of RANTES by skin cells. Production by keratinocytes was shown to be stimulated by TNF-α or IFN-γ[143], whereas its production was inhibited by IL-17[239]. Synergy in up-regulation of RANTES by TNF-α and IFN-γ has been shown in keratinocytes and dermal endothelial cells[112;143]. In dermal fibroblasts, TNF-α,

IL-1α, and IL-1ß have been shown to induce expression of RANTES[64].

Because of its chemotactic and cell-stimulating properties, RANTES may play a significant role in the inflammatory processes of the skin. In psoriasis, increased amounts of RANTES in epidermis have been reported[115;240] which provide an explanation for migration of activated T cells to the epidermis of the psoriatic lesions. The factor responsible for up-regulation of RANTES production by psoriatic keratinocytes is not known. In allergic skin diseases such as atopic dermatitis, eosinophils migrate from the circulation to the skin; RANTES may be one of the factors responsible for this phenomenon because of its chemotactic properties for eosinophils[142;241].

Monocyte chemoattractant protein 1

Monocyte chemoattractant protein 1 (MCP-1) is a chemokine comprised of 76 amino acids. It belongs to the CC family of chemokines. MCP-1 specifically binds to chemokine receptor CCR2, which is expressed on monocytes, basophils, activated T cells, dendritic cells, and natural killer cells but not on eosinophils, neutrophils, and resting T cells[218]. Non-chemotactic activities of MCP-1 include the induction of histamine release from basophils[242] and the enhancement of target cell lysis by blood derived natural killer cells[243].

Many cell types of several organs have been shown to synthesize MCP-1. As regard skin cells, keratinocytes, fibroblasts, dermal endothelial cells, and melanocytes have been shown to synthesize MCP-1[244]. Only a limited number of studies have been carried out on the regulation of MCP-1 synthesis in these cells. IL-1α was shown to be a strong stimulator for MCP-1 synthesis in keratinocytes[244]. In dermal endothelial cells, TNF-α, IL-1α and IL-1ß have been shown to induce the expression of MCP-1[112;244]. IL-1α, IL-1ß, IL-2, IFN-γ, and TGF-ß have been shown to induce expression in human fibroblasts[188;244-246]. It is not known if CD40 - CD40L interaction also plays a role in the regulation of MCP-1 in the skin. In at least one epithelial cell-type not belonging to the skin, namely cervical carcinoma cells, this interaction has been shown to induce MCP-1 production[247].

Because MCP-1 has been shown to be one of the most efficient chemokines in recruiting T cells into the skin it is thought to play an important role in the pathogenesis of several inflammatory conditions of the skin[248;249]. In psoriasis, the role of MCP-1 is evident from its increased immunostaining for MCP-1 in all layers of the epidermis, except the stratum granulosum[250]. It is assumed that keratinocyte-derived MCP-1 also plays a role in the genesis of contact hypersensitivity by inducting the recruitment of dendritic and Langerhans cells to the epidermis[251], although it remains to be shown that MCP-1 is excreted in response to antigen stimulation. In cutaneous delayed hypersensitivity MCP-1 was shown to be relevant in recruitment of T cells and monocytes towards the lesional skin[252].

CONCLUDING REMARKS

From the first part of the above survey of literature, it appears that the importance of local synthesis of complement in different organs is beginning to be realized. Synthesis of complement and its regulation in various cell types is currently under investigation. Complement biosynthesis and its regulation in keratinocytes has also begun to be studied. It is beginning to emerge that keratinocytes may be the main producers of complement in the epidermal compartment of the skin. Keratinocytes have been shown to synthesize C3 and factor B. Regulation of synthesis of C3 by cytokines has been studied at protein level but the regulation of expression of C3 transcripts by cytokines has not yet been studied. Nothing is known about the regulation of synthesis of factor B protein and transcripts by cytokines in keratinocytes. Whether keratinocytes synthesize other components of complement remains an open question. The same is true for synthesis and regulation of synthesis of fluid phase regulators of complement. How inflammatory mediators including cytokines influence the expression of membrane embedded inhibitors of complement and thus influence their survival under inflammatory conditions remains unknown. Regulation of expression of complement receptors has also not been studied. Another gap in our current knowledge on the regulation of synthesis of complement proteins is whether their synthesis is regulated by ligations formed during cell-cell interactions such as by CD40 and CD40-ligand interaction, as has been seen for some chemokines in some cell types. Studies on the regulation of complement components and fluid phase and membrane embedded complement proteins by external factors known to stimulate keratinocytes such as UV-radiation (see **Figure 1**) have not been carried out. In short, a vast amount of work on complement biosynthesis and its regulation in keratinocytes, the major cell type in the epidermis, is needed to fill up the gaps in the knowledge in the field of 'skin as a complement synthesizing organ'.

AIMS OF THE STUDIES

This thesis describes studies on the regulation of production of some inflammatory mediators, namely complement components and chemokines, by keratinocytes. It also investigates the regulation of complement regulatory proteins, which protect autologous cells against complement attack.

The complement system plays an important role in host defense and inflammation. Evidence is emerging that locally synthesized complement performs these functions at tissue and organ level. In the epidermis, the keratinocyte is the major cell type and is known to produce at least two complement components, C3 and factor B. Since keratinocytes also produce cytokines

which are known to regulate the production of complement in many cell-types, they are expected to regulate the synthesis of complement in keratinocytes as well. In **Chapter 2**, the regulation of synthesis of C3 and factor B in keratinocytes by cytokines, some of them known to be produced by inflammatory cells and some by keratinocytes, was investigated.

Expression of complement regulatory proteins on cells in areas of inflammation may be altered by mediators released by inflammatory cells during inflammation. In **Chapter 3**, we studied the regulation of expression of complement regulatory proteins on human keratinocytes in response to mediators of inflammatory cells. Supernatant of activated mononuclear cells was used as a source of inflammatory mediators of inflammatory cells, and recombinant forms of several cytokines, known to be present in the supernatant, were studied for their ability to regulate the expression of DAF, MCP and CD59. The aim was to obtain an insight into whether protection of keratinocytes against complement at the site of inflammation is altered. The results indicated that expression of MCP and CD59 on keratinocytes is up-regulated by the supernatant of activated mononuclear cells, TGF-β and an as yet unidentified additional factor(s). Thus, keratinocytes at the site of inflammation appear to be better protected against autologous complement than keratinocytes in normal non-inflamed skin.

UVB irradiation causes infiltration of inflammatory cells into the skin. From the results described in **Chapter 4** it is clear that some cytokines released from inflammatory cells and some from UV-stimulated keratinocytes may strongly up-regulate the synthesis of C3 and factor B by keratinocytes. It is therefore logical to expect that UVB exposure of the skin may increase the constitutive synthesis of complement components by keratinocytes and that in order to protect themselves from their own complement produced in excess, keratinocytes may be endowed with the ability to up-regulate complement regulatory proteins (MCP, DAF, and CD59) in response to UVB. *In vivo,* UVB may up-regulate these proteins by direct effect or via cytokines released by keratinocytes or infiltrating cells. *In vitro*, UVB may up-regulate the synthesis of these proteins only directly, because of the dilution of cytokines released by keratinocytes in the medium. In **Chapter 4**, effects of direct UVB exposure of cultured human keratinocytes on the constitutive release of C3 and factor B and on surface expression of DAF, MCP and CD59 were studied. The results of *in vitro* studies in **Chapter 4** indicate that UVB does not directly increase the synthesis of C3 and factor B but can directly up-regulate the expression of DAF, MCP, and CD59. UVB induced and cytokine mediated up-regulation of C3 and factor B synthesis *in vivo* was not ruled out.

Keratinocytes have so far been shown to synthesize two complement components, C3 and factor B. This suggests that they may also be able to synthesize fluid phase regulators, namely factor H and factor I, which regulate the activities of C3 and factor B. **Chapter 5** was aimed at finding out whether keratinocytes can synthesize factor H and, if yes, how the synthesis

of factor H in keratinocytes is regulated. The results show that both known molecular species of factor H are synthesized by keratinocytes, and that the synthesis was regulated by IFN-γ but not by many other cytokines tested.

In psoriatic epidermis, both activated T cells and CD40 expressing keratinocytes are seen. Because activated T-cells can express CD40L, it is expected that T cells in psoriatic lesions may interact with keratinocytes through CD40 - CD40L interaction. In analogy with other cell types in which this interaction causes the release of inflammatory mediators, this ligation on keratinocytes may cause the release of chemokines. Production of complement proteins in response to this ligation may also be expected. In **Chapter 6**, this possibility was explored *in vitro* by inducing CD40 expression on cultured human keratinocytes followed by ligating them either with soluble CD40L or with CD40L transfected cells. The results showed that the synthesis of chemokines, IL-8, MCP-1, and RANTES, thought to be involved in pathogenesis of psoriasis, was appreciably up-regulated but the production of C3 and factor B and the expression of MCP, DAF and CD59 on keratinocytes, after CD40 - CD40L interaction, was not significantly changed.

Finally, **Chapter 7** summarizes and briefly discusses the results obtained in this study.

Chapter Two

Synthesis of complement components C3 and factor B in human keratinocytes is differentially regulated by cytokines

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J. Invest. Dermatol. 2000. 114: 78-82

Abstract

The complement system plays an important role in host defense and inflammation. Locally synthesized complement may perform these functions at tissue and organ level. In the epidermis the keratinocyte is the major cell type known to produce two soluble complement components, C3 and factor B. In the present study we investigated the regulation of synthesis of these components in foreskin keratinocytes by cytokines.

Human keratinocytes were cultured in the presence of supernatant of activated peripheral blood mononuclear cells, IL-1α, IL-2, IL-6, TGF-β1, TNF-α, or IFN-γ. C3 and factor B proteins were measured in culture supernatant by ELISA and C3 and factor B transcripts in harvested cells by reverse-transcriptase polymerase chain reaction.

Cultured keratinocytes constitutively produced C3 and factor B. Supernatant of activated mononuclear cells up-regulated C3 and factor B production by 27- and 15-fold, respectively. IL-1α, IFN-γ, and TNF-α up-regulated C3 synthesis by 7-, 8-, and 22-fold, and IL-1α, IL-6, and IFN-γ up-regulated factor B synthesis by 3-, 3-, and 34-fold, respectively. TNF-α induced production of C3 and IFN-γ induced production of factor B were inhibited by cycloheximide. Cytokine induced up-regulation of C3 and factor B proteins was always associated with the up-regulation of levels of C3 and factor B mRNAs. This indicated that, as expected, cytokine-induced enhancement in C3 and factor B levels was due to increase in synthesis rather than their possible release from intracellular stores.

In conclusion, synthesis of C3 and factor B in keratinocytes is regulated by some cytokines, known to be produced by inflammatory cells and keratinocytes.

INTRODUCTION

The complement system is comprised of a large number of proteins which include components of classical and alternative pathways[3;13]. Complement components C3 and factor B occupy the central position in the alternative pathway as, following activation, they become constituents of the C5-convertase ($C3b_n$.Bb). C3 is also central to the classical pathway as it provides the catalytic subunit of classical pathway C5-convertase (C4b.C2a.C3b). C5-convertases of both pathways can eventually generate the membrane attack complex (C5b-9; MAC). Generation of MAC on foreign cells, such as microbes, can lead to their killing, but under certain circumstances its generation on self cell can lead to effects such as release of inflammatory mediators and cell-proliferation[3;27].

Although the liver is the primary source of plasma complement, other cells of various organs also produce complement proteins. The list of tissues and cells capable of producing complement components is growing[253-258]. Human keratinocytes, the most abundant cell-type in epidermis, have been studied for the biosynthesis of two complement components, C3 and factor B. Biosynthetic labeling and pulse chase studies with human keratinocytes and a human keratinocyte cell line A431 have shown that these cells synthesize a 195-kD pro-C3[34] and a 100-kD pro-factor B[35] molecule. The C3 precursor molecule is processed intracellularly to a mature C3 molecule which consists of disulphide linked 120- and 75-kD C3 alpha and beta chains. The 100-kD factor B precursor molecule is processed intracellularly to a 105-kD extracellular mature factor B. It was also shown by these authors that when mature C3 and factor B were subject to specific cleavage by appropriate enzymes of classical and alternative pathways, respectively, they were cleaved and activated in a manner analogous to that of circulating C3 and factor B. Hence skin epithelial cells were seen as a potential source of biologically active C3 and factor B. Northern blot analysis from human keratinocytes and A431 revealed the presence of a 5.1 kb C3 mRNA and 2.8 kb factor B mRNA in these cells. These results convincingly demonstrated that human keratinocytes are local source of C3 and factor B and of their important cleavage products.

Demonstration of constitutive synthesis of C3 and factor B led to two main questions, (1) are keratinocytes, like some other cell types[256;259], also capable of synthesizing all other components of complement and (2) under what conditions or biological stimuli, synthesis of C3 and factor B (and of other components if produced) in keratinocytes is regulated? Studies aimed at answering these questions are currently underway. As regards the latter question, Terui *et al.* recently studied a large number of cytokines and growth factors and showed that TNF-α and IFN-γ are the main cytokines involved in regulation of production of C3 in human keratinocytes[36]

although IL-1α and IL-1β also up-regulated C3 production to some extent. They also showed that protein kinase C plays a stimulatory role whereas protein tyrosine kinase an inhibitory role in C3 synthesis and release. State of keratinocyte differentiation also influenced constitutive and cytokine mediated release of C3 to some extent; differentiated keratinocytes were more active in producing C3. These workers did not study pretranslational regulation of C3 by the above cytokines. They also did not include the regulation of synthesis of factor B in their studies. In the current study we investigated the cytokine regulation of C3 and factor B production both at protein and mRNA levels.

MATERIALS AND METHODS

Chemicals and reagents

Human recombinant cytokines IFN-γ, IL-1α, IL-2, IL-6, TGF-ß1 and TNF-α were purchased from Boehringer Mannheim (Mannheim, Germany). Normal rabbit IgG, neutralizing rabbit antibodies to IFN-γ and cycloheximide were purchased from Sigma (St. Louis, MO, USA). Supernatant of activated mononuclear cells was prepared from stimulated peripheral blood mononuclear cells as described[260]. Briefly, peripheral blood mononuclear cells (PBMC; 50 x 10^6/ml) from normal donors were stimulated for 2 h at 37°C with 0.1 μg/ml PMA in Iscove's Modified Dulbecco's Modified Eagles Medium (IMDM) (supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin and 100 μg/ml streptomycin). PMA-treated cells were washed extensively and cultured (50 x 10^6/ml) for 48 h with 15 μg/ml Con A in supplemented IMDM. Con A was neutralized by addition of 50 mM α-methyl-mannoside for 30 min at 37°C. Supernatant was obtained by centrifugation. Cytokines, supernatant of activated mononuclear cells and neutralizing antibodies were aliquoted in small portions and stored at -20°C and diluted in keratinocyte serum free medium (keratinocyte SFM; GibcoBRL, Breda, The Netherlands) just before use. The sources of other chemicals and reagents are indicated below.

Keratinocyte culture

Human keratinocytes were isolated from foreskin obtained by circumcision of children (< 5 years). Foreskin was incubated with thermolysin (0.50 mg per ml, Sigma, St. Louis, Mo) at 4°C for 16 h and subsequently trypsinized (0.025%) for 5 min at 37°C. Trypsin (Sigma) was then neutralized by an excess volume of heat inactivated fetal calf serum (GibcoBRL, Breda, The Netherlands). Cells were separated from debris by filtering through a nylon mesh, centrifuged, and resuspended in keratinocyte SFM supplemented with penicillin/streptomycin (100 IU per ml, 100 μg per ml; GibcoBRL). The keratinocytes were plated onto 100 mm plastic Petri dishes at a density of 400,000 cells per Petri dish and were incubated at 37°C in humidified, 5% CO_2, tissue culture incubator. Medium was changed every 2-3 d, and at 70% confluence cultures were split after a 5 min exposure to 0.025% trypsin, 1.5 mM EDTA and recultured.

Stimulation of keratinocytes

For stimulation of keratinocytes with cytokines, cells were seeded at a density of 100,000 per well in 2000 μl of medium. When cultures reached 60-80% confluence, medium was removed and monolayers were washed twice with PBS. Keratinocyte SFMs containing different cytokines was added to the cells. The concentrations and ranges

of concentrations of cytokines used in different experiments are mentioned in the section '*Results*'. After stimulation with cytokines for a predetermined suitable period of 72 h (unless indicated otherwise), culture supernatants were collected for C3 and factor B analysis by ELISA. As controls, supernatants were collected from keratinocyte cultured in keratinocyte SFM without cytokines. Cells in representative wells were counted by hematocytometer before the experiment and the cells in all wells were counted after finishing the experiment. Cells in passage 2-5 were used.

In some experiments supernatant of activated mononuclear cells or lipopolysaccharide (LPS), at concentrations indicated in the section '*Results*', were used instead of cytokines.

Enzyme-linked immunosorbent assay (ELISA) for measurement of C3 and factor B

The concentrations of C3 and factor B in culture supernatant of keratinocytes were estimated as follows. For quantification of C3, a previously described sandwich ELISA[261] was used with some modifications. Briefly, wells of 96-well flat-bottom microtiter plates were coated with 0.7 µg polyclonal goat IgG anti-human C3 (Cappel, Boxtel, The Netherlands) per ml in 100 µl carbonate buffer (pH 9.6) overnight at 4°C. After thorough washing with Tween-80 (0.05%) (Sigma) in demineralized water, the wells were blocked for 1 h at room temperature with 250 µl PBS containing 2% bovine serum albumin (Sigma) and 1 mM EDTA. Washing was repeated and wells were incubated with 100 µl of sample diluted in the same buffer that was used for blocking. Plates were incubated for 2 h at 37°C. The wells were then washed and incubated with 100 µl peroxidase labeled goat anti-human C3 IgG (0.05 µg per ml) (Cappel) for 1 h at 37°C. After washing, the wells were incubated with 100 µl 3,3',5,5'tetramethylbenzidine (Sigma) in dimethylsulfoxide (Merck, Hohenbrunn, Germany)-citrate buffer for 10 min. Reaction was stopped with 100 µl H_2SO_4 (2 M). Optical density (OD) was measured at 450 nm. The detection limit of this ELISA was 1 ng per ml of C3.

Factor B was assayed by a previously described sandwich ELISA[262] with several modifications. Briefly, wells were coated overnight at 4°C with 3 µg polyclonal goat-anti-human factor B IgG (ATAB, Stillwater, MN, USA) per ml in carbonate buffer. After thorough washing with PBS/Tween-80 (0.05%), wells were blocked with PBS/milk powder (2%) (Nutricia, Zoetermeer, The Netherlands) for 1 h at room temperature, and then washed again. Wells were then incubated for 2 h at 37°C with 100 µl sample, diluted in same buffer that was used for blocking, and washed. They were then incubated with biotinylated goat anti-human factor B IgG (1.25 µg per ml) for 1 h at 37°C. After washing, the wells were incubated for another h at 37°C with peroxidase conjugated poly streptavidin (1:1000; DAKO, Glostrup, Denmark). The wells were then repeatedly washed. Incubation of the wells with the peroxidase substrate, termination of the reaction, and measurement of OD was carried out as described for C3. The detection limit of this ELISA was 100 pg per ml. Standard curves for both ELISA's were made using Human Complement Calibrator CA1 (ATAB).

Isolation of RNA and semi-quantitative reverse-transcriptase polymerase chain reaction

Total RNA was isolated from human keratinocytes grown in 100 mm Petri dishes in medium alone or medium supplemented with IL-1α (200 U/ml), IL-6 (1000 U/ml), IFN-γ (100 U/ml) or TNF-α (750 U/ml) for 6 h and 24 h using Trizol (Life Technologies, Paisley, UK). The RNA pellet was dissolved in formamide and the amount of RNA was determined by a spectrophotometer at 260 nm and 280 nm.

Reverse-transcriptase polymerase chain reaction (RT-PCR) was carried out as described[87], with some minor modifications. Briefly, 5 µg of the extracted total cellular RNA was reverse transcribed in a reaction volume of 20 µl and 1 µl of the resulting cDNA solution was used to amplify cDNA by C3-, or factor B-specific PCR. The PCR were performed in 50 µl per well in polyethylene reaction tubes and applying cycles consisting of denaturation step at 94°C for 30 seconds, annealing for 1 min at 59°C, and extension for 1 min at 72°C. The PCR incubation

mixture, in a total volume of 50 µl, contained 50 mM KCl, 10 mM Tris-HCl pH 8.1, 2.0 mM $MgCl_2$, 0.01% gelatin, 1.25 unit Taq polymerase (Gibco), 250 µM dNTP mix (Pharmacia, Uppsala, Sweden), and 140 ng of the sense and anti-sense primer each. The following specific primer sets were synthesized in our laboratory by an oligo-synthesizer: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward primer 5'-CGAGATCCCTCCAAAATCAA-3' (nt 298-317); and GAPDH reverse primer 5'-AGGTCAGGTCCACCACTGAC-3' (nt 799-780)[87]; factor B forward primer 5'-CAACAGAAGCGGAAGATCGTC-3' (nt 766-786); and factor B reverse primer 5'-TATCTCCAGGTCCCGCTTCTC-3' (nt 1630-1650)[263]; and C3 forward primer 5'-TCGGATGACAAGGTCACCCT-3' (nt 4627-4646); and C3 reverse primer 5'-GACAACCATGCTCTCGGTGA-3' (nt 5015-5034)[264]. 12.5 microliters of each PCR product was mixed with 5 microliters stop layer mix and run on a 1.7% agarose gel in tris/borate/EDTA buffer. After electrophoresis the gel was scanned by an Eagle Eye imager (Stratagene Europe, Amsterdam, The Netherlands) and the signal strength was integrated to obtain a densitometric value for each amplification product. To enable semi-quantitative analysis, the number of PCR cycles was chosen in such a way that a linear relationship was achieved between PCR product formation (plotted on a log scale) and cycle number (28 to 34 cycles for C3 and factor B and 26 to 32 cycles for GAPDH), without having reached saturation of the product formation.

Statistical analysis

Statistical analysis was performed using the Student's *t*-test for unpaired samples and a *p* value of less than 0.05 was considered significant.

RESULTS

Human keratinocytes constitutively produce C3 and factor B

Keratinocytes produce small amounts of C3 and factor B in culture as determined by ELISA (**Figure 1**). When keratinocytes were cultured up to passage six, and the production of C3 and factor B was monitored in the culture supernatant of each passage, a significant increase of both components with increasing number of passages was observed in cultures derived from three different foreskins (C3 $p<0.002$, and factor B $p<0.02$; passage 6 compared to passage 1; **Figure 1**). Because of these differences we used only cultures from passage 2 to 5 in subsequent studies.

Mediators released from activated mononuclear cells up-regulate the production of C3 and factor B from keratinocytes

Keratinocytes cultured in absence of supernatant of activated mononuclear cells produced 2.1 ± 0.4 ng C3 and 0.4 ± 0.07 ng factor B per 10^6 cells per 24 h. In the presence of 10% supernatant, production of C3 was 56.4 ± 20.7 and of factor B was 6.5 ± 1.4 ng per 10^6 cells per 24 h. In all cultures, supernatant of activated mononuclear cells induced a significant increase in production of both C3 ($p<0.005$) and factor B ($p<0.001$) in a dose dependent fashion.

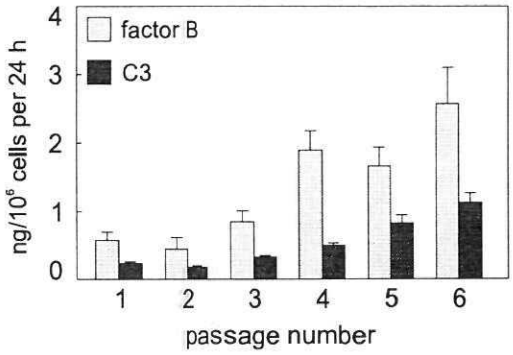

Figure 1. Constitutive release of C3 and factor B by keratinocytes is passage dependent. Subconfluent keratinocytes were cultured for 72 h. Supernatants were collected and cells were rinsed, harvested and counted (passage 1). They were again passaged to sub-confluence, fresh medium was added (at time 0) and the whole process was repeated (passage 2) until passage 6. Supernatants collected after each passage were analyzed for C3 and factor B by ELISA. Three independent experiments with cultures derived from different foreskins were carried out. Values are the mean ± SD for triplicate determinations of one representative culture.

Cytokines differentially regulate the production of C3 and factor B from keratinocytes

The supernatant of activated mononuclear cells is known to contain a number of cytokines, which include IL-1α, IL-2, IL-6, TGF-ß, TNF-α and IFN-γ[265;37]. We tested recombinant forms of these individual cytokines to find if one or more of these mimics the effects seen with supernatant of activated mononuclear cells.

IL-2 (0-1000 U per ml), TGF-ß1 (0-10 ng per ml) and IL-6 (0-1000 U per ml) had no effect on the production of C3 (data not shown). IL-1α, IFN-γ and TNF-α showed a dose-dependent up-regulation of C3 production (**Figure 2**). These cytokines at doses of 100 U per ml, 50 U per ml, and 750 U per ml, respectively, up-regulated C3 production 7-, 8-, and 22-fold, compared to the basal production. Further increase in dose did not cause additional increase in response.

IL-2 (0-1000 U per ml), TNF-α (0-1000 U per ml), and TGF-ß1 (0-10 ng per ml) did not show any significant effect on the production of factor B. IL-1α, IL-6, and IFN-γ caused dose-dependent up-regulation of factor B. IL-1α and IL-6 up-regulated factor B production to about three-fold of the basal release at 200 U per ml and 1000 U per ml, respectively, and IFN-γ about 34-fold at 75 U/ml (**Figure 2**). Further increase in dose did not cause further increase in factor B production.

To confirm that the regulation of C3 or factor B production by normal human keratinocytes was a specific property of the above mentioned cytokines, antibody blocking

experiments were performed. Keratinocytes were cultured in medium alone or in medium containing optimal concentration of individual cytokines in the presence and absence of neutralizing antibodies or control rabbit or mouse IgG (**Figure 2**). The results showed that neutralization of IL-1α with anti-IL-1α, IFN-γ with anti-IFN-γ, or TNF-α with anti-TNF-α abolished the induction of C3 production. Neutralization of IL-1α with anti-IL-1α, IFN-γ with anti-IFN-γ, and IL-6 with anti-IL-6, abolished the induction of factor B production.

The batches of FCS, supernatant of activated mononuclear cells, and keratinocyte medium did not show C3 or factor B reactivity in the respective ELISA assays.

Up-regulation of production of C3 and factor B is inhibited by cycloheximide

TNF-α and IFN-γ which were the strongest up-regulators of C3 and factor B production from keratinocytes, respectively, were selected for further studies to find out whether they caused enhanced production by inducing *de novo* synthesis. This was investigated by observing the effect of cycloheximide on TNF-α and IFN-γ mediated up-regulation of C3 and factor B, respectively. Keratinocytes were grown in medium alone, medium containing IFN-γ (100 U per ml) or TNF-α (750 U per ml) with and without 2.0 μg per ml cycloheximide. Higher concentrations of cycloheximide could not be used because of irreversible toxic effects on the cells. At definite time intervals, supernatants were harvested for C3 and factor B analysis by ELISA. These experiments revealed that cycloheximide significantly inhibited the TNF-α induced C3 and the IFN-γ induced factor B production after 24 h and 48 h (**Figure 3**). This indicated that increased production of C3 in response to TNF-α, and of factor B in response of IFN-γ were due to increased *de novo* synthesis. Removal of cycloheximide by washing the cells and again culturing them in the medium containing TNF-α and IFN-γ restored C3 and factor B production, respectively (data not shown).

Cytokines regulate the production of C3 and factor B at pretranslational level

C3 and factor B mRNA transcripts obtained from cytokine treated and untreated cells were analyzed by RT-PCR. Keratinocytes were incubated for 6 h and 24 h in medium containing IFN-γ (100 U per ml), IL-1α (200 U per ml), IL-6 (1000 U per ml) or TNF-α (750 U per ml). After RT-PCR and gel electrophoresis as described in *'Materials and Methods'* the Eagle Eye analysis revealed 2-, 8-, and 11-fold increase of the ratio C3/GAPDH message after stimulation with IL-1α, IFN-γ and TNF-α, respectively (**Figure 4**). The ratios of factor B/GAPDH were increased 7-, 4-, and 23-fold, by stimulation of keratinocytes with IL-1α, IL-6, and IFN-γ, respectively (**Figure 4**). These semi-quantitative data suggest that the up-regulation of C3 synthesis by IFN-γ, IL-1α and TNF-α and of factor B by IL-1α, IL-6 and IFN-γ was at a pretranslational level.

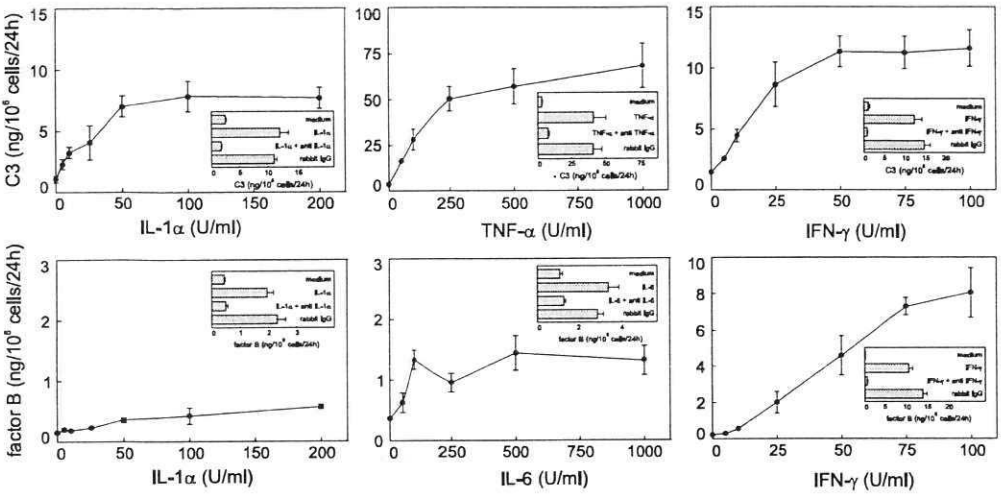

Figure 2. Release of C3 and factor B by keratinocytes is differentially regulated by cytokines. Keratinocytes were cultured for 72 h in the presence of indicated concentrations of cytokines and supernatants were collected and assessed for C3 and factor B concentrations by ELISA. Insets show the effects of cytokine (indicated) specific neutralizing rabbit antibodies on cytokine (indicated) induced C3 and factor B release by keratinocytes. Keratinocytes were cultured with an optimal concentration of indicated cytokine alone, cytokine plus specific neutralizing antibodies, or control IgG. After 72 h, supernatant were collected and assessed for C3 and factor B. The data in main figures and insets are expressed as the mean ± SD of duplicate measurements of three cultures.

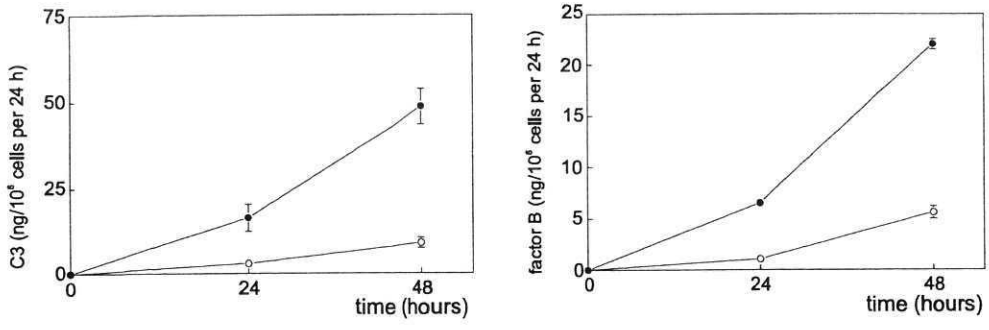

Figure 3. Cycloheximide inhibits C3 and factor B synthesis by keratinocytes. Sub-confluent keratinocytes were cultured in medium containing TNF-α (750 U per ml) or IFN-γ (100 U per ml) in the presence and absence of 2 μg cycloheximide per ml. At 24 h and 48 h, culture supernatant were collected and assayed for C3 and factor B. Results obtained with cultures without (●) and with cycloheximide (○) are shown. Values are the mean ± SD for duplicate determinations of triplicate cultures.

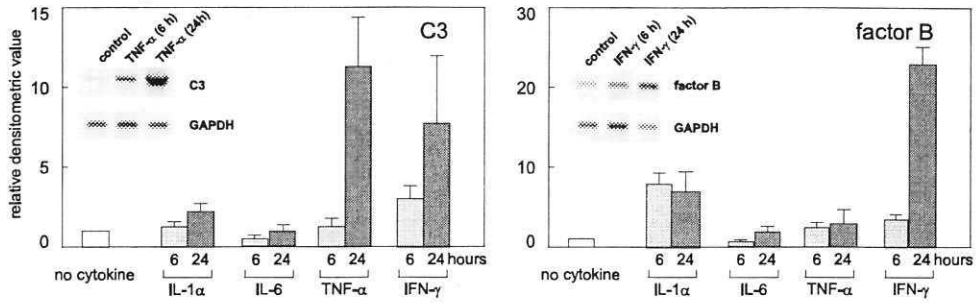

Figure 4. Expression of C3 and factor B in keratinocytes is differentially regulated by cytokines at pretranslational level. RNA was isolated from keratinocytes at 0 h, 6 h, and 24 h after stimulation with indicated cytokines at concentrations given below, and subjected to semi-quantitative C3- or factor B specific RT-PCR as described in the text. After electrophoresis, the densitometric values of the products were determined and corrected for the value of GAPDH, a household gene. The relative densitometric value obtained with untreated keratinocytes (0 h) was arbitrarily set at 1 and were related to those of cytokine-treated keratinocytes (6 h; 24 h).

Inset in the *left figure* shows up-regulation of C3 transcripts by TNF-α (representative example; effects of other cytokines are not shown). RNA was isolated from keratinocytes after 0 h, 6 h, or 24 h of stimulation and subjected to a C3 RT-PCR. After electrophoresis the products were visualized in ethidium bromide solution. Inset in the *right figure* shows up-regulation of factor B transcripts by IFN-γ (representative example; effects of other cytokines are not shown) under the conditions identical to those as for C3 (upper figure).

Concentrations of cytokines: IL-1α = 200 U per ml; IL-6 = 1000 U per ml; TNF-α = 750 U per ml; IFN-γ = 100 U per ml

DISCUSSION

The epidermal compartment of skin is known to express immunity[266]. Because the epidermis is in frequent contact with foreign antigens it is expected to possess, besides components of cellular immunity, a complement synthesizing apparatus to form the first line of immunological defense. The most abundant cells in human epidermis, the keratinocytes, indeed have been shown to express C3[34;267], factor B[35;267], several complement receptors[28], and complement regulatory proteins[11;37] and like some other cell types[256], may produce all the components of classical and alternative pathways. The complement synthesized in the epidermis can perform some important immunological functions at tissue level as is begining to be envisaged in other organs[109].

Keratinocytes are known to synthesize low levels of C3[34;36] and factor B[35], but if local synthesis of complement plays a role in immunological defense and inflammatory conditions of the skin, their production should be amplified when it is most required, *e.g.* during microbial

invasion and under inflammatory conditions of the skin. This amplification should most likely be mediated by cytokines which are produced by keratinocytes and infiltrating inflammatory cells and which are known to regulate the synthesis of complement-components in many cell types differentially and in tissue specific manner[258;268;56;129].

In the present study we investigated the regulation of synthesis of C3 and factor B by the supernatant of activated mononuclear cells, a source of mediators derived from inflammatory cells, and by recombinant forms of several cytokines known to be present in this supernatant. Constitutive production of C3 was greatly up-regulated by the supernatant of activated mononuclear cells and TNF-α but also appreciably by IL-1α and IFN-γ (**Figure 2**). IL-2, IL-6 and TGF-ß had no effect. Constitutive production of Factor B was greatly up-regulated by the supernatant of activated mononuclear cells and IFN-γ but also appreciably by IL-1α and IL-6 (**Figure 2**). IL-2, TNF-α and TGF-ß had no effect. These results show differential effects of some cytokines on the production of C3 and factor B by human keratinocytes.

Since several cell types such as neutrophils store large amounts of some complement components and secrete them upon stimulation without stimulating their synthesis[269] we investigated whether the cytokine induced release of C3 and factor B was due to their export from intracellular reserves or was associated with increase in their synthesis. The possibility of export of intracellular reserves was ruled out by the facts that (1) cycloheximide inhibited cytokine induced enhancement of C3 and factor B production and (2) IL-1α, TNF-α, and IFN-γ mediated enhancement of production of C3 and IL-1α, IL-6, and IFN-γ mediated enhancement of production of factor B was associated with the up-regulation of transcription of C3 and factor B genes as seen by semi-quantitative RT-PCR (**Figure 4**). LPS was also tested for its ability to induce the synthesis of C3 and factor B by keratinocytes but was found to have no effects at 100 ng per ml, a concentration 10 to 100 times higher than that needed to affect complement synthesis in human fibroblasts[270].

The up-regulation of C3 synthesis in keratinocytes by IL-1α and IL-6 does not appear to be mediated by autocrine release of TNF-α by keratinocytes in culture medium. Most likely TNF-α released in the medium is diluted to concentrations insufficient to induce the synthesis of C3 and factor B by keratinocytes. The support for this postulation comes from the following observation. In our laboratory, the concentration of TNF-α in culture medium of stimulated keratinocytes was found to be very low (<1 U per ml)[267] whereas that required for up-regulation of C3 in our system in this study was much higher (**Figure 2**). Similarly, the enhancement of factor B synthesis in response to IL-1α and IL-6 appears not to have been caused by any other cytokine released in autocrine manner from keratinocytes.

IL-1α and IL-6 had comparatively weak stimulating effects on the synthesis of C3 and factor B, respectively, by human keratinocytes. This was in contrast to the findings of Katz *et*

al.[93], and Katz and Strunk[62] in human skin fibroblasts, who showed that IL-1α and IL-6 play an important role in the regulation of both C3 and factor B synthesis. In glomerular epithelial cells IFN-γ does not increase C3 gene expression[271], which is also in sharp contrast to the strong up-regulation of C3 caused by IFN-γ in keratinocytes. Comparison of our results with those obtained with other cell types confirmed the widely held view that regulation of complement synthesis by cytokines is highly cell type specific.

If the observations made in the present study could be extrapolated to *in vivo* situations, several cytokines produced locally by keratinocytes and infiltrated inflammatory cells, during an inflammatory response, can up-regulate the synthesis of complement components *in vivo*. Because complement is known to be continuously activated, complement produced by keratinocytes in higher than usual amounts in response to some cytokines may damage keratinocytes. To prevent this there should exist a mechanism(s) which can protect keratinocytes from this autologous complement. One such mechanism could be the differential up-regulation of complement-regulatory molecules, which protect autologous cells from activated complement, by keratinocytes or inflammatory cells derived cytokines. This has indeed been shown in a recent study in which up-regulation of complement regulatory membrane proteins, membrane cofactor protein (CD46) and CD59, by supernatants of activated mononuclear cells and TGF-ß has been shown[37].

In conclusion, we provide data which confirm that keratinocytes constitutively produce low levels of C3 and factor B. TNF-α, IFN-γ and IL-1α regulate the synthesis of C3 and IFN-γ, IL-1α and IL-6 that of factor B. Local complement protein synthesis may contribute to local immunological defenses. Other possible roles of local synthesis of complement by keratinocytes have yet to be investigated.

Chapter Three

Transforming Growth Factor-β isoforms regulate the surface expression of membrane cofactor protein (CD46) and CD59 on human keratinocytes

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Eur. J. Immunol. 1999. 29: 100-108

Abstract

We studied the regulation of the expression of complement regulatory proteins, membrane cofactor protein (MCP), decay accelerating factor (DAF) and CD59, on human keratinocytes by supernatant of activated mononuclear cells and by some individual cytokines present therein. Cultured keratinocytes expressed MCP, DAF and CD59. Supernatant of activated mononuclear cells and recombinant forms of transforming growth factor (TGF)-ß variants (β1, β2 and β3) up-regulated MCP and CD59 but not DAF. Recombinant IL-1α, IL-2, IL-6, TNF-α and IFN-γ had no influence. TGF-ß present in the supernatant was likely responsible for up-regulation of MCP and CD59. A monoclonal anti-TGF-β antibody, which neutralized TGF-ß1, -ß2 and -ß3 did not inhibit the up-regulation of MCP and CD59 by the supernatant. These results indicated that TGF-β and an additional factor(s) present in the supernatant may be responsible for up-regulating the expression of MCP and CD59 on keratinocytes; both may be acting non-synergistically.

INTRODUCTION

The complement system is constantly activated *in vivo* and may potentially damage autologous cells[2]. The complement-mediated damage of autologous cells is, however, prevented by complement-regulatory proteins that are expressed on their surfaces[14;13;2;272]. These proteins include decay accelerating factor (DAF), membrane cofactor protein (MCP) and CD59. They protect cells on which they are expressed by inhibiting different steps of complement activation. DAF combines with C4b and C3b bound to the cell membrane and interferes with the interaction of C2 with C4b and of factor B with C3b[273]. DAF also accelerates the dissociation of C2a from C4b and of Bb from C3b within C3-convertases of classical and alternative pathway, respectively[274]. Thus, DAF interferes with the formation of C3- and C5-convertases of the classical and alternative pathways and accelerates their decay. MCP combines with C4b and C3b bound to the cell membrane and then acts as a cofactor for the enzyme factor I. This enzyme cleaves C4b and C3b and generates the inactive derivatives iC4b and iC3b[275]. Thus, MCP interferes with the assembly of C3- and C5-convertases of both pathways. CD59 binds to an epitope on the α-chain of the α-γ-subunit of C8. This epitope is exposed when C8 is incorporated into the C5b-7 complex on the cell membrane[276]. CD59 also binds to C9 in which a CD59 binding site is exposed after surface adsorption[277]. Thus, CD59 inhibits the transmembrane channel formation by late components by binding to C8 and C9[277;276]. DAF, MCP and CD59 may be coexpressed on a self cell and act synergistically to inhibit complement activation on it[278;279].

Activation of complement on or around keratinocytes occurs in many inflammatory conditions of the skin (*e.g.* pemphigus, pemphigoid)[280] and poses particular risk for keratinocytes to be damaged. Since complement-regulatory proteins are endowed with the task of protecting keratinocytes during complement-mediated inflammation, there should exist a mechanism(s) which can up-regulate their expression on the keratinocyte surface during complement attack. This mechanism could be as follows. complement attack can release several cytokines from keratinocytes as has been shown with many cell types[3;27] and some of these can up-regulate complement-regulatory proteins which can provide increased protection to keratinocytes against complement attack. Cytokines capable of up-regulating complement-regulatory proteins on keratinocytes may also be released by inflammatory cells that infiltrate the epidermis during complement-mediated inflammation.

In the present study we investigated the regulation of expression of DAF, MCP and CD59 on human keratinocytes by supernatant of activated peripheral blood mononuclear cells and by several individual cytokines known to be present therein[265]. In our laboratory TGF-β was also found to be present in the supernatant. We therefore included recombinant forms of TGF-β

variants, *i.e.* TGF-β1, TGF-β2 and TGF-β3, in this study. Some artificial stimuli of protein kinase C (PKC) and protein kinase A (PKA) were also studied to see if their effects mimic the effects of TGF-ß in the supernatant of mononuclear cells.

MATERIALS AND METHODS

Chemicals and reagents

Dibutyryl-adenosine-cyclic-monophosphate (cAMP), dibutyryl-guanoside-cyclic-monophosphate (cGMP), phorbol 12-myristate 13 acetate (PMA), calcium ionophore A23187, concavalin A, dimethylmannoside, normal mouse IgG and human recombinant TGF-ß3 were purchased from Sigma (St. Louis, MO). Penicillin/streptomycin, keratinocyte serum free medium (keratinocyte SFM), Iscove's modified Dulbecco's medium (IMDM) and fetal calf serum (FCS) were obtained from Gibco BRL (Breda, The Netherlands). Human recombinant IL-2 and isotype control mAb (clone 203) were purchased from the Central Laboratory of Blood Transfusion Services (Amsterdam, The Netherlands). Human recombinant cytokines IFN-γ, IL-1α, IL-6, TGF-ß1, and TGF-ß2 were purchased from Boehringer Mannheim (Mannheim, Germany). Human recombinant TNF-α was purchased from Genzyme (Genzyme, Cambridge, MA). FITC-labeled mouse anti-human MCP IgG1 (clone 122-2) and FITC-labeled mouse anti-human DAF IgG1 (clone Bric 110) were obtained from Instruchemie (Hilversum, The Netherlands). Anti-TGF mAb (clone 2G7)[281], was a gift from Dr. Stevin Schoenberger (Department of Immunohematology, University of Leiden, Leiden, The Netherlands). Mouse anti-human CD59 IgG1 (1F5) was a kind gift from Dr. Noriko Okada (Nagoya City University School of Medicine, Nagoya, Japan) and was biotinylated according to routine technique[282]. Supernatant of activated mononuclear cells was prepared as described[260]. Briefly, peripheral blood mononuclear cells (PBMC; 50 x 10^6/ml) from normal donors were stimulated for 2 h at 37°C with 0.1 µg/ml PMA in IMDM (supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin and 100 µg/ml streptomycin). PMA-treated cells were washed extensively and cultured (50 x 10^6/ml) for 48 h with 15 µg/ml Con A in supplemented IMDM. Con A was neutralized by addition of 50 mM α-methylmannoside for 30 min at 37°C. Aliquots were stored at -20°C.

Keratinocyte culture

Human keratinocytes were isolated by incubation of foreskins with 0.50 mg/ml thermolysin (Sigma) at 4°C for 16 h and subsequent trypsinization (0.025%) for 5 min at 37°C. Trypsin (Sigma) was then neutralized by FCS. Cells were separated from debris by filtering, centrifuged and resuspended in keratinocyte SFM supplemented with 100 IU/ml penicillin/100 µg/ml streptomycin. The keratinocytes were plated onto 100 mm plastic Petri dishes at a density of 400,000 cells per Petri dish and were incubated at 37°C in a humidified, 5% CO_2, tissue culture incubator. Medium was changed every 2-3 days, and at 70% confluency cells were split with trypsin (0.25%) and recultured. For use in flow cytometry experiments, cells were seeded in 6-well tissue culture plates at a density of 100,000 cells per well. Cells in passage 2-5 were used for experiments as soon as 60-80% confluency was achieved. For studying the effect of various additives, cells were incubated in 1500 µl medium alone or 1500 µl medium containing the additive.

Immunohistochemistry

Keratinocytes were grown for 48 h on a microscopic slide, rinsed in PBS and then fixed for 10 min using a 3% paraformaldehyde solution in PBS. Endogenous peroxidase activity was inactivated by 30 min incubation in

0.3% H_2O_2 in PBS. The immunohistochemical staining was then performed by a three-step immunoperoxidase technique[283]. The slides were incubated with 20 µg/ml monoclonal anti-DAF, 2 µg/ml anti-MCP or 10 µg/ml anti-CD59 antibody or 20 µg/ml mouse IgG1 (DAKO A/S, Glostrup, Denmark) for 60 min followed by incubation for 60 min with a 1:200 dilution of a biotinylated rabbit anti-mouse Ig (DAKO) in PBS/human AB serum (10%; DAKO). They were then incubated for 30 min with horseradish peroxidase-labeled polystreptavidin (ABC Complex, DAKO). Peroxidase activity was visualized by incubation of the slides with 0.05% 3-amino-9-ethylcarbazole in acetate buffer for 10 min followed by a counterstaining with hematoxylin for 20-30 s. All incubations were at room temperature.

Flow cytometry

Keratinocytes were detached by a trypsin solution (0.025% trypsin, 1 mM EDTA) for 3-5 min. This treatment did not affect DAF, MCP and CD59 as preliminary experiments with combinations of different concentrations of trypsin and EDTA had shown. Trypsin was inactivated by FCS and cells were washed and resuspended in FACS buffer (PBS, 2% FCS, 0.1% sodium azide). Approximately 10^5 cells were incubated with biotinylated anti-CD59 mAb or the biotinylated isotype control for 30 min at 4 °C. Cells were washed twice and incubated for 30 min with streptavidin-RPE-CY5 (DAKO). Cells for analysis of MCP and DAF were incubated with FITC-labeled specific antibodies or isotype control for 30 min at 4 °C. Cells were washed twice and 10,000 cells were analyzed immediately using a FACSCalibur (Becton Dickinson, San José, CA).

MFI was calculated with WinMDI software. The MFI values of untreated cells were taken as 100%. The MFI values obtained from cells treated with a test material (*e.g.* cytokine) are presented in terms of percent of untreated cells.

ELISA for the measurement of TGF-β

The concentration of TGF-β in the supernatant of activated mononuclear cells was estimated by a sandwich ELISA as described[284]. Briefly, wells were coated with 2 µg/ml mouse anti-human TGF-β (2G7; described above) in PBS by incubating the plates for 1 h at 37 °C and then overnight at 4 °C. After washing, wells were blocked for 1 h at room temperature with PBS, 0.2% Tween-20, 2% bovine serum albumin (BSA). Washing was repeated and samples were added in triplicate to the wells for 2 h at room temperature. After washing wells were incubated with chicken anti-human TGF-β antibodies coupled to digoxigenin (DIG) for 1 h at room temperature. Plates were washed and incubated with anti-DIG $F(ab')_2$ fragments conjugated with horseradish peroxidase for 1 h at room temperature. Peroxidase activity was visualized by addition of the peroxidase substrate 2,2-azino-bis-3-ethylbenzthiazolin (Sigma) prepared in a 100 mM citrate/100 mM phosphate buffer, pH 4.2. Optical density was measured at 415 nm. A standard curve for TGF-β was made using human rTGF-β1 as standard for every plate. TGF-β was activated by pH.

Statistical analysis

Statistical analysis was performed using the Student's *t*-test for paired samples and a *p* value of less than 0.05 was considered significant.

RESULTS

Cultured human keratinocytes express MCP, DAF and CD59

Previous *in situ* immunohistochemical studies in our laboratory have demonstrated that MCP, DAF and CD59 are present on several structures on human skin including keratinocytes[11]. When the expression of MCP, DAF and CD59 on cultured keratinocytes was studied immunohistochemically, all the three proteins were found to be present on their membranes (**Figure 1**). Further flow cytometric analysis confirmed that all these complement-regulatory proteins were expressed on nonstimulated keratinocytes (**Figure 2**).

Expression of MCP and CD59 on human keratinocytes is up-regulated by mediators released from activated mononuclear cells

Complement-mediated inflammation is almost invariably associated with infiltration by inflammatory cells. Therefore it was considered likely that mediators released by inflammatory cells may have profound effects on the expression of complement regulatory proteins on keratinocytes. We analyzed the regulation of surface expression of MCP, DAF and CD59 by supernatant of mononuclear cells activated as described in '*Materials and Methods*'. As shown in **Figure 2**, supernatant at a final concentration of 10% caused an up-regulation of MCP and CD59 but not of DAF. Time-response studies with 10% supernatant (data not shown) revealed that there was no up-regulation of MCP in the first 24 h and only slight up-regulation of CD59 at 24 h; up-regulation of both was maximal at 72 h and 144 h, respectively.

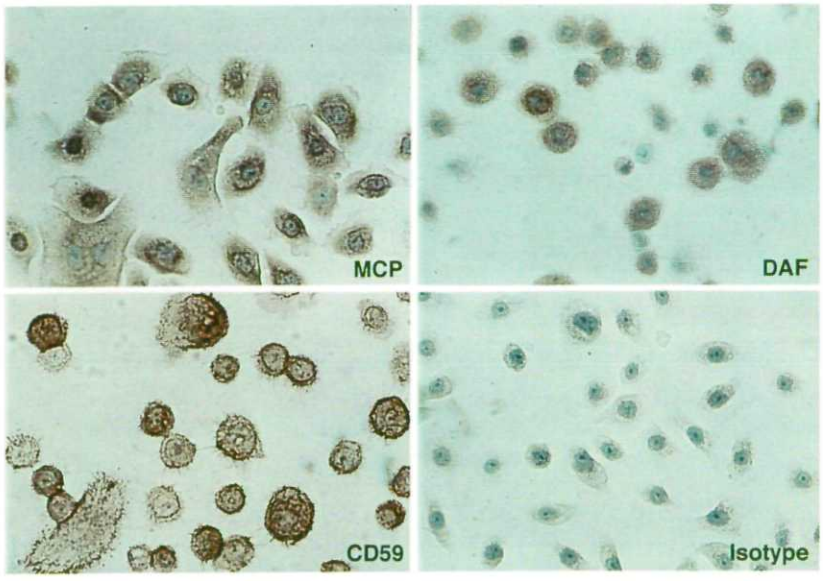

Figure 1. Surface staining of DAF, MCP and CD59 on cultured human keratinocytes. The cells were stained by a immunoperoxidase technique using primary antibodies to MCP, DAF, and CD59 and IgG1 as isotype control. All views are 40x.

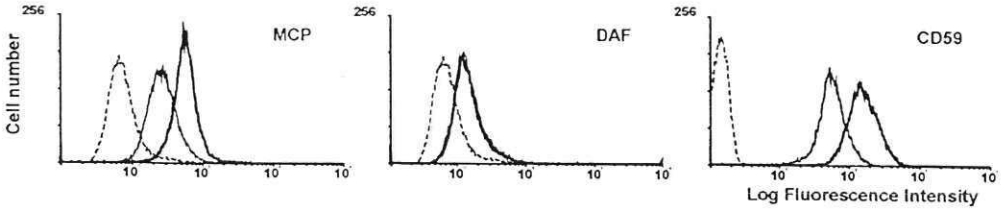

Figure 2. Expression of DAF, MCP and CD59 on cultured human keratinocytes and their up-regulation by supernatant of activated mononuclear cells. Expression of DAF, MCP and CD59 on 10^4 keratinocytes was analyzed by flow cytometry after culturing in medium containing 10% supernatant of activated mononuclear cells. Histograms for expression of DAF, MCP and CD59 are shown. Bold lines represent expression in the presence and thin lines in the absence of supernatant. Broken lines represent results obtained with isotype controls. The data are representative of three independent staining experiments. Supernatant at 0-5% concentrations had no significant effect on expression of DAF, MCP or CD59.

To test if up-regulation of MCP and CD59 in response to supernatant of activated mononuclear cells was due to *de novo* synthesis, the effect of cycloheximide on the expression and up-regulation of these molecules by the supernatant was studied. Expression of MCP and CD59 in the presence of a predetermined sublethal dose of cycloheximide (2 μg/ml) was lower than in its absence both in supernatant-treated as well as untreated cells (**Figure 3**). This was probably because cycloheximide not only inhibited supernatant-induced up-regulation but also normal constitutive synthesis of these molecules. The effect of cycloheximide was reversible: expression of MCP and CD59 was restored in cells which after incubation with cycloheximide for 72 h were washed and subsequently cultured for another 72 h (data not shown). These results suggest that up-regulation caused by supernatant was due to *de novo* synthesis. Cycloheximide also caused a decrease in expression of DAF in absence of supernatant [40% decrease in mean fluorescence intensity (MFI)] and in its presence (50% decrease in MFI). Expression of MCP, DAF and CD59 in the presence of cycloheximide did not reach zero level, likely because pre-existing molecules on the surface of keratinocytes were not catabolized completely during 72 h culture.

Several pro-inflammatory cytokines known to be secreted by activated mononuclear cells do not regulate the expression of MCP, DAF and CD59 in human keratinocytes

Supernatant of activated mononuclear cells contains a number of cytokines, including IL-1, IL-2, IL-6, TNF-α and IFN-γ[265]. One or more of these cytokines could have been responsible for up-regulation of MCP and CD59 caused by supernatant of activated mononuclear cells. We tested the effect of their recombinant forms on the expression of MCP, DAF and CD59.

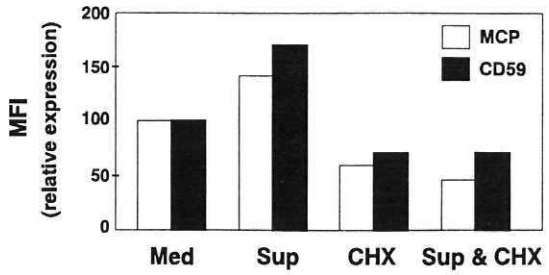

Figure 3. Effects of cycloheximide (CHX) on up-regulation of expression of MCP and CD59 by keratinocytes. Keratinocytes were cultured for 72 h in medium alone (Med) or medium containing 10% supernatant of activated mononuclear cells (Sup), CHX (2 μg/ml) or Sup and CHX. MCP and CD59 expression was determined by flow cytometry analysis and results were expressed as a percentage of MFI value obtained with keratinocytes cultured in medium alone.

Keratinocytes (60-80% confluent) were cultured for 72 h in the presence and absence of 2-200 U/ml IL-1α, 10-1,000 U/ml IL-2, 10-1,000 U/ml IL-6, 5-200 U/ml IFN-γ and 10-1,000 U/ml TNF-α and analyzed for the expression of MCP, DAF and CD59 by flow cytometry. None of these cytokines significantly up-regulated any of the three complement-regulatory proteins (three independent experiments with each cytokine; data not shown).

TGF-β is present in the supernatant of mononuclear cells and can up-regulate the expression of MCP, DAF and CD59 on keratinocytes

After having failed to identify any specific mediator, known to be present in the supernatant of activated mononuclear cells responsible for up-regulation of MCP and CD59, we investigated the presence of TGF-β in the supernatant. TGF-β isoforms TGF-β1 and TGF-β2, are produced by activated macrophages[285]. Measurement of TGF-β in the supernatant of activated mononuclear cells by an ELISA, which measures all the three variants combined, showed that TGF-β was present in the supernatant at a concentration of 3.5 ng/ml.

To find out whether TGF-β variants in concentrations present in the supernatant (0.35 ng/ml in 10% supernatant) could be responsible for up-regulating MCP and CD59, the ability of recombinant TGF-β1 and TGF-ß2 (0.1-10 ng/ml) to up-regulate MCP and CD59 on keratinocytes was investigated. TGF-β3, which is produced by cells of mesenchymal origin but has 79% homology with TGF-β2, was also included in the study. Keratinocytes (60-80% confluent) were cultured for 72 h in the presence and absence of TGF-β1, -β2, or -β3 and analyzed for the expression of MCP, DAF and CD59 by flow cytometry. Like the supernatant, all the three

variants of TGF-β (at 1.0 and 10 ng/ml) up-regulated the expression of MCP and CD59 (**Figure 4**) but did not affect the expression of DAF significantly (data not shown). In independent experiments TGF-β1 and TGF-β2, at concentrations 0.35 ng/ml and higher, were found to up-regulate MCP and CD59 significantly (data not shown here). TGF-β3 required higher concentrations than 0.35 ng/ml for significant effect. These results suggest that TGF-β1 and/or TGF-β2 was the factor or factors in the supernatant responsible for up-regulation of MCP and CD59.

TGF-β1, TGF-β2 and TGF-β3 mediated up-regulation of MCP and CD59 was blocked by the anti-TGF-β mAb 2G7 (**Figure 5**)[281]. This showed that up-regulation caused by these TGF-β variants was specific. The anti-TGF-β could not block up-regulation of MCP and CD59 induced by supernatant of activated mononuclear cells, even at a concentration of anti-TGF-β as high as 200 μg/ml (**Figure 5**). This indicated the presence, in addition to TGF-ß1 and TGF-β2, of a factor(s) in the supernatant responsible for causing up-regulation of MCP and CD59 on keratinocytes in a non-synergistical fashion.

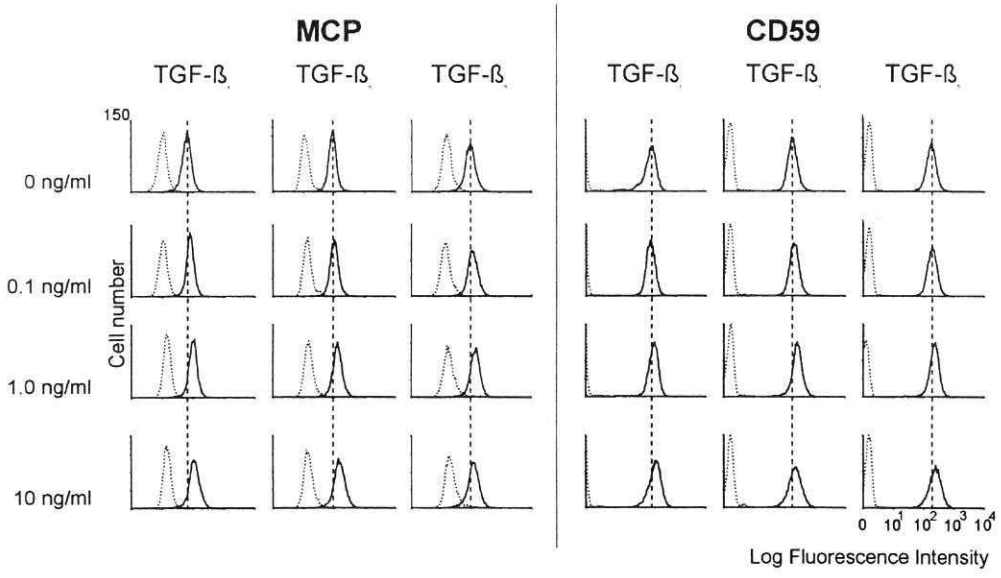

Figure 4. Effect of TGF-β variants on the expression of complement-regulatory proteins by keratinocytes. Expression of DAF, MCP and CD59 on 10^4 keratinocytes after culturing in medium containing the indicated concentrations of TGF-β1, TGF-β2 or TGF-β3 was analyzed by flow cytometry. Bold lines represent MCP or CD59 expression in the presence of TGF-β isoforms. Broken lines show isotype controls. None of the TGF-β isoforms had significant influence on expression of DAF (data not shown). The results are representative of three staining experiments carried out in duplicate.

Expression of MCP, DAF and CD59 on human keratinocytes is up-regulated by activators of PKC and PKA

Since TGF-β is known to activate PKC[286], it was interesting to investigate whether activators of PKC (PMA and calcium ionophore A23187) can mimic the effects of TGF-β variants on expression of MCP, DAF and CD59. An activator of PKA, dibutyryl-cAMP, was also included. Keratinocytes (60-80% confluent) were stimulated with these activators for 72 h and analyzed by flow cytometry. PMA (0.5-5.0 nM), calcium ionophore A23187 (5.0-50 mM) and dibutyryl-cAMP (0.1-1.0 mM) caused a dose-dependent increase in surface expression of MCP, DAF and CD59 (**Figure 6**; data with highest concentrations are shown here). To rule out the effect of dibutyryl part of dibutyryl-cAMP, we used dibutyryl-cGMP as a control. Dibutyryl-cGMP had no effect on the surface expression of MCP, DAF and CD59 (data not shown). These results indicate that PKC and PKA activation can up-regulate the expression of MCP, DAF and CD59 on human keratinocytes.

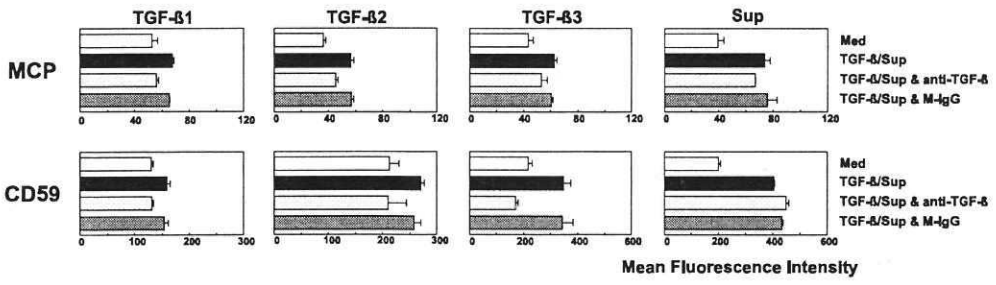

Figure 5. Neutralization of MCP- and CD59-up-regulating capability of TGF-β1, TGF-β2 and TGF-β3 but not that of supernatant of activated mononuclear cells by an anti-TGF-β mAb. Keratinocytes were cultured in medium containing 1.0 ng/ml of TGF-β1, TGF-β2 or TGF-β3 or 10% supernatant (Sup) of activated mononuclear cells in the presence or absence of an anti-TGF-β mAb or a control mouse-IgG (M-IgG). Keratinocytes cultured in medium alone were also examined (Med). Expression of MCP and CD59 was determined using flow cytometry analysis. Up-regulation of MCP and CD59 by 1.0 ng/ml of TGF-β1, TGF-β2 or TGF-β3 could be completely neutralized with 20 μg/ml, 100 μg/ml and 200 μg/ml anti-TGF-β antibody, respectively. Up-regulation of MCP and CD59 by 10 % supernatant could not be neutralized by a final concentration as high as 200 μg/ml of the anti-TGF-ß antibody. M-IgG did not show neutralizing effects on TGF-ß induced up-regulation of MCP and CD59.

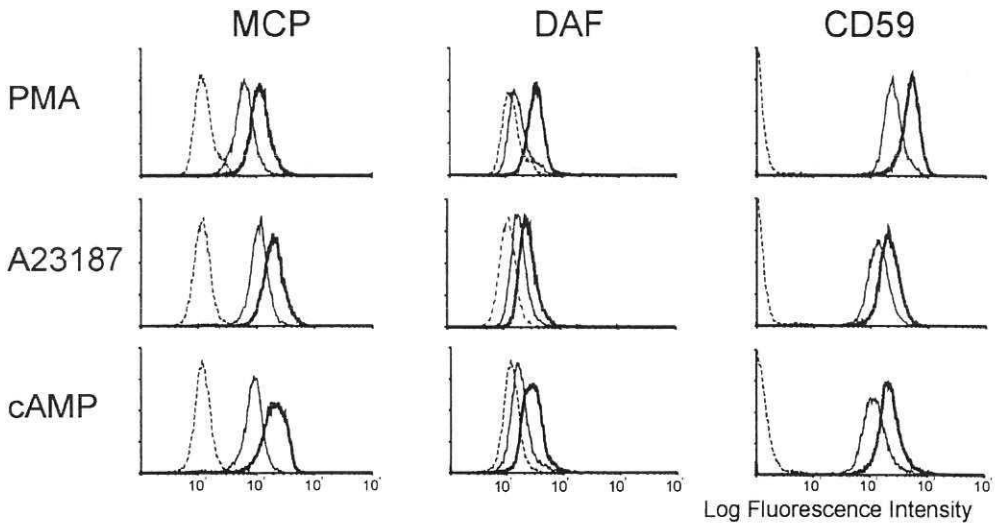

Figure 6. Effect of activators of PKC and of PKA on the expression of DAF, MCP and CD59 by keratinocytes. Expression after culturing in medium containing 1mM dibutyryl-cAMP, 5mM PMA or 50mM calcium ionophore A23187 was analyzed by flow cytometry (data only with the highest concentration tested are presented here; see the text). The results are representative of two independent experiments carried out in triplicate. Bold lines represent DAF, MCP or CD59 expression in the presence of an activator of PKC or PKA, thin lines in the absence of the activator and broken lines represent isotype controls.

DISCUSSION

This study shows that human keratinocytes express complement regulatory molecules DAF, MCP and CD59 on their surface. The co-expression of all these molecules suggests that keratinocytes may possess a high degree of resistance against autologous complement. Indeed, human keratinocytes[38] and a squamous carcinoma cell line, SCC-12F[39], are remarkably resistant to complement-mediated lysis. In diseases such as pemphigus in which there is a strong complement attack directed against keratinocytes[287], keratinocyte death is not a predominant feature. Thus, keratinocytes are strongly protected against autologous complement attack. They need strong protection against complement because epidermis is under a continuous threat of exposure to foreign microbes and other materials which can activate complement and cause bystander complement attack on keratinocytes.

The protective role of complement-regulatory molecules against autologous complement demands that their expression on keratinocytes be up-regulated in inflammatory conditions of epidermis in which complement attacks keratinocytes. The mechanism of increased expression could be the up-regulation by cytokines produced keratinocytes attacked by C^3 or by infiltrating cells at the site of complement-mediated inflammation. To test this, we used supernatant of activated mononuclear cells as a source of inflammatory cell-derived cytokines[265] and investigated its ability to up-regulate the expression of complement-regulatory proteins on keratinocytes. The expression of MCP and CD59 was indeed up-regulated by this supernatant but the expression of DAF was not appreciably altered. Results of experiments with cycloheximide (**Figure 3**) showed that increased expression of MCP and CD59 was likely due to increased *de novo* synthesis. If these *in vitro* findings could be extrapolated to the *in vivo* situation, this would mean that mediators released from mononuclear cells in inflammatory conditions *in vivo* will up-regulate MCP and CD59 on keratinocytes. Thus, keratinocytes in areas of inflammation could be better protected from complement-mediated lysis than those present in areas free of inflammation.

Attempts were made to identify the factor(s) present in the supernatant responsible for causing up-regulation of MCP and CD59. IL-1, IL-2, IL-6, TNF-α, and IFN-γ are known to be present in the supernatant of activated mononuclear cells[265]. Recombinant forms of none of these cytokines up-regulated any of the three complement-regulatory proteins on keratinocytes. This suggested the presence of some other active principle in the supernatant. We argued that TGF-ß (TGF-β1 and TGF-β2), which is produced by activated macrophages[285], might be present in the supernatant of activated mononuclear cells and may be responsible for up-regulation of MCP and CD59 on keratinocytes. Further experiments showed that recombinant TGF-β1, TGF-β2 and TGF-β3, like the supernatant, up-regulated MCP and CD59 but not DAF. The concentration of TGF-β found in the supernatant, and the concentrations of TGF-β1 and TGF-β2 required for up-regulation, suggested that TGF-β present in the supernatant may be causing the up-regulation of surface expression of MCP and CD59 on keratinocytes.

Addition of anti-TGF-ß in amounts in excess of those needed to neutralize all the TGF-ß present in the 10% supernatant did not abolish the up-regulation of MCP and CD59 caused by the supernatant (**Figure 5**). This suggests that an additional factor other than TGF-ß may also be present in the supernatant of activated mononuclear cells which can up-regulate MCP and CD59 in a non-synergistical manner. This may be postulated to be a member of the TGF-ß superfamily.

TGF-β variants perform a variety of functions. They are involved in proliferative,

inductive and regulatory effects on a wide variety of cell types[288;289]. For instance, they regulate the production of acute phase proteins[290]. TGF-β inhibits inflammatory cytokine-induced C3 production in astrocytes[291]. It up-regulates MCP and CD59 in keratinocytes as has been demonstrated in this study. Further studies may show similar effects of TGF-β in other cell types. If so, TGF-ß may be assigned the new function of protection of cells against complement attack by inhibiting complement production and up-regulating complement-regulatory proteins on cells.

Activators of PKC (PMA and A23187) of PKA (dibutyryl-cAMP) can up-regulate MCP, DAF and CD59. This raises questions such as (1) why IL-1[292-294] and TNF-α[294-295], which can also activate PKC and PKA, cannot up-regulate any of the three complement-regulatory proteins and (2) why TGF-ß, which is known to activate PKC[286] can up-regulate only MCP and CD59. These observations provide rationale to investigate up-regulating and down regulating factors in pro-inflammatory cytokines- and TGF-ß mediated signalling in keratinocytes.

In conclusion, the expression of MCP and CD59 but not DAF on human keratinocytes is up-regulated by the supernatant of activated mononuclear cells. This supernatant was found to contain TGF-β. TGF-β (β1, β2 and β3) also up-regulates MCP and CD59 but not DAF. TGF-β present in the supernatant could be responsible for up-regulation of MCP and CD59. An additional factor(s) appears to be present in the supernatant which can up-regulate MCP and CD59 on keratinocytes in a non-synergistical manner. Expression and up-regulation of complement-regulatory proteins are important for protection of keratinocytes during complement-mediated inflammation.

Chapter Four

Effects of UVB on the synthesis of complement proteins by keratinocytes

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J. Invest. Dermatol. 1998. 111: 683-688

Abstract

UVB-exposure of the skin results in increased production of several cytokines by keratinocytes and infiltration of inflammatory cells. We hypothesized that UVB may increase the expression of complement components and complement regulatory proteins by keratinocytes. *In vivo*, UVB may up-regulate these proteins by direct effects or via cytokines released by keratinocytes or infiltrating inflammatory cells. *In vitro*, UVB may up-regulate these proteins only directly, because of dilution of released cytokines in the medium. To test this, we exposed cultured human keratinocytes to UVB (0-64 J per m^2) and monitored C3 and factor B release in the medium by enzyme-linked immunosorbent assay, and surface expression of membrane cofactor protein, decay accelerating factor, and CD59 by flow cytometry. Keratinocytes produced small amounts of C3 and factor B, which remained unaffected by UVB. UVB (32 J per m^2) caused a transient up-regulation of all three complement regulatory proteins. Decay accelerating factor expression was maximal at 48 h (1.81 ± 0.06-fold increase in mean fluorescence intensity over non exposed cells), membrane cofactor protein at 72 h (2.13 ± 0.09-fold increase in mean fluorescence intensity), and CD59 at 120 h (1.96 ± 0.09-fold increase in mean fluorescence intensity), returning to baseline values within 96, 192 and 192 h, respectively. Exposure to 64 J per m^2 resulted in significant cell death; cells surviving this dose up to 48 h expressed a higher level of all the three proteins than those surviving 32 J per m^2.

In conclusion, UVB up-regulated membrane cofactor protein, decay accelerating factor, and CD59 on keratinocytes without affecting the constitutive release of C3 and factor B. Thus, UVB can increase the resistance of keratinocytes against their own complement known to be produced excessively in response to cytokines of inflammatory cells which infiltrate the skin following UVB exposure.

INTRODUCTION

Human keratinocytes, the major cell type in the epidermis, have been recognized as initiators of inflammation[278]. They are immunocompetent cells: they act as accessory cells in T cell responses[296] and synthesize a number of cytokines[297]. They also synthesize complement components C3[34] and factor B[35]. Recently it has been shown that some of the cytokines produced by keratinocytes and inflammatory cells can differentially enhance the synthesis of C3[36;298] and factor B[298] by keratinocytes. Keratinocytes are also expected to produce other components of complement whose synthesis is also likely to be regulated by cytokines. Therefore, under inflammatory conditions of the epidermis, keratinocytes may be considered a local source of complement.

C3 and factor B produced by keratinocytes are beneficial to the host in that they participate in the first line of defence against invasion by foreign cells such as microbes in the skin. These components can eliminate foreign cells via the alternative pathway of complement by generating the C3/C5-convertases (C3b.Bb)/[$(C3b)_n$.Bb)] of the alternative pathway on them[14]. C5-convertase in the presence of C5 through C9, can cause the assembly of the C5b-9 complex, the membrane attack complex, on the cell surface that lyzes the foreign cells.

Although complement produced by keratinocytes is beneficial in host defence against foreign cells, it potentially can damage autologous keratinocytes as well, through the membrane attack complex assembly on them. Keratinocytes have complement regulatory proteins embedded in their membranes to prevent this damage[299;28;11]. These include decay accelerating factor (DAF; CD55), membrane cofactor protein (MCP; CD46), and CD59[13]. These proteins inhibit different steps of complement activation on cell surfaces. DAF reversibly interferes with the formation of C3/C5-convertases of both pathways of complement and accelerates their decay[300]. MCP acts as a cofactor for the C3 cleaving enzyme, Factor I, and interferes irreversibly with the assembly of C3/C5-convertases of both pathways[301]. CD59 is incorporated into the membrane attack complex during its assembly on the cell membrane and makes the membrane attack complex noncytolytic[23]. Because DAF, MCP and CD59 are mostly coexpressed, they act synergistically to inhibit complement activation on autologous cells to protect them.

UV light exposure of the skin induces dramatic changes *in vivo*, among them the release of a large number of cytokines from keratinocytes, including IL-1, IL-3, IL-4, IL-5, IL-6, IL-8, TNF-α, GM-CSF, M-CSF and MCAF[302]. Some of these cytokines can attract other cell types, such as monocytes and T cells. As a result inflammatory cells infiltrate the epidermis after UVB exposure[303;304]. These cells secrete cytokines, some of which are not secreted by keratinocytes, *e.g.* T cells can secrete IL-2 and IFN-γ[305;306]. Thus, UVB exposure results in accumulation in the skin of different cytokines, which have profound effects on cellular immune responses.

From the foregoing and from other studies showing suppression of delayed

hypersensitivity it is clear that UVB exposure exerts important effects on cellular immune reactions[307]; however, the effects of UVB exposure on humoral immune system, including the synthesis of complement components, has not yet been studied. We argued that UVB exposure of the skin may increase synthesis of complement components by keratinocytes either directly or indirectly via some cytokines released by keratinocytes or by inflammatory cells infiltrating the skin in response to UVB. If synthesis of components of complement is increased after UVB exposure, increased levels of complement can damage keratinocytes. Therefore, a mechanism(s) must exist to protect keratinocytes from complement, excessively produced by them, in response to UVB. This mechanism could be the increased expression of complement regulatory proteins on keratinocyte, either directly by UVB or indirectly via some cytokines released by keratinocytes or by inflammatory cells infiltrated following exposure to UVB. We investigated whether direct UVB exposure of cultured human keratinocytes can increase (1) the constitutive release of C3 and factor B and, (2) the surface expression of DAF, MCP and CD59.

MATERIALS AND METHODS

Keratinocyte culture

Human keratinocytes were isolated by incubation of foreskin with thermolysin (0.50 mg per ml, Sigma, St. Louis, Mo) at 4°C for 16 h and subsequent trypsinization (0.025%) for 5 min at 37°C. Trypsin (Sigma) was then neutralized by an excess volume of heat inactivated fetal calf serum (GibcoBRL, Breda, The Netherlands). Cells were separated from debris by filtering through a nylon mesh, centrifuged, and resuspended in keratinocyte serum free medium (GibcoBRL) supplemented with penicillin/streptomycin (100 IU per ml, 100 µg per ml; GibcoBRL). The keratinocytes were plated onto 100 mm plastic Petri dishes at a density of 400,000 cells per Petri dish and were incubated at 37°C in humidified, 5% CO_2, tissue culture incubator. Medium was changed every 2-3 d, and at 70% confluence cultures were split after a 5 min exposure to trypsin (0.025%)/ethylenediamine tetraacetic acid (EDTA) (1.5 mM) and recultured. For use in experiments, cells were seeded in 6 well tissue culture plates (Costar) at a density of 100,000 cells per well in 2000 µl of medium. Cells in passage 2-5 were used for experiments as soon as 60-80% confluence was achieved. Cells in representative wells were counted by a hemacytometer before the experiment and cells in all wells were counted after finishing the experiment.

UV exposure and cell viability

Subconfluent cultures (60-80%) of keratinocytes in 6 well culture plates were washed twice with phosphate-buffered saline (PBS), exposed to increasing doses of UVB (0-64 J per m^2), brought to 2 ml of culture medium and cultured for 48 or 72 h in two sets of experiments. After incubation, keratinocytes were counted with a hemacytometer and after propidium iodide staining viability of these cells was determined by flow cytometer. Cell survival after UVB exposure was expressed as the percentage of control nonexposed alive cells. Doses that were not lethal to the majority of the exposed keratinocytes at a given time after exposure (48 or 72 h) were used in subsequent experiments.

The UVB source consisted of a bank of two Philips TL-12 lamps (Philips, Eindhoven, The Netherlands).

These lamps emit UV in the range of 250-400 nm, primarily in the UVB region (280-320 nm), with a peak at 315 nm. The UVB output was 1 J per m^2 per second at a target distance of 40 cm and was monitored by an IL443 radiometer with a SEE 1240 UVB photo detector (International Light Inc., Newburyport, MA, USA).

Enzyme-linked immunosorbent assay (ELISA) for measurement of C3, factor B, and IL-8

The concentrations of C3, factor B, and IL-8 in culture supernatants of UVB exposed and non-exposed keratinocytes were estimated as follows.

For quantification of C3 a previously described sandwich ELISA[261] was used with some modifications. Briefly, wells of 96-well flat-bottom microtiter plates were coated with 0.7 µg polyclonal goat IgG anti-human C3 (Cappel, Boxtel, The Netherlands) per ml in 100 µl carbonate buffer (pH 9.6) overnight at 4°C. After thorough washing with Tween-80 (0.05%) (Sigma) in demineralized water the wells were blocked for 1 h at room temperature with 250 µl PBS containing 2% bovine serum albumin (Sigma) and 1 mM EDTA. Washing was repeated and wells were incubated with 100 µl of sample, diluted in the same buffer that was used for blocking. Plates were incubated for 2 h at 37°C. The wells were then washed and incubated with 100 µl peroxidase labeled goat anti-human C3 IgG (0.05 µg per ml) (Cappel) for 1 h at 37°C. After washing the wells were incubated with 100 µl 3,3',5,5'tetramethylbenzidine (Sigma) in dimethylsulfoxide (Merck, Hohenbrunn, Germany)-citrate buffer for 10 min. Reaction was stopped with 100 µl H_2SO_4 (2 M). Optical density (OD) was measured at 450 nm. The detection limit of this ELISA was 1 ng per ml of C3.

Factor B was assayed by a previously described sandwich ELISA[262] with several modifications. Briefly, wells were coated overnight at 4°C with 3 µg polyclonal goat-anti-human factor B IgG (ATAB, Stillwater, MN, USA) per ml in carbonate buffer. After thorough washing with PBS/Tween-80 (0.05%), wells were blocked with PBS/milk powder (2%) (Nutricia, Zoetermeer, The Netherlands) for 1 h at room temperature, and then washed again. Wells were then incubated for 2 h at 37°C with 100 µl sample, diluted in same buffer that was used for blocking, and washed. They were then incubated with biotinylated goat anti-human factor B IgG (1.25 µg per ml) for 1 h at 37°C. After washing, the wells were incubated for another h at 37°C with peroxidase conjugated poly streptavidin (1:1000; DAKO, Glostrup, Denmark). The wells were then repeatedly washed. Incubation of the wells with the peroxidase substrate, termination of the reaction, and measurement of OD was carried out as described for C3. The detection limit of this ELISA was 100 pg per ml.

For IL-8 ELISA, wells were coated with 1 µg monoclonal mouse-anti-human IL-8 (Biosource, Breda, The Netherlands) per ml in 100 µl carbonate buffer (pH 9.6) overnight at 4°C. After thorough washing with Tween-80 (0.05%) in PBS the wells were blocked for 1 h at 37°C with 200 µl of PBS containing 1% bovine serum albumin. Washing was repeated and wells were incubated with 100 µl of sample, diluted in the same buffer that was used for blocking. Plates were incubated for 2 h at 37°C. The wells were then washed and incubated with 100 µl biotinylated mouse anti-human IL-8 (0.1 µg per ml) (Biosource) for 1 h at 37°C. After washing, the wells were incubated for another h at 37°C with peroxidase conjugated poly streptavidin (1:1000; DAKO) in PBS/Tween (0.05%)/milkpowder (1%). The wells were then repeatedly washed. Incubation of the wells with the peroxidase substrate, termination of the reaction, and measurement of OD was carried out as described for C3. The detection limit of this ELISA was 5 pg per ml.

Standard curves for C3 and factor B ELISA were made using human complement calibrator CA1 (ATAB). Standard curve for IL-8 ELISA was made using IL-8 Calibrator (Biosource).

Flow cytometry

Keratinocytes were detached with the trypsin/EDTA solution for 3-5 min. Trypsin was inactivated by fetal calf serum and detached cells were washed and resuspended in fluorescence-activated cell sorter (FACS) buffer (PBS, 2% fetal calf serum, 0.1% sodium azide). Approximately 10^5 cells were incubated with specific monoclonal antibodies to MCP (clone J4-48, CLB, Amsterdam, The Netherlands), DAF (clone BRIC 110, CLB) or CD59 (1F5)[308] or isotype control (Becton Dickinson, San Jose, CA) for 30 min at 4°C. Cells were washed two times and incubated for 30 min with fluorescein-conjugated $F(ab')_2$ fragments of goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). Hereafter, cells were washed two times with FACS buffer. Propidium iodide (1 mg per ml) (Sigma) was added to identify dead cells and 20,000 cells were analyzed immediately by FACSCalibur (Becton Dickinson). Dead cells were excluded.

The detachment of keratinocytes with trypsin (0.025%)/EDTA (1.5 mM) at 37°C for 3-5 min did not cause degradation of any of the three complement regulatory proteins, as expression of these molecules on cells detached by this procedure was the same as on those detached with EDTA (2 mM) alone at 42 °C for 30 min. The former procedure was selected because it did not cause loss of cell viability, whereas latter procedure caused a high degree of loss of cell viability.

Mean fluorescence intensity (MFI) was calculated with WinMDI software. MFI was corrected for isotype MFI and values of cells not exposed to UVB were taken as 100%. MFI values obtained from cells exposed to UVB were presented in terms of percent of nonexposed cells.

Statistical analysis

Statistical analysis was performed using Wilcoxon signed rank test to determine significance in FACS experiments and a Student *t* test was used for data from ELISA experiments. A p value of less than 0.05 was considered significant.

RESULTS

Increasing doses of UVB cause progressive loss of cell viability

In order to study the effect of UVB exposure on the expression of complement and complement regulatory proteins by keratinocytes, suitable doses of UVB were determined as described in *'Material and Methods'*. Cell survival decreased with increasing doses of UVB (0-64 J per m^2) both 48 h and 72 h cultures (**Figure 1**). Exposure of keratinocytes to 64 J per m^2 resulted in the death of 45% of the cells in 48 h cultures. The surviving cells were not able to recuperate. Exposure to 32 J per m^2 resulted in about 10% cell death in 48 h and 50% cell death in 72 h. The cells that survived after 72 h (32 J per m^2) were healthy as assessed by the fact that they were able to proliferate (as seen up to 192 h; data not shown). Therefore, a dose range of 0-32 J per m^2 and a culture time of 72 h were used in most experiments. In some experiments a dose of 64 J per m^2 and a culture time of 48 h were used to mimic the situation *in vivo* in which UV exposure results in keratinocyte death, *e.g.* sunburn cell formation.

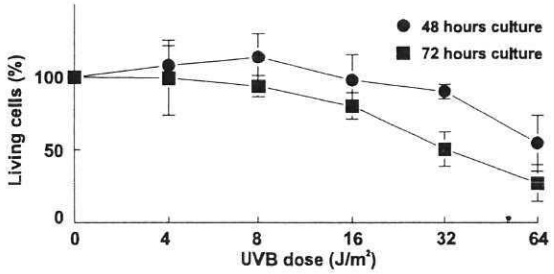

Figure 1. Increasing doses of UVB cause progressive loss of cell viability Subconfluent keratinocyte monolayers were exposed to UVB (0-64 J per m^2) and were cultured for 48 h and 72 h. Cells were counted with the haemacytometer and cell survival was analyzed with the flow cytometer after propidium iodide staining. Cells living after exposure with each dose are presented as a percentage of unexposed living cells. Data are averages ± SD of three experiments.

UVB exposure has no influence on the synthesis of C3 and factor B by keratinocytes

To see if UVB influences the constitutive production of C3 and factor B, keratinocytes were exposed to increasing doses of UVB (4-32 J per m^2). Non-exposed cells served as controls. Both exposed and nonexposed cells were cultured for 72 h and supernatants were collected. The concentrations of C3, factor B, and IL-8 were measured by ELISA in the supernatants.

In supernatants harvested from cultures of nonexposed keratinocytes C3, factor B, and IL-8 were found to be present in low concentrations. In supernatants of cultures of keratinocytes exposed to increasing doses of UVB, concentrations of C3 and factor B remained low but the concentration of IL-8 was significantly increased (**Figure 2**). IL-8 production was highest at 32 J per m^2. The maximal IL-8 level in the supernatant was 609 pg per ml. To exert biological activity much higher concentrations are required[309]. These results show that UVB activates keratinocytes, as could be concluded from the IL-8 production, but does not directly increase the production of C3 and factor B.

Cultured keratinocytes express DAF, MCP and CD59

Previous immunohistochemical studies in our laboratory have shown that DAF, MCP and CD59 were present *in situ* on several structures of human skin, including keratinocytes[11]. Flow cytometric analysis of cultured keratinocytes in this study confirmed that all three proteins were expressed on these cells (**Figure 3**).

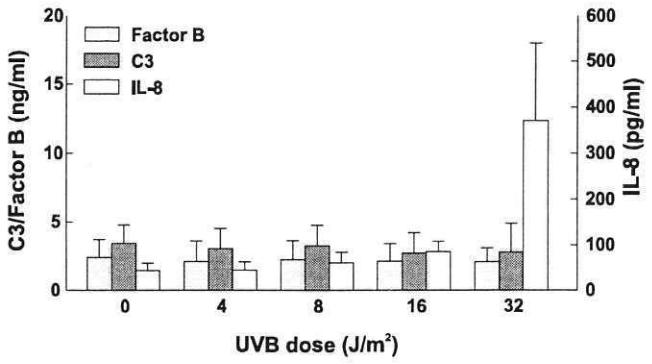

Figure 2. UVB exposure has no influence on the synthesis of C3 and Factor B by human keratinocytes Subconfluent keratinocyte cultures were exposed to indicated doses of UVB. Supernatants were harvested at 72 h and concentrations of C3 and Factor B determined by ELISA. IL-8 was included as a positive control. The figure shows C3, Factor B and IL-8 protein concentrations in supernatants of three independent experiments. In each experiment three wells were exposed to each dose and the contents of each well were analyzed in quadruplicate. Mean values and SD are shown.

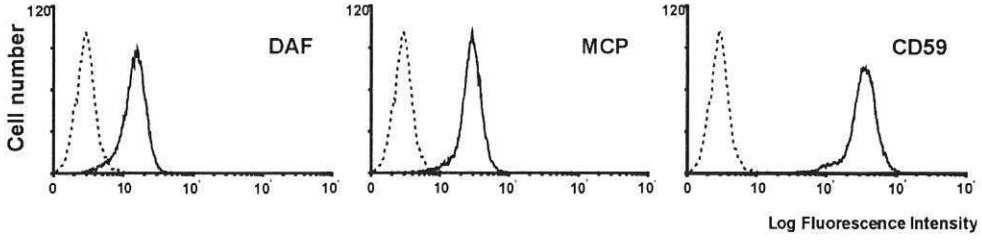

Figure 3. Cultured human keratinocytes express DAF, MCP and CD59 Cultured keratinocytes were washed and 10^4 cells were analyzed for the expression of C regulatory proteins by FACS. Continuous lines show keratinocyte staining of MCP, DAF or CD59. Broken lines show staining with isotype controls. For all parts, the data are shown as cell number *versus* the log of fluorescence and are representative of three staining experiments each carried out in triplicate.

A single dose of UVB causes a prolonged increase in the surface expression of DAF, MCP and CD59

When cultured keratinocytes were exposed to UVB (32 J per m^2) and surface expression of the complement regulatory proteins was analyzed at several time points (0-192 h), expression of all the three proteins was found to be transiently increased (**Figure 4**). DAF expression increased rapidly, reaching its maximum at 48 h after UVB exposure (1.81 ± 0.06-fold increase

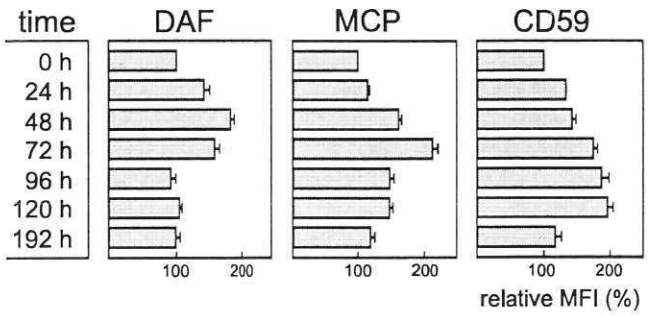

Figure 4. A single dose of UVB causes prolonged increase in the surface expression of DAF, MCP and CD59
Subconfluent keratinocyte cultures were exposed to 32 J per m^2 and expression of DAF, MCP and CD59 was monitored by flow cytometer at the indicated time points. MFI, corrected for isotype control, is shown. Data represent one of two independent experiments. In each experiment three wells were exposed to UVB for each time point and cells in each well were analyzed in duplicate. Mean values and SD are shown.

in MFI over nonexposed cells). Thereafter, DAF expression decreased rapidly, returning to control values within 96 h. MCP expression increased, reaching its maximum at 72 h (2.13 ± 0.09-fold increase in MFI). Expression of MCP gradually returned to control values in 192 h. CD59 expression increased rather slowly, reaching a plateau at 72-120 h (1.74 ± 0.07-fold and 1.96 ± 0.09-fold increase in MFI, respectively). Expression of CD59 returned to almost baseline values in 192 h.

Increasing doses of UVB cause a dose-dependent increase in surface expression of complement regulatory proteins

Time-response studies described above showed that with 32 J per m^2 surface expression of DAF was maximal at 48 h of culture after irradiation and surface expression of MCP and CD59 was maximal at 72 h. Therefore, dose-response studies with a moderate dose of up to 32 J per m^2 were carried out at 48 h of culture after irradiation for DAF and at 72 h of culture after irradiation for MCP and CD59. In the case of the expression of DAF, there was no effect of UVB doses up to 16 J per m^2 but the expression was significantly increased with 32 J per m^2 (**Table**). In the case of MCP and CD59, expression gradually increased from 8 J per m^2 onwards and reached its maximum at 32 J per m^2.

A high dose of UVB results in a high surface expression of DAF, MCP and CD59

Further studies were aimed at comparing the effects of moderate (32 J per m^2) and high dose of UVB (64 J per m^2) on the expression of all the three complement regulatory proteins.

Table. *Effect of increasing doses of UVB on the expression of MCP, DAF and CD59 by keratinocytes*[a].

	Mean Fluorescence Intensity (% of untreated cells)		
dose (J per m^2)	DAF	MCP	CD59
0	100	100	100
8	97.4 ± 8.0	123.2 ± 3.7***	114.4 ± 11.7*
16	102.1 ± 1.7	125.8 ± 3.3***	131.1 ± 7.3**
32	151.0 ± 29.9**	158.6 ± 18.0**	183.4 ± 18.4***

[a]Subconfluent cultures were exposed to various doses of UVB. After 48 h (DAF) or 72 h (MCP and CD59) membrane expression of complement regulatory proteins on 20,000 cells was determined by FACS analysis. MFI values of unexposed keratinocytes were taken as 100%. All values represent mean ± SD of six experiments; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

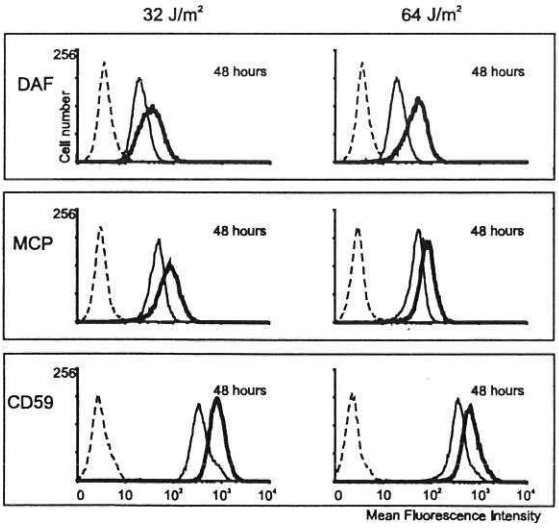

Figure 5. Increasing doses of UVB cause progressive increase in surface expression of DAF, MCP and CD59
Subconfluent keratinocyte cultures were exposed to a moderate dose (32 J per m^2) and a high dose (64 J per m^2) of UVB. Expression of DAF, MCP and CD59 was analyzed by flow cytometer at 48 h. Single parameter histograms for DAF, MCP and CD59 expression are shown. Thin lines show the expression of DAF, MCP and CD59 on unexposed keratinocytes and bold lines show that on UVB-exposed keratinocytes. Broken lines show the expression obtained with isotype controls. The data are representative of at least two independent experiments carried out in triplicate. MFI after exposure to 64 J per m^2 was significantly higher for DAF ($p<0.01$) and CD59 ($p< 0.001$) but not for MCP ($p=0.18$) than after exposure to 32 J per m^2.

These studies were carried out at 48 h of culture because of the low degree of cell survival at 72 h of cells exposed to 64 J per m^2 (see above). The results showed that expression of all the three complement regulatory proteins was higher with 64 J per m^2 than with 32 J per m^2, although the difference in expression of MCP did not reach significant levels (p=0.18) (**Figure 5**).

DISCUSSION

UVB radiation is the mid range portion (290-320 nm) of the ultraviolet radiation spectrum (200-400 nm). UVB is present in biologically significant amounts at the earth's surface and exhibits pleomorphic effects both on the whole organism and on the skin. It penetrates the stratum basale and reaches the keratinocytes. UVB increases production of several cytokines by keratinocytes. Some of these cytokines recruit inflammatory cells and thereby induce inflammation at the site of UVB exposure of the skin[303;219;304]. Alteration in production of some cytokines by UVB induces immunosuppression[307].

In many inflammatory disorders, like psoriasis, atopic dermatitis, seborrhoeic dermatitis, and lamellar ichthyosis, complement deposits are found in the epidermis without the presence of autoantibodies[310]. In some autoimmune diseases, such as pemphigus, pemphigoid, discoid lupus erythematosus, and photosensitive lupus erythematosus, complement deposits on keratinocytes are seen in lesional skin but can also be induced in nonlesional skin by UVB exposure[311;312]. In some diseases of unknown etiology such as photoallergy, polymorphous light eruptions, solar urticaria, solar eczema, and actinic reticuloid, the disease is exacerbated by light but the role of complement remains unknown. In none of these diseases is the origin of complement in the skin known. And, in none of these diseases, except aforementioned autoimmune diseases, has the effect of UVB on the development of complement deposits been studied. In spite of these gaps in knowledge, studies on the effects of UV exposure on the expression of complement and complement regulatory proteins by keratinocytes isolated from normal individuals and from patients has not been carried out.

We argued that UVB exposure can probably increase the constitutive production of complement components by keratinocytes. In evolutionary terms, this may be to compensate for local immunosuppression induced by UVB. We also argued that UVB exposure can increase the surface expression of complement regulatory proteins on keratinocytes to protect them from their own complement. We raised the possibility that UVB can up-regulate the synthesis of both complement and complement regulatory proteins, either directly or indirectly via cytokines released by keratinocytes or infiltrated inflammatory cells.

We observed that keratinocytes constitutively produced small amounts of C3 and factor B. This was in agreement with previous observations[34;35]. Exposure to UVB caused stimulation

of keratinocytes, as evidenced by the production of IL-8, but did not increase production of C3 and factor B (**Figure 2**). This proved that direct stimulation of keratinocytes by UVB could not induce an increase in production of C3 and factor B. This also raised the question why those C3 and factor B up-regulating cytokines that are released from keratinocytes upon UVB exposure do not increase the production of C3 and factor B in our system. One of the possible explanations is that they may be released in the medium in concentrations not high enough to stimulate the keratinocytes. The concentrations of TNF-α needed to up-regulate C3 in our system were 50-1000 U per ml. But the concentrations of TNF-α in culture medium of UVB exposed keratinocytes (at 72 h) was found to be very low (< 1 U per ml; data not shown here). The same appears to be true for IL-8, whose effects on the expression of C3, factor B, and complement regulatory proteins is not known. The level of IL-8 in the culture medium of UVB exposed keratinocytes was always lower than 610 pg per ml (**Figure 2**). Biologically effective IL-8 concentrations vary from 5 to 50 ng per ml [309]. These results indicate that cytokines released from keratinocytes in culture medium after UVB exposure probably become too diluted to be effective. It remains to be seen if cytokines released from keratinocytes upon UVB exposure can up-regulate C3 and factor B *in vivo*.

DAF, MCP and CD59 are present on keratinocytes in the human skin as seen *in situ* in previous immunohistochemical studies[28][11]. This study shows surface expression of DAF, MCP and CD59 on cultured keratinocytes. The coexpression of DAF, MCP and CD59 on human keratinocytes suggests that these molecules collectively offer a high degree of protection to keratinocytes against complement attack. Indeed, human keratinocytes[38] and a keratinocyte-derived squamatous carcinoma cell line, SCC-12F[39], are remarkably resistant to complement mediated lysis. In addition, cell death is not a prominent feature in diseases like pemphigus in which strong complement attack against keratinocytes occurs. Thus, keratinocytes *in vivo* are strongly protected against autologous complement. Keratinocytes do need strong protection against complement attack under physiologic conditions, because the epidermis is under a continuous threat of exposure to microbes and other agents that can activate complement and cause bystander complement attack on keratinocytes.

This study also shows that the expression of DAF, MCP and CD59 on keratinocytes is increased for several days by UVB exposure in a dose-dependent manner. Because these molecules act synergistically, increases in all three implies that UVB exposure results in a prolonged and a high degree of protection against autologous complement. We did not try to correlate increased expression with increased protection against complement attack because increased expression of DAF, MCP and CD59 in response to UVB exposure could be accompanied by increased or decreased expression of other complement regulatory proteins, such as complement receptor 1, C1q receptor and homologous restriction factor. Possible changes in expression of these latter complement regulatory proteins would have made results difficult to

interpret. For example, an increase in the expression of CD59 in the EA.hy926 cell line caused by inducers of protein kinase complement and protein kinase A could not be correlated with the increase in resistance to complement mediated lysis[313].

The increased expression of DAF, MCP and CD59 seen *in vitro* could have been due either to a direct effect of UVB on keratinocytes or an indirect effect in response to mediators released by keratinocytes. The latter possibility appears to be less likely because the concentrations of cytokines released from UVB-exposed keratinocytes into the culture medium do not appear to be high enough to be able to up-regulate complement regulatory proteins significantly.

If these findings may be extrapolated to the *in vivo* situation, keratinocytes in UVB exposed areas may be better protected against complement mediated lysis than keratinocytes in nonexposed areas. The situation *in vitro,* however, may differ from the situation *in vivo* in which infiltrating cells may also influence the expression of complement regulatory proteins through the release of their cytokines.

UVB is known to activate protein kinase complement in keratinocytes[314]. Increase in expression of DAF, MCP and CD59 by activators of protein kinase C, phorbol myristate acetate, and calcium ionophore A23187, and an activator of protein kinase A, butyryl-cAMP, has been shown in our laboratory (data not presented here). Protein kinase complement and protein kinase A signalling may perhaps be involved in the up-regulation of DAF, MCP and CD59 by UVB.

In conclusion, this study demonstrates that cultured human keratinocytes constitutively release low amounts of C3 and factor B, which remains unaffected by UVB. They also express DAF, MCP and CD59. Expression of these complement regulatory proteins is increased by UVB. Because UVB may increase complement production *in vivo* through cytokines of keratinocytes and infiltrated cells, this increase in complement regulatory proteins may be important for protecting keratinocytes from becoming bystander victims of complement during UVB-mediated inflammation.

Chapter Five

Human keratinocytes synthesize factor H: Synthesis is regulated by Interferon-γ

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Manuscript submitted

Abstract

Evidence is emerging that locally synthesized complement plays an important role in host defense and inflammation at tissue and organ level. In the epidermis, the major cell type is the keratinocyte which has so far been shown to synthesize two soluble complement components, C3 and factor B. We investigated whether factor H, which controls the activities of C3 and factor B, is also synthesized and released by keratinocytes. Regulation of synthesis of factor H by some inflammatory mediators was also studied.

Keratinocytes were grown to near confluence and then cultured for additional 72 h in the presence and absence of supernatant of activated peripheral blood mononuclear cells, IL-1α, IL-2, IL-6, TGF-ß1, TNF-α, or IFN-γ. Factor H was then measured in culture supernatants by ELISA. Molecular species of released factor H were identified by Western blotting. Factor H transcripts in harvested keratinocytes and A431 cells were detected by RT-PCR. Regulation of factor H message by individual cytokines was studied by a semi-quantitative RT-PCR.

Human keratinocytes constitutively released factor H which was strongly up-regulated by the supernatant of activated mononuclear cells. None of the above-mentioned cytokines, except IFN-γ, influenced this release. IFN-γ up-regulated factor H release strongly. IFN-γ mediated release of factor H was inhibited by cycloheximide. Factor H mRNA was constitutionally expressed in keratinocytes and was strongly up-regulated by IFN-γ.

In conclusion, keratinocytes are capable of synthesizing factor H and this synthesis is regulated by IFN-γ at pretranslational level.

INTRODUCTION

Two types of mechanisms protect self cells from complement mediated lysis. These are fluid phase and cell surface mechanisms. Fluid phase mechanisms involve several inhibitors and inactivators (*e.g.* C1-inhibitor, C4-binding protein, factor H, factor I) which tend to keep complement dormant in fluid phase while allowing it to be activated on foreign invading cells. These regulators minimize the formation of complement fragments and complement complexes in their active forms in fluid phase, so that a minimum of them collide with self cells. However, under certain circumstances a proportion of them may escape from fluid phase regulation, get fixed on self cells and cause complement activation on them. This cell surface activation of complement is, however, prevented by cell surface complement regulatory mechanisms. These mechanisms involve complement regulatory proteins (MCP, DAF, CD59, etc.), embedded in the membranes of the self cells, which inactivate the above-mentioned fragments and complexes[13;14]. In the membranes of foreign invading cells these regulators are not present and subsequent complement activation takes place on them resulting in their lysis.

Of the above mentioned fluid phase complement regulators, factor H plays a pivotal role. It prevents the assembly of C3/C5-convertase ($C3b.Bb/(C3b)_n.Bb$) of the alternative pathway in the fluid phase by (1) binding to C3b in competition with factor B[315], (2) accelerating the dissociation of Bb from the convertase complexes[316], and (3) combining with freshly formed C3b and then serving as a cofactor for the enzyme factor I which cleaves the α-chains of C3b to inactivate it[317]. By virtue of being able to inactivate C3b, factor H is also a restrictive factor in the assembly of C3/C5-convertase ($C4b.C2a.(C3b)_n$) of the classical pathway[318].

Two monomeric molecular species of human factor H, 155-kD and 45-kD, are known. The 155-kD isoform is made up of 1213 amino acid residues[319]. Its entire sequence is built up from 20 contiguous short consensus repeats (SCRs), which are repeating domains of approximately 60 amino acids joined by short linkers[25]. This gives factor H the shape of a string of beads (see **Chapter 1, Figure 3**)[320]. The 45-kD isoform is made up of the 7 N-terminal SCRs of the 155-kD isoform of factor H. In both isoforms, SCRs 3-5 of factor H are involved in binding to C3b[316;321]. Larger molecules arise from a 4.3-kb factor H mRNA and the shorter from a 1.8-kb mRNA; these transcripts are generated by alternative splicing of factor H mRNA[18;322;4]. Both mRNA species are present in the liver, the major site of factor H expression, and in several extra-hepatic tissues. Both isoforms of factor H are expressed in many cell types. These include human monocytes[323], umbilical vein endothelial cells[324], brain microvessel endothelial cells[325], proximal tubular epithelial cells[326], mesangial cells[327;265], myoblasts[328], amnion[329] astrocytes[330], skeletal microtubes[331], B-cell lines[332] and fibroblasts[333].

The keratinocyte is the major cell type in the epidermis and is believed to be the initiator

of inflammation in the skin[1]. They synthesize complement components C3[34] and factor B[35], and have been shown to express several complement receptors and complement regulatory proteins[28;11;37]. Keratinocytes are also expected to produce other complement proteins. Production of C3 and factor B suggests that keratinocytes may also be able to produce factor H to regulate the activation of these components in fluid phase as a part of the mechanism of prevention of epidermal cell damage by the complement synthesized by them.

The role of factor H in suppression of complement at C3/C5-convertase stage demands that, if produced by keratinocytes, its production be up-regulated when there is up-regulation of C3 and factor B production. C3 and factor B synthesis appears to be up-regulated under inflammatory conditions as mediators released from activated mononuclear cells as well as several individual proinflammatory cytokines have been shown to enhance the synthesis of these components differentially (see **Chapter 2**)[298]. Thus, the synthesis of factor H, if occurs in keratinocytes, should also be enhanced by mediators released from inflammatory cells as well as by some individual proinflammatory cytokines to suppress the activation of C3 and factor B and protect keratinocytes from damage.

In the present study we investigated whether normal human keratinocytes are capable of synthesizing factor H. We also tested the impact of supernatant of activated mononuclear cells as a source of mediators of inflammation as well as of various recombinant cytokines on the synthesis of factor H by keratinocytes.

MATERIALS AND METHODS

Chemicals and reagents

Human recombinant cytokines IFN-γ, IL-1α, IL-2, IL-6, TGF-ß1 and TNF-α were purchased from Boehringer Mannheim (Mannheim, Germany). Normal rabbit IgG, neutralizing rabbit antibodies to IFN-γ and cycloheximide were purchased from Sigma (St. Louis, MO, USA). Polyclonal rabbit anti-serum to human factor H was a kind gift of dr. M.R. Daha, Department of Nephrology, University of Leiden, the Netherlands[324]. Supernatant of activated mononuclear cells was prepared from stimulated peripheral blood mononuclear cells as described[260]. Briefly, peripheral blood mononuclear cells (50 x 10^6/ml) from normal donors were stimulated for 2 h at 37°C with 0.1 µg/ml PMA in IMDM (supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin and 100 µg/ml streptomycin). PMA-treated cells were washed extensively and cultured (50 x 10^6/ml) for 48 h with 15 µg/ml Con A in supplemented IMDM. Con A was neutralized by addition of 50 mM α-methyl-mannoside for 30 min at 37°C. Supernatant was obtained by centrifugation. Cytokines, supernatant of activated mononuclear cells and neutralizing antibodies were aliquoted in small portions and stored at -20°C and diluted in keratinocyte serum free medium (keratinocyte SFM; GibcoBRL, Breda, The Netherlands) just before use. The sources of other chemicals and reagents are indicated below.

Keratinocyte and A431 cultures

Human keratinocytes were isolated by incubation of foreskin with thermolysin (0.50 mg per ml, Sigma) at 4°C for 16 h and subsequent trypsinization (0.025%) for 5 min at 37°C. Trypsin (Sigma) was then neutralized by an excess volume of heat inactivated fetal calf serum (GibcoBRL). Cells were separated from debris by filtering through a nylon mesh, centrifuged, and resuspended in keratinocyte SFM supplemented with penicillin/streptomycin (100 IU per ml, 100 µg per ml; GibcoBRL). A431 cells were purchased from American Type Culture Collection (Manassas, VA) and cultured in Dulbecco's modified eagle medium, supplemented with 10% fetal calf serum, penicillin/streptomycin (100 IU per ml, 100 µg per ml), and glutamine. The keratinocytes and A431 cells were plated onto 100 mm plastic Petri dishes at a density of 400,000 cells per Petri dish and were incubated at 37°C in humidified, 5% CO_2, tissue culture incubator. Medium was changed every 2-3 d, and at 70% confluence cultures were split after a 5 min exposure to 0.025% trypsin, 1.5 mM EDTA and recultured. For use in experiments, keratinocytes were seeded in 6 well tissue culture plates (Costar) at a density of 100,000 cells per well in 2000 µl of medium. Cells in passage 2-4 were used for experiments when 60-80% confluence was achieved. Cells in representative wells were counted by a hemacytometer before the experiment and cells in all wells were counted after finishing the experiment.

Immunohistochemical staining of cultured keratinocytes for factor H

Keratinocytes were grown for 72 h on a microscopic slide in the presence or absence of 100 U per ml of IFN-γ, rinsed in PBS, dried in the air for 5 h, and then fixed for few minutes in acetone. Slides were stored at -20°C till staining. Endogenous peroxidase activity was inactivated by 30 min incubation in 0.3% H_2O_2 in PBS. The immunohistochemical staining was then performed by a three-step immunoperoxidase technique[283]. The slides were incubated with 3 µg polyclonal rabbit anti-factor H or normal rabbit serum per ml for 60 min followed by incubation for 60 min with a 1:100 dilution of a biotinylated swine anti-rabbit Ig (DAKO) in PBS/human AB serum (10%; DAKO). They were then incubated for 60 min with horseradish peroxidase-labeled polystreptavidin (ABC Complex, DAKO). Peroxidase activity was visualized by incubation of the slides with 0.05% 3-amino-9-ethylcarbazole in acetate buffer for 10 min followed by a counterstaining with hematoxylin for 20-30 s. All incubations were performed at room temperature.

ELISA for measurement of factor H

The concentration of factor H in culture supernatants of cytokine-stimulated and non-stimulated keratinocytes were estimated by ELISA as described[324].

Wells of 96 well flat-bottom microtiter plates were coated with 50 µl of 5.2 µg affinity purified polyclonal rabbit anti-human factor H per ml in carbonate buffer overnight at 4°C. After thorough washing with Tween-80 (0.05%) (Sigma) in PBS the wells were blocked for 1 h at room temperature with 150 µl of PBS containing 2% bovine serum albumin (Sigma) and 0.05% Tween-80. Washing was repeated and wells were incubated with 50 µl of sample, diluted in the same buffer that was used for blocking. Plates were incubated for one hour at 37°C. The wells were then washed and incubated with 50 µl digoxigenin-labeled rabbit anti-human factor H (0.3 µg per ml) for 1 h at 37°C. After washing, the wells were incubated for another h at 37°C with 33 mU peroxidase conjugated F(ab')2 fragments of sheep anti-digoxigenin per ml (Boehringer Mannheim). After washing, the wells were incubated with 50 µl 3,3',5,5'tetramethylbenzidine (Sigma) in dimethylsulfoxide (Merck, Hohenbrunn, Germany)-citrate buffer for 10 min. The reaction was stopped with 50 µl H_2SO_4 (2 M). Optical density (OD) was measured at 450 nm. The detection limit of this ELISA was 80 pg per ml of factor H. Standard curves for factor H were made using Human Complement Calibrator CA1 (ATAB).

Western blot analysis of keratinocyte supernatant for factor H protein

Five microliters of ten-fold concentrated supernatants of unstimulated and IFN-γ stimulated (100 U per ml) keratinocytes were added to 2.5 µl of SDS sample buffer and 2.5 µl of H_2O and run on a 7.5% SDS-PAGE gel. Gels were blotted for 90 min in a semi-dry electroblot apparatus on to PVDF membrane. Membranes were blocked for 30 min in Blotto (5% non-fat milk powder (Biorad, Hercules, CA) in 0.05% tween-20 containing PBS) at room temperature. Blots were incubated with polyclonal goat antibody to human factor H (Quidel, San Diego, CA) for 1 h at room temperature in 1% Blotto. They were washed three times in 0.05% tween-20 containing PBS and subsequently incubated with biotinylated rabbit anti-goat antibody (DAKO) for 1 h at room temperature. They were washed again three times in 0.05% tween-20 containing PBS and incubated with polystreptavidin-horse radish peroxidase (DAKO) for 1 h at room temperature. They were washed again three times in 0.05% tween-20 containing PBS and incubated with peroxidase substrate ECL (Amersham Life Science, Buckinghamshire, England). Luminescence was determined with a Lumi-Imager (Boehringer Mannheim).

Isolation of RNA and semi-quantitative reverse-transcriptase polymerase chain reaction

Total RNA was isolated from human keratinocytes and A431 cells grown in 100 mm Petri dishes in medium with and without supplementation with IFN-γ (100 U/ml), IL-1α (200 U/ml), IL-6 (1000 U/ml), or TNF-α (750 U/ml), respectively, for 6 h and 24 h using Trizol (Life Technologies, Paisley, UK). The RNA pellet was dissolved in formamide and the amount of RNA was determined by a spectrophotometer at 260 nm and 280 nm. RNA obtained from normal human liver tissue was prepared similarly and used as a positive control for factor H RNA.

Reverse-transcriptase polymerase chain reaction (RT-PCR) was performed as described[87] with some minor modifications. Briefly, 5 µg of the extracted total cellular RNA was reverse transcribed in a reaction volume of 20 µl and 1 µl of the resulting cDNA solution was used to amplify cDNA by factor H-specific PCR. The PCR were performed in 50 µl per well in polyethylene reaction tubes and applying cycles consisting of denaturation step at 94°C for 30 seconds, annealing for 1 min at 59°C, and extension for 1 min at 72°C. The PCR incubation mixture in a total volume of 50 µl contained 50 mM KCl, 10 mM Tris-HCl pH 8.1, 2.0 mM $MgCl_2$, 0.01% gelatin, 1.25 unit Taq polymerase (GibcoBRL), 250 µM dNTP mix (Pharmacia, Uppsala, Sweden), and 140 ng of the sense and anti-sense primer each. The following specific primer sets were synthesized in our laboratory by an oligo-synthesizer: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward primer 5'-CGAGATCCCTCCAAAATCAA-3' (nt 298-317); and GAPDH reverse primer 5'-AGGTCAGGTCCACCACTGAC-3' (nt 799-780); factor H forward primer 5'-CTGATCGCAAGAAAGACCAG-3' (nt 1812-1831), and factor H reverse primer 5'-TTGGGTCCATACTCCATGAA-3' (nt 2274-2293). To confirm purity of the keratinocyte cultures, several cDNA samples obtained after reverse transcription of keratinocyte RNA were checked for the presence of non-keratinocyte cDNA using the following primers: Tyrosinase forward primer 5'-AATGAAAAATGGATCAACACCC-3' (nt 976-997), and tyrosinase reverse primer 5'-GTTTCCAGGATTACGCCGTA-3' (nt 1392-1411); HLA-DR forward primer 5'-GCCAACATAGCTGTGGACAA-3' (nt 283-302), and HLA-DR reverse primer 5'-ATAATGATGCCCACCAGACC-3' (nt 706-725); CD18 forward primer 5'-GACTCCATTCGCTGCGACAC-3' (nt 148-167)[334], and CD18 reverse primer 5'-CACGGTCTTGTCCACGAAGG-3' (nt 485-504)[334]; CD3 forward primer 5'-TCTCCATCTCTGGAACCACA-3' (nt 167-186), and CD3 reverse primer 5'-GTTCGCATCTTCTGGTTTGC-3' (nt 362-382) and parathyroid hormone receptor (PTH-R) forward primer 5'-CAATGAGACTCGTGAACGGG-3' (nt 553-572), and PTH-R reverse primer 5'-AAGTTGAGCACAATGGAGGC-3' (nt 1133-1152). Each PCR product (12.5 µl) was mixed with 5 µl stop layer mix and run on a 1.7% agarose gel in tris/borate/EDTA buffer. After electrophoresis the gel was scanned by an Eagle Eye imager (Stratagene Europe, Amsterdam, The Netherlands) and

the signal strength was integrated to obtain a densitometric value for each amplification product. To enable qualitative analysis of cDNA, the number of PCR cycles was chosen at 36. To enable semi-quantitative analysis of cDNA obtained from cytokine stimulated keratinocytes, the number of PCR cycles was chosen in such a way that a linear relationship was achieved between PCR product formation (plotted on a log scale) and cycle number, without having reached saturation of the product formation.

RESULTS

Cultured Human keratinocytes express factor H *in situ*

Expression of factor H on cultured human keratinocytes was first studied immunohistochemically as described in '*Material and Methods*'. Since the expression of several complement proteins is enhanced by IFN-γ, IFN-γ stimulated keratinocytes were also included. There was virtually no staining of control unstimulated and IFN-γ stimulated keratinocytes treated with normal rabbit IgG (**Figure 1**). Staining of IFN-γ stimulated keratinocytes for factor H treated with rabbit anti-human factor H IgG was, however, much stronger than that of unstimulated keratinocytes. Although from this experiment it was not established whether the expression of factor H in unstimulated and IFN-γ stimulated keratinocytes was cell surface associated[335] or due to internal pool destined for release, these results provided rationale for further studies aimed at investigating the release and synthesis of factor H by keratinocytes.

Human keratinocytes constitutively release factor H

Keratinocytes released small amounts of factor H in culture medium as determined by ELISA . When keratinocytes were cultured up to passage six and the release of factor H was monitored in the culture supernatant of each passage, a significant increase with increasing number of passages was observed in cultures derived from three different foreskins (**Figure 2**). Because of these differences we used only cultures from passage 2 to 4 in subsequent studies.

More than one molecular species of factor H is secreted by keratinocytes

To investigate the isoforms of factor H released by normal human keratinocytes, supernatants of keratinocytes cultured in the absence and presence of IFN-γ (100 U/ml) for 72 hours were subjected to SDS-PAGE followed by Western blotting as described in `*Material and Methods*'. Polyclonal anti-factor H antibody detected a lightly stained band of apparent molecular weight of 155-kD in supernatant of unstimulated keratinocytes (**Figure 3**).

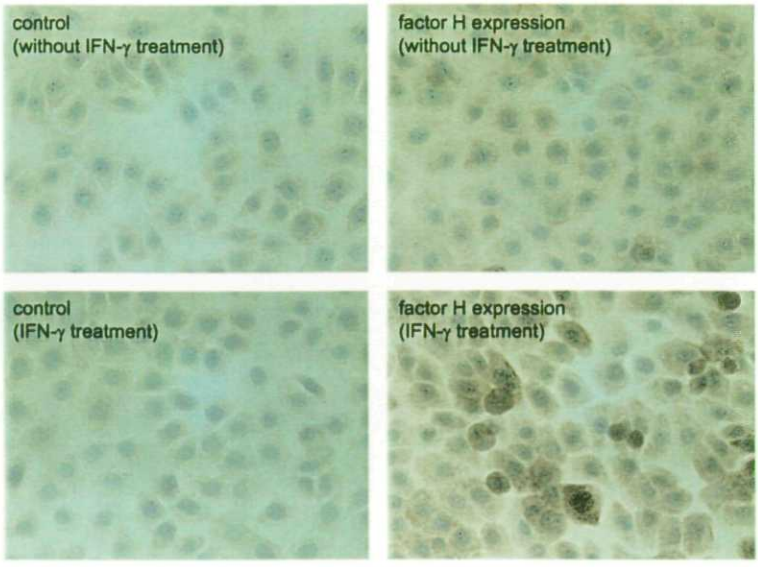

Figure 1. Surface staining of factor H on cultured human keratinocytes Unstimulated and IFN-γ atimulated keratinocytes were stained by an immunoperoxidase technique using polyclonal rabbit anti-factor H IgG and rabbit IgG controls. All views are 40 x.

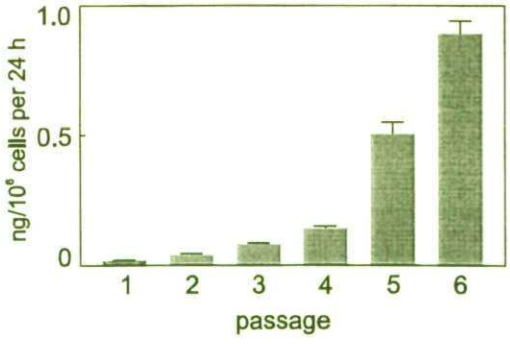

Figure 2. Human keratinocytes release factor H Sub-confluent keratinocytes were cultured for 72 h. Supernatants were collected and cells were rinsed, harvested and counted (passage 1). They were again passaged to sub-confluence, fresh medium was added (at time 0) and the whole process was repeated (passage 2) until passage 6. Supernatants collected after each passage were analyzed for factor H by ELISA. Three independent experiments with cultures derived from different foreskins were carried out. Values are the mean ± SD for triplicate determinations of one representative culture.

This band was strongly stained in the supernatant of IFN-γ treated keratinocytes, indicating strong up-regulation of 155-kD Factor H by IFN-γ. Supernatant of unstimulated keratinocytes showed three lightly stained bands presumably those of other SCRs bearing proteins. Because expression of these proteins was not up-regulated by IFN-γ stimulation, they were considered to be the translational products of transcripts other than those of factor H. In the supernatant of unstimulated keratinocytes, 45-kD band was not visible but in that of IFN-γ stimulated keratinocytes, this band representing the smaller isoform of factor H was visible though weekly stained. Thus, unstimulated keratinocytes produced detectable amounts of 155-kD factor H and undetectable amounts of 45-kD factor H but IFN-γ stimulated keratinocytes produced both molecular species of factor H.

Mediators released from activated mononuclear cells up-regulate the release of factor H from keratinocytes

Keratinocytes were cultured for 72 h in the presence of increasing concentrations of supernatant of activated mononuclear cells and the release of factor H in culture medium was monitored by ELISA. Three cultures of keratinocytes were analyzed, each in duplicate. In all cultures, supernatant of activated mononuclear cells induced a dose-dependent increase in release of factor H (**Figure 4**).

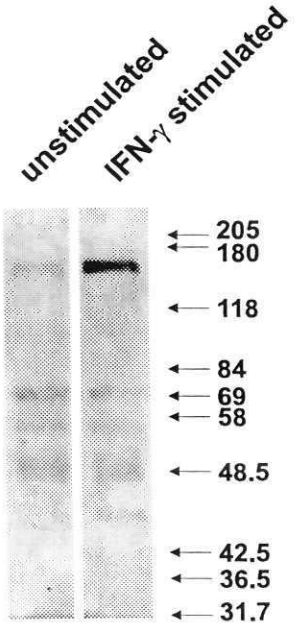

Figure 3. Human keratinocytes release both 155-kD and 45-kD factor H Concentrated supernatants of 72 h cultures of unstimated and IFN-γ (100 U/ml) stimulated keratinocytes were subjected to SDS-PAGE and Western blotting. Western blotting was performed using polyclonal rabbit anti-human factor H antibodies, followed by biotinylated swine anti-rabbit IgG and streptavidin-horseradish peroxidase. Peroxidase activity was visualized by CLE luminescrence and recorded by a lumi-imager.

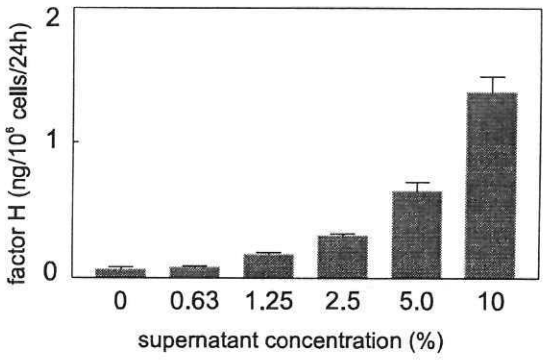

Figure 4. Supernatant of activated mononuclear cells enhances the release of factor H by keratinocytes Sub-confluent keratinocytes were cultured in media containing various concentrations of supernatant of activated mononuclear cells as indicated. At 72 h, culture supernatants were harvested and factor H concentrations determined. Values are the mean ± SD for duplicate determinations of three cultures.

Interferon-γ regulates the release of factor H from keratinocytes

The supernatant of activated mononuclear cells is known to contain a number of cytokines, including IL-1α, IL-2, IL-6, TGF-ß, TNF-α and IFN-γ[265;37]. Therefore, we tested recombinant forms of these individual cytokines in several concentrations mentioned in '*Materials and Methods*' to find out which of these cytokines were able to mimic the effects seen with supernatant of activated mononuclear cells.

None of these, except IFN- γ, showed any significant effect on the release of factor H. IFN-γ up-regulated the release of factor H 24 and 65 times the basal release, at concentrations of 31.25 and 1000 U per ml, respectively (**Figure 5**).

To confirm that the regulation of factor H release by normal human keratinocytes is a specific property of IFN- γ, antibody blocking experiments were performed. Keratinocytes were cultured for 72 h in medium alone or in medium containing 100 U per ml of IFN- γ in the presence and absence of neutralizing anti- IFN-γ antibodies or control rabbit IgG (**Figure 5**). The results showed that neutralization of IFN-γ with anti-IFN-γ abolished the induction of factor H release.

The batches of FCS, supernatant of activated mononuclear cells, and keratinocyte medium did not show factor H reactivity in the ELISA assay.

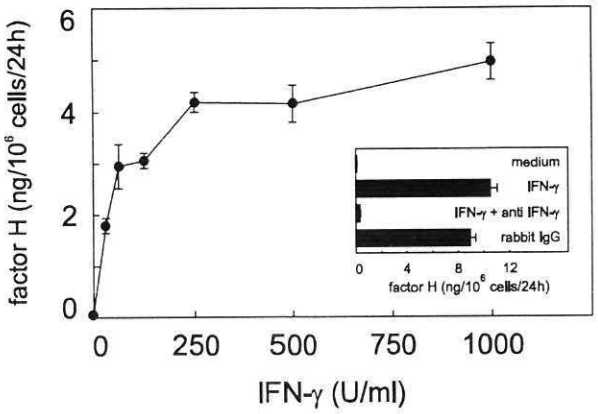

Figure 5: Release of factor H by keratinocytes is regulated by IFN-γ Keratinocytes were cultured for 72 h in the presence of indicated concentrations of IFN-γ and supernatants were collected and assessed for factor H concentrations by ELISA. Insets show the effects of IFN-γ-specific neutralizing rabbit antibodies on IFN-γ induced factor H release by keratinocytes. Keratinocytes were cultured with 100 U IFN-γ per ml alone, IFN-γ plus specific neutralizing antibodies, or control IgG. After 72 h, supernatant were collected and assessed for factor H. The data in main figures and insets are expressed as the mean ± SD of duplicate measurements of three cultures.

Up-regulation of release of factor H is inhibited by cycloheximide

To find out whether IFN-γ caused enhancement in release by inducing *de novo* synthesis, we investigated the effect of cycloheximide on IFN-γ mediated up-regulation of factor H. Keratinocytes were grown in medium alone or medium containing IFN-γ (100 U per ml), with and without 2.0 µg per ml cycloheximide. Higher concentrations could not be used because of irreversible toxic effects on the cells. At definite time intervals, supernatants were harvested for factor H analysis by ELISA. These experiments revealed that cycloheximide significantly inhibited the IFN-γ induced factor H release after 24 h and 48 h (**Figure 6**). This indicated that increased release of factor H in response of IFN-γ was due to increased *de novo* synthesis. Removal of cycloheximide by washing the cells and again culturing them in the medium containing IFN-γ restored factor H release (data not shown).

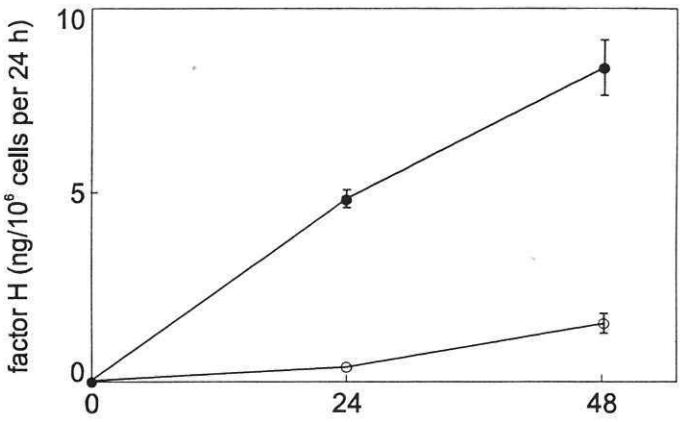

Figure 6: Cycloheximide inhibits factor H synthesis by keratinocytes Sub-confluent keratinocytes were cultured in medium containing IFN-γ in the presence and absence of 2 μg cycloheximide per ml. At 24 h and 48 h, culture supernatants were collected and assayed for factor H. Results obtained with cultures without (●) and with cycloheximide (○) are shown. Values are the mean ± SD for duplicate determinations of triplicate cultures.

Keratinocytes constitutively express factor H transcript

As shown in **Figure 7**, specific transcript for factor H were detected by RT-PCR in unstimulated normal human keratinocytes and A431 cells. cDNA derived from human liver was used as a positive control. Such specific transcripts were found in all samples examined - several cultures of keratinocytes from different foreskins and three different passages of A431 cells - demonstrating that these cells constitutively express factor H mRNA. The size of the product coincided with the predicted base pair (481 Bp). No signals were found in the negative control water blanks indicating that the results obtained were not the results of crossover or DNA contamination. Finally, PCR amplification of GAPDH mRNA after 28 cycles is also shown as a positive control for equivalent loading and integrity of the RNA preparations used in the analysis.

To confirm that cultured keratinocytes used in the above experiments were devoid of contaminanting cells, RT-PCR using primers specific for HLA-DR (macrophages, dendritic cells, B-cells), CD3 (T-cells), tyrosinase (melanocytes) and PTH-R (dermal fibroblasts) was performed under identical conditions on different passages of several cultures of keratinocytes. All cultures in first passage were found to be positive for one or more contaminating cell type but most cultures in third passage onwards were devoid of any contaminating cells (results not shown). The above experiments were performed with cultures which were verified to be negative for above mentioned cell types.

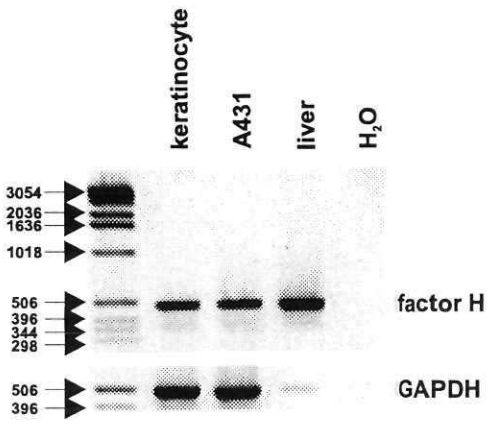

Figure 7. Human keratinocytes and A431 cells express mRNA for factor H RNA was isolated from cultured human keratinocytes and A431 cells and subjected to 36 cycles of a factor H specific RT-PCR as described in the text. Liver cDNA was used as a positive control. Keratinocyte cultures which were found to be negative at 36 cycles in a qualitative RT-PCR for tyrosinase, HLA-DR, CD3, PTH-R, and CD19 were used (see the text).

Interferon-γ regulates the synthesis of factor H at pretranslational level

Factor H mRNA transcripts obtained from IFN-γ treated and untreated cells were analyzed by RT-PCR. Keratinocytes were incubated for 6 h and 24 h in medium containing IFN-γ (100 U per ml). After RT-PCR and gel electrophoresis as described in '*Materials and Methods*' the Eagle Eye analysis revealed a 3.5 and 9.1-fold increase of the ratio factor H/GAPDH message after stimulation with IFN-γ for 6 and 24 hours, respectively (**Figure 8**). These semi-quantitative data showed increases in factor H transcripts in response to IFN-γ suggesting that the up-regulation of factor H by IFN-γ was at a pre-translational level. IL-1α, IL-6, and TNF-α were also analyzed, but none of these cytokines showed increase in levels of factor H transcripts.

DISCUSSION

The epithelial compartment of skin is known to express cellular immunity[266]. Because the epidermis is in frequent contact with foreign antigens it is expected to possess, besides components of cellular immunity, a complement synthesizing apparatus to form the first line of immunological defense. Indeed, the most abundant cells in human epidermis, keratinocytes, have been shown to produce C3[34;267], and factor B[35;267]. Keratinocytes, like some other cell types[256], may also produce other components of classical and alternative pathways. They have already been shown to express several complement receptors[28], and complement regulatory proteins[11;37]. The production of C3 and factor B by keratinocytes suggests that these cells may also be able to produce factor H which control the activities of these components[18]. We envisaged that if

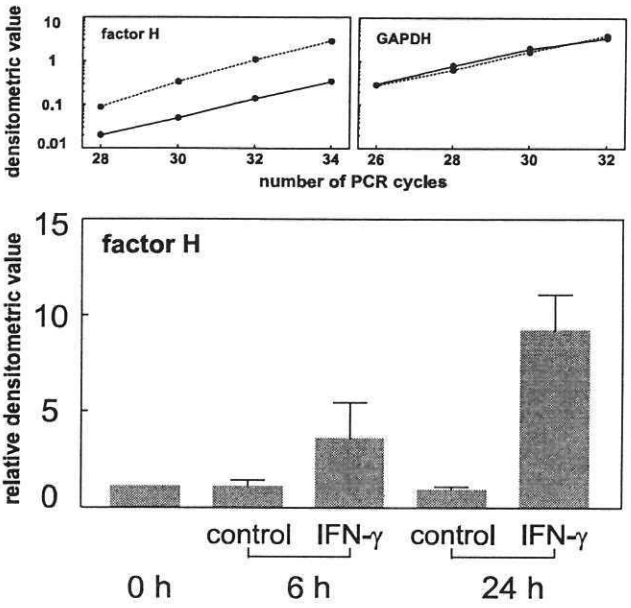

Figure 8: Expression of factor H in keratinocytes is regulated by IFN-γ at pretranslational level RNA was isolated from keratinocytes at 0 h, 6 h, and 24 h after stimulation with 100 U IFN-γ per ml, and subjected to semi-quantitative factor H specific RT-PCR as described in the text. The relative densitometric values obtained with untreated keratinocytes (0 h) were arbitrarily set at 1 and were related to those of cytokine-treated keratinocytes (6 h; 24 h). *Upper left diagram* shows representative examples of densitometric values obtained as a function of PCR cycle number for factor H and *upper left diagram* shows the same for GAPDH. Continuous lines; RNA isolated from untreated keratinocytes (0 h), and broken lines: RNA isolated 24 h after IFN-γ treatment.

keratinocytes do synthesize factor H, synthesis of factor H may also be regulated by inflammatory cytokines which regulate the synthesis of C3 and factor B[298] (see **Chapter 3**).

Early experiments showed that human keratinocytes constitutively release small amounts of factor H in culture. Western blotting revealed that unstimulated keratinocytes release 155-kD factor H protein in detectable amounts; 45-kD isoform, if released constitutively, was not detectable under the experimental conditions. IFN-γ stimulated keratinocytes released both molecular species of factor H.

Experiments aimed at studying the regulation of synthesis of factor H showed that the supernatant of activated mononuclear cells which was used as a source of mediators derived from inflammatory cells was able to induce the release of factor H strongly at a concentration of 10% (**Figure 4**). IL-2, IL-1α, IL-6, TNF-α, IFN-γ, and TGF-β, are known to be present in this supernatant. Of these only IFN-γ up-regulated the release of factor H significantly. Strong

inhibition by cycloheximide of IFN-γ induced release of factor H showed that enhanced release was due to increased *de novo* synthesis, rather than possible increased release from intracellular stores. Semi-quantitative RT-PCR experiments (**Figure 5**) showed that IFN-γ mediated enhancement of release of factor H was due to up-regulation of the factor H gene at pre-translational level.

The present study shows that factor H synthesis in keratinocytes is up-regulated by IFN-γ but not by IL-1α. In glomerular mesangial cells and proximal tubular cells, however, the synthesis of factor H was regulated by IFN-γ as well as IL-1α[265;336]. These results support the widely held view that regulation of synthesis of complement proteins by cytokines is highly cell type specific.

If the observations made in the present study and those in previous studies could be extrapolated to *in vivo* situations, following could be stated. IFN-γ produced locally by infiltrated T cells, during an inflammatory response, can up-regulate the synthesis not only of C3[36] and factor B[298] but also of factor H. Because complement is known to be continuously activated, complement produced by keratinocytes in excessive amounts in response to some cytokines may damage keratinocytes. Up-regulation of factor H parallel to C3 and factor B by IFN-γ appears to be the fluid phase mechanism to minimize complement-mediated damage of autologous keratinocytes. The other mechanism for protection of keratinocytes from keratinocyte-derived complement under the inflammatory conditions of excessive complement production could be a cell surface mechanism. This may involve up-regulation of complement regulatory molecules, membrane cofactor protein (CD46) and CD59, by TGF-ß present amongst the mediators released from activated mononuclear cells, as has recently been shown[37].

In conclusion, we provide data that support the concept that the keratinocytes have important immunological functions including production of complement proteins. They synthesize and release factor H to control the activities of C3 and factor B also known to be produced by keratinocytes. Local synthesis of factor H may contribute to the prevention of local epidermal immune damage caused by locally synthesized C3 and factor B.

Chapter Six

CD40 mediated activation of keratinocytes induces the production of chemokines but not of complement components and complement regulatory proteins

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To be submitted

Abstract

Keratinocytes are CD40 expressing immunocompetent cells. In some inflammatory conditions of the skin, keratinocytes express increased amounts of CD40 and epidermis contains activated T cells which transiently express CD40 ligand (CD40L). It is believed that CD40 on keratinocytes may ligate with CD40L on T cells. This ligation may induce the release of some inflammatory mediators and may contribute to inflammation. We tested by ELISA whether CD40 activation of IFN-γ pre-treated cultured human keratinocytes ($CD40^+$ keratinocytes) by means of CD40L transfected cells or soluble CD40L can result in enhanced production of chemokines IL-8, RANTES and MCP-1 and of complement components C3 and factor B by keratinocytes. We also tested the effect of CD40 activation of $CD40^+$ keratinocytes on the expression of complement regulatory proteins, namely membrane cofactor protein (MCP), decay accelerating factor (DAF), and CD59 by flow cytometry.

CD40 activation of $CD40^+$ keratinocytes up-regulated the release of IL-8 and RANTES greatly, and that of MCP-1 moderately. The production of C3 and factor B and the expression of MCP, DAF, and CD59 was not altered. Specificity of the results with CD40L transfected cells was confirmed using untransfected cells as controls, co-culturing $CD40^+$ keratinocytes and transfected cells with and without physical contact with each other in a Transwell system, and inhibiting CD40 activation with neutralizing anti-CD40 monoclonal antibodies.

In conclusion, CD40 activation on cultured human $CD40^+$ keratinocytes up-regulated their release of the chemokines IL-8, RANTES and MCP-1 without affecting the release of complement proteins C3 and factor B and without altering the expression of MCP, DAF, and CD59. If these *in vitro* results may be extrapolated to *in vivo* situations they suggest that an interaction of activated T cells with keratinocytes via CD40-CD40L interaction may play a role in inflammatory conditions of the skin.

INTRODUCTION

CD40 is a 50-kD cell membrane glycoprotein expressed on B-cells, monocytes, dendritic cells and T-cells[337]. The ligand of CD40 (CD40 ligand; CD40L; CD154) is a 35-kD glycoprotein which is transiently expressed on activated $CD4^+$ T-cells, mast cells, eosinophils, and basophils[337;338]. Both CD40 and CD40L belong to the tumour necrosis factor (TNF) receptor superfamily and have been reviewed extensively[337;338].

CD40-CD40L ligation between CD40 on B and CD40L on T cells was shown to play a role in T cell dependent B cell activation and in isotype switching of IgM producing B cells[338-340]. Absence of CD40-CD40L ligation due to mutations in the CD40L gene in humans results in X-linked hyper-IgM syndrome in which there is deficient isotype switching characterized by lack of circulating IgG and IgA and absence of germinal centers[341]. Recent studies have, however, shown that CD40 is also expressed on non-lymphoid cells. These include fibroblasts[342;343], endothelial cells[344;345], dendritic cells[346], mesangial cells[347], and cortical and medullary thymic epithelial cells[348]. Interaction of CD40 expressed on these cells with CD40L has been shown to cause release of mediators of inflammation and induction of expression of pro-inflammatory cell surface molecules on them[343-347].

Recently, human keratinocytes have also been shown to express functional CD40[90;147;349]. Keratinocytes are the major cell-type in the epidermis which by virtue of their ability to synthesize and secrete inflammatory mediators such as cytokines[296] and complement components[267] have been recognized as initiators of inflammation in the skin[1]. CD40-CD40L ligation has been shown to activate keratinocytes resulting in the release of IL-6 and IL-8[90;147] and induction of proinflammatory cell adhesion molecule ICAM-1[147;350]. This ligation also enhances the differentiation of keratinocytes and inhibits their proliferation[349]. The role which CD40-CD40L interaction plays in regulation of the production of other inflammatory proteins in keratinocytes is not known.

CD40-CD40L interaction on keratinocytes may be involved in the pathogenesis of some inflammatory conditions of the skin. It has been shown that in lesional skin of patients with psoriasis and atopic dermatitis, CD40 expression on keratinocytes is elevated. In psoriatic lesions, increased CD40 expression in epidermal keratinocytes[344;147;351] is associated with increased production of the chemokines IL-8, RANTES, and MCP-1[115;250;213], increased accumulation of complement activation products (*e.g.* C5a des-arg, C5b-C9), dramatic cellular infiltration, and altered expression of complement regulatory proteins DAF and CD59[352]. It is likely that the interaction of CD40L on activated T cells with CD40 on keratinocytes may be one of the factors responsible for inducing these abnormalities in the epidermal compartment. From this point of view we investigated the release of chemotactic cytokines IL-8, RANTES and MCP-

1 and of complement proteins C3 and factor B by keratinocytes, which were induced to express optimal amounts of CD40 ($CD40^+$ keratinocytes), in response to CD40L transfected J558L cells and soluble recombinant CD40L. We also investigated the influence of CD40L on $CD40^+$ keratinocytes on the expression of complement regulatory proteins MCP, DAF, and CD59.

MATERIALS AND METHODS

Keratinocyte cultures

Human keratinocytes were isolated by incubation of foreskin with thermolysin (0.50 mg per ml, Sigma, St. Louis, MO) at 4 °C for 16 h and subsequent trypsinization (0.025%) for 5 min at 37 °C. Trypsin (Sigma) was then neutralized by an excess of heat inactivated fetal calf serum (GibcoBRL, Breda, The Netherlands). Cells were separated from debris by filtering through a nylon mesh, centrifuged and resuspended in keratinocyte serum free medium (GibcoBRL) supplemented with 100 IU per ml penicillin/100 µg per ml streptomycin (GibcoBRL). The keratinocytes were plated onto 100 mm plastic Petri dishes at a density of 400,000 cells per Petri dish and incubated at 37 °C in humidified, 5% CO_2, tissue culture incubator. Medium was changed every 2-3 d and at 70% confluence, cultures were split after a 5 min exposure to trypsin (0.025%)/ethylene diamine tetra-acetic acid (EDTA) (1.5 mM) and recultured. For use in different experiments, cells were seeded in 6 well tissue culture plates (Costar) at a density of 100,000 cells per well in 1500 µl of medium or in 12 well plates at a density of 50,000 cells per well in 750 µl of medium. Cells in passage 2-5 were used for experiments as soon as 60-80% confluence was achieved.

Keratinocyte interaction with CD40L

CD40L transfected J558L hybridoma cells (kindly supplied by Dr. P. Lane, University of Birmingham, Birmingham, UK) and control untransfected J558L cells were cultured in IMDM supplemented with FCS[353]. In all experiments they were irradiated with 50 Gy and washed extensively before use. For ligation studies, subconfluent keratinocyte cultures were always pre-treated with IFN-γ (10 U per ml; Pharma Biotechnology Hannover, Hannover, Germany) for 72 h at 37°C in a humidified, 5% CO_2 cell culture incubator. These cells pre-treated with IFN-γ expressed high levels of CD40 as described in '*Results*' and were referred to as $CD40^+$ keratinocytes throughout this manuscript. $CD40^+$ keratinocytes were then incubated with CD40L transfected or control J558L cells in a 1:1 ratio, unless indicated otherwise, and subsequently cultured in medium supplemented with IFN-γ (10 U per ml). After 72 h, supernatants were harvested, centrifuged and frozen in aliquots for the analysis of chemokines and complement components. Keratinocytes were detached with trypsin/EDTA, as described above, and used in flow cytometry experiments.

In some experiments instead of CD40L transfected cells, an 18-kD soluble recombinant CD40L (sCD40L; a gift of Dr. J-Y Bonnefoy, GlaxoWellcome Institute for Molecular Biology, Geneva, Switzerland)[354] was used for CD40 triggering. Briefly, $CD40^+$ keratinocytes were cultured with sCD40L in medium supplemented with IFN-γ (10 U per ml). After 72 h, supernatants were harvested, centrifuged and frozen in aliquots for the analysis of chemokines and complement proteins. sCD40L treated $CD40^+$ keratinocytes were detached and used in flow cytometry experiments.

Culture of CD40$^+$ keratinocytes and CD40L transfected cells in a Transwell System

In experiments aimed at investigating the requirement of contact between CD40L transfected J558L cells and CD40$^+$ keratinocytes for the activation of CD40$^+$ keratinocytes a Transwell System (Costar) was used. CD40$^+$ keratinocytes were cultured to subconfluence on the bottom of the lower wells of 24 mm diameter Transwell system. Cell number was estimated as 5-7 X 10^5 keratinocytes per well. After removal of medium, 1500 µl fresh medium was added to the lower well. In wells in which direct contact between transfected J558L cells and keratinocytes was not desired, CD40L transfected J558L cells or control J558L cells in 500 µl medium supplemented with IFN-γ (10 U per ml) were added to the upper wells with bottoms mounted with a 0.4 µm pore size polycarbonate membrane, in a 1:1 ratio. In wells in which direct contact of the cells was desired, transfected or control J558L cells in 500 µl medium containing IFN-γ were added to the lower well, in a 1:1 ratio. After 72 h, supernatants were harvested, centrifuged and frozen in aliquots for the analysis of chemokines. Concentration of chemokines in supernatants in wells containing transfected or control J558L cells separated from CD40$^+$ keratinocytes by the porous membrane were compared with those in supernatants of wells containing transfected or control cells not separated through the membrane.

Blocking of CD40 activation of CD40$^+$ keratinocytes by specific antibody

CD40$^+$ keratinocytes were cultured to subconfluence on the bottom of 6 well tissue culture plates and pre-treated for 2 h with 10 µg per ml of anti-CD40 monoclonal antibody 5 D12 (a kind gift of Tanox Pharma, Amsterdam, The Netherlands) in IFN-γ containing medium (10 U per ml). Hereafter, CD40L transfected J558L cells (or control J558L cells) were added in a 1:1 ratio to the CD40$^+$ keratinocytes (5-7 X 10^5 cells per well) in IFN-γ and anti-CD40 containing medium. After an incubation period of 72 h supernatants were harvested, centrifuged and frozen in aliquots for the analysis of chemokines.

Semi-quantitative determination of cell surface proteins by flow cytometry

Keratinocytes were detached with trypsin (0.025%)/EDTA (1.5 mM) for 3-5 min. For analysis of MCP, DAF, and CD59, CD40L treated CD40$^+$ keratinocytes were used (see above). Trypsin was inactivated by fetal calf serum and detached cells were washed and resuspended in fluorescence-activated cell sorter (FACS) buffer (PBS, 2% fetal calf serum, 0.1% sodium azide). Approximately 10^5 cells were incubated with specific monoclonal antibodies to CD40 (clone 5D12), MCP (clone J4-48, CLB, Amsterdam, The Netherlands), DAF (clone BRIC 110, CLB) or CD59 (1F5)[308] or isotype control (Becton Dickinson, San Jose, CA) for 30 min at 4°C. Cells were washed two times and incubated for 30 min with fluorescein-conjugated $F(ab')_2$ fragments of goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). Hereafter, cells were washed two times with FACS buffer. Propidium iodide (1 mg per ml) (Sigma) was added to identify dead cells and 20,000 cells were analyzed immediately by FACSCalibur (Becton Dickinson). Dead cells were excluded.

The detachment of CD40$^+$ keratinocytes with the trypsin/EDTA solution at 37°C for 3-5 min did not cause degradation of CD40 or any of the three complement regulatory proteins, as expression of these molecules on cells detached by this procedure was the same as on those detached with EDTA (2 mM) alone at 42 °C for 30 min. The former procedure was selected because it did not cause loss of cell viability, whereas latter procedure caused a high degree of loss of cell viability.

ELISA for measurement of C3, factor B, IL-8, RANTES and MCP-1

For quantification of C3 and factor B, previously described sandwich ELISAs developed in our laboratory were used[267] (described in **Chapter 2**). The detection limits of these ELISAs were 1 ng per ml for C3 and 100 pg per ml for factor B. Standard curves for both ELISAs were made using Human Complement Calibrator CA1 (ATAB).

The concentrations of IL-8, RANTES and MCP-1 in culture supernatants of CD40L stimulated and non-stimulated keratinocytes were estimated by ELISA as follows.

For quantification of IL-8, wells of 96 well flat-bottom microtiter plates were coated with 100 µl of 0.5 µg monoclonal mouse-anti-human IL-8 IgG1 (Biosource, Breda, The Netherlands) per ml in carbonate buffer overnight at 4°C. After thorough washing with Tween-80 (0.05%) (Sigma) in PBS the wells were blocked for 1 h at room temperature with 200 µl of PBS containing 2% bovine serum albumin (BSA; Sigma) and 0.05% Tween-80. Washing was repeated and wells were incubated with 100 µl of sample, diluted in the same buffer that was used for blocking. Plates were incubated for 2 h at 37°C. The wells were then washed and incubated with 100 µl biotinylated mouse anti-human IL-8 IgG1 (0.05 µg per ml) (Biosource) for 1 h at 37°C. After washing, the wells were incubated for another h at 37°C with peroxidase conjugated poly streptavidin (1:8000; Central Laboratory of the Netherlands Red Cross and Blood Transfusion Services, Amsterdam, The Netherlands). Wells were thoroughly washed and incubated with 100 µl 3,3',5,5'tetramethylbenzidine (Sigma) in dimethylsulfoxide (Merck, Hohenbrunn, Germany)-citrate buffer for 10 min. The reaction was stopped with 100 µl H_2SO_4 (2 M). Optical density (OD) was measured at 450 nm. The detection limit of this ELISA was 1 pg per ml of IL-8.

For determination of RANTES, wells were coated overnight at 4°C with 50 µl of 2 µg monoclonal mouse-anti-human RANTES IgG (R&D Systems, Minneapolis, MN) per ml in PBS. After thorough washing, blocking and application of samples (100 µl) essentially as described above for IL-8, the wells were incubated with biotinylated polyclonal goat anti-human RANTES IgG (5 ng per ml) (R&D Systems) for 1 h at 37°C. After further washing, the wells were incubated for 1 h at 37°C with peroxidase conjugated poly streptavidin (1:8000). The wells were then repeatedly washed. Incubation of the wells with the peroxidase substrate, termination of the reaction, and measurement of OD was carried out as described for IL-8. The detection limit of this ELISA was 30 pg per ml.

For MCP-1 ELISA, wells were coated with 50 µl of 2 µg monoclonal mouse-anti-human MCP-1 IgG1 (Pharmingen, San Diego, CA) per ml in carbonate buffer overnight at 4°C. After thorough washing with Tween-80 (0.05%) in PBS, the wells were blocked with 200 µl blocking/dilution buffer (PBS containing Tween-80 (0.05%) and milk powder (2%) (Nutricia, Zoetermeer, The Netherlands) for 1 h at room temperature. Washing was repeated and wells were incubated with 50 µl sample for 2 h at 37°C. The wells were then washed and incubated with 50 µl biotinylated polyclonal rabbit anti-human MCP-1 IgG (1 µg per ml) (Pharmingen) for 1 h at 37°C. Treatment of the wells with peroxidase conjugated polystreptavidin, incubation of the wells with the peroxidase substrate, termination of the reaction and measurement of OD was carried out as described for IL-8. The detection limit of this ELISA was 7 pg per ml.

Standard curves for IL-8, RANTES and MCP-1 were made using recombinant human IL-8 (Biosource), recombinant human RANTES (R&D Systems) or recombinant human MCP-1 calibrator (Pharmingen), respectively.

Statistical analysis

Statistical analysis was performed using a Students' *t*-test for data from ELISA experiments. A p value of less than 0.05 was considered significant.

RESULTS

IFN-γ at low concentrations can up-regulate the constitutive expression of CD40 on keratinocytes

Cultured human keratinocytes are known to express low but detectable amounts of CD40[90;147;350;349]. In ligation studies, expression of CD40 is first up-regulated by treatment with IFN-γ, usually at a dose of 100 U per ml and for a culture period of 48 - 72 h. Keratinocytes treated with IFN-γ in doses as high as 100 U per ml become significantly stimulated[298]. To find out the lowest concentration of IFN-γ which can cause optimal expression of CD40, the effect of IFN-γ (10-300 U per ml; 72 h) on the expression of CD40 on cultured human keratinocytes was analyzed by flow cytometry as described in '*Materials and Methods*'. From 10 U per ml onwards we observed a very low dose-dependent up-regulation of the constitutive CD40 expression. A dose of 10 U per ml induced an up-regulation in CD40 expression of the keratinocytes which was more than 80% of the up-regulation induced by as high a dose of IFN-γ as 300 U per ml. (**Figure 1**). A dose of 10 U per ml and a time period of 72 h was therefore selected for pre-treatment of keratinocytes ($CD40^+$ keratinocytes as described in '*Materials and Methods*') in CD40-CD40L ligation studies to induce optimal CD40 expression and at the same time to prevent major stimulation of the keratinocytes.

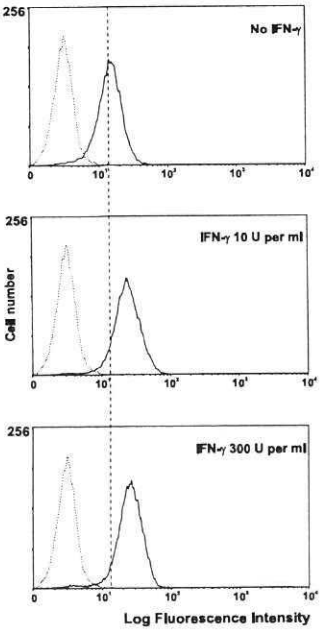

Figure 1. The constitutive expression of CD40 on human keratinocytes is up-regulated by low concentrations of IFN-γ. Keratinocytes were cultured in the presence of increasing concentrations of IFN-γ (10-300 U per ml). After 72 h, 2 X 10^4 cells were analyzed for the expression of CD40 by flow cytometry. Histograms for expression of CD40 are shown. Continuous line represent expression of CD40. Broken lines represent results obtained with isotype controls.

Activation of CD40⁺ keratinocytes by CD40L transfected J558L cells enhances chemokine production

We tested whether CD40 ligation can activate keratinocytes to produce enhanced amounts of chemokines. For this reason, CD40⁺ keratinocytes were co-cultured with CD40L transfected J558L cells or control J558L cells as described in *'Materials and Methods'* and the concentrations of IL-8, RANTES and MCP-1 were determined in the culture supernatants. **Figure 2** shows that CD40⁺ keratinocytes produce low levels of IL-8, RANTES and MCP-1 which was not altered upon co-culturing with increasing numbers of control non-transfected J558L cells but was markedly increased upon co-culturing with increasing numbers of CD40L transfected cells. At a CD40L transfected J558L cells : CD40⁺ keratinocyte ratio of 1:1 the CD40 activation caused 9-, 65- and 2.4-fold increase in production of IL-8, RANTES and MCP-1 whereas at a ratio of 5:1 of these cells CD40 activation caused 16-, 125-, and 1.4- fold increase in production of these chemokines, respectively. These results are representative of one of the two independent experiments with almost identical results. Decrease in MCP-1 production at a cell ratio of 5:1 was not a strange finding as after an optimal increase at 1:1 ratio, similar decrease in MCP-1 production with increasing ratio of transfected cells has been observed with proximal tubular epithelial cells[347].

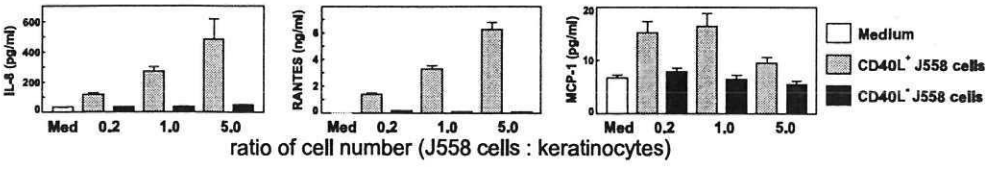

Figure 2. Chemokine production by CD40⁺ keratinocytes in co-cultures is dependent on the dose of CD40L transfected cells Subconfluent CD40⁺ keratinocyte cultures were co-cultured with CD40L transfected (CD40L⁺) J558L cells or control (CD40⁻) J558L cells in an increasing ratio with the keratinocytes (0.2, 1.0, and 5.0). Supernatants were harvested at 72 h and the concentrations of IL-8, RANTES and MCP-1 were measured by ELISA. Data represent one of two independent experiments, each in triplicate. Mean values and SD are shown.

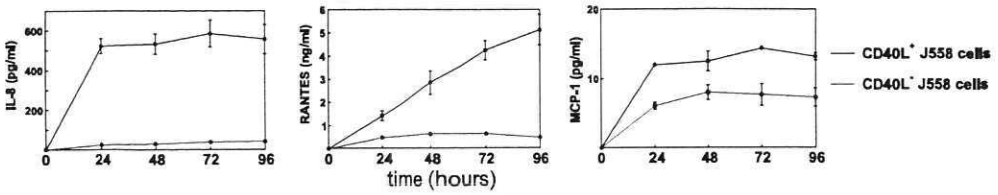

Figure 3. Chemokine production by CD40⁺ keratinocytes in response to CD40L transfected cells is time-dependent CD40⁺ keratinocytes were co-cultured with CD40L transfected (CD40L⁺) J558L cells or control (CD40L⁻) J558L cells in a 1:1 ratio and supernatants were harvested at 24, 48, 72 or 96 h. The concentrations of IL-8, RANTES and MCP-1 in supernatants were measured by ELISA. Data represent one of two independent experiments, each in triplicate. Mean values and SD are shown.

When cultures were followed in time, strong enhancement in release of IL8, RANTES and MCP-1 by CD40⁺ keratinocytes after CD40 activation could already be detected at 24 h (**Figure 3**). In case of IL-8 and MCP-1, active production subsided after 24 hours whereas in case of RANTES production continued over a long period of time and was still seen at 96 hours.

To prove that contact between CD40⁺ keratinocytes and CD40L transfected J558L cells was necessary for keratinocyte activation and to rule out the possibility that CD40L transfected J558L cells may be releasing any soluble mediator which might be causing the activation of keratinocytes to release chemokines, following experiment was performed in a Transwell system. CD40⁺ keratinocytes were placed in the lower well and CD40L transfected or control J558L cells in upper wells in the same medium but separated from each other through a porous polycarbonate membrane. In a parallel set CD40⁺ keratinocytes as well as CD40L transfected J558L cells or control J558L cells were cultured under identical conditions in lower wells without physical separation as described in '*Materials and Methods*'. After 72 h of culture neither supernatant of the wells containing transfected cells nor the supernatants of wells containing control J558L cells showed any increase in production of chemokines from CD40⁺ keratinocytes in wells in which both cell types were cultured without cell-cell contact (**Figure 4**). In supernatants harvested from CD40⁺ keratinocytes cultured in direct contact with CD40L transfected J558L cells, concentrations of IL-8, RANTES, and MCP-1 were greatly increased. This suggested that contact of CD40⁺ keratinocytes with CD40L transfected cells was essential for keratinocyte activation to produce enhanced amounts of chemokines.

After confirming that cell to cell contact was necessary for CD40L transfected J558L cells to activate CD40⁺ keratinocytes to produce chemokines, attempt was made to show that during cell-cell contact it was the CD40L molecule on CD40L transfected J558L cells which triggered the CD40 molecule on keratinocytes. To demonstrate this, experiments were performed

in which interaction was selectively blocked by means of blocking antibodies directed against the CD40 molecule. CD40$^+$ keratinocytes and CD40L expressing J558L cells were co-cultured for 72 h in the medium containing non-activating anti-CD40 antibodies or irrelevant mouse IgG1. The results showed that neutralization of CD40 on CD40$^+$ keratinocytes with anti-CD40 abolished the induction of IL-8 and MCP-1 production, and reduced the induction of RANTES production (**Figure 5**). Similar concentrations of a control IgG1 showed no effect. This experiment was performed in triplicate and in all three independent experiments induction of RANTES was reduced but not completely inhibited. The specificity of CD40 activation of CD40$^+$ keratinocytes by CD40L transfected J558L cells was confirmed by almost complete inhibition of IL-8 and MCP-1 after addition of blocking antibodies against CD40 but the reason for incomplete inhibition of production of RANTES remained unclear.

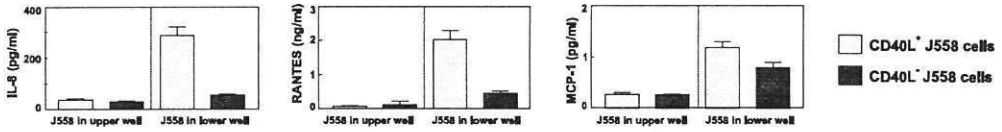

Figure 4. Physical contact between CD40L transfected cells and CD40$^+$ keratinocytes is necessary to activate keratinocytes. CD40$^+$ keratinocytes cultures were grown to subconfluence on the bottom of the lower wells of Transwell tissue culture plates. CD40L transfected (CD40L$^+$) J558L cells or control (CD40L$^-$) J558L cells were added to the upper well in a 1:1 ratio (no direct contact of the transfected or control J558L cells with the CD40$^+$ keratinocytes) or to the lower well (with direct contact of the J558L cells with the CD40$^+$ keratinocytes). Supernatants were harvested at 72 h and the concentrations of IL-8, RANTES and MCP-1 were determined by ELISA. Data represent one of two independent experiments, each in triplicate. Mean values and SD are shown.

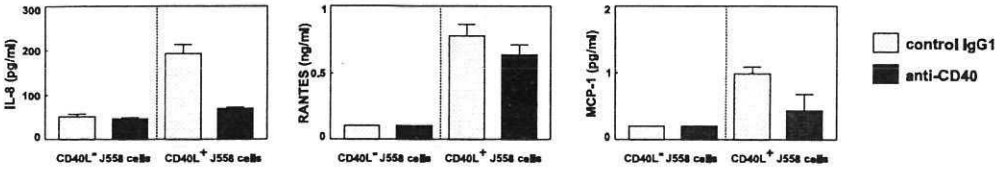

Figure 5. Chemokine production by keratinocytes in co-cultures of CD40$^+$ keratinocytes and CD40L transfected cells is inhibited by antibody specific for CD40. CD40$^+$ keratinocytes were grown to subconfluence followed by a 2 h incubation with 10 µg per ml of anti-CD40 antibodies (5D12). Hereafter, CD40L transfected (CD40L$^+$) or control (CD40L$^-$) J558L cells were added in a 1:1 ratio to the keratinocytes and cultured. Supernatants were harvested at 72 h and the concentrations of IL-8, RANTES and MCP-1 were determined by ELISA. Data represent one of two independent experiments, each in triplicate. Mean values and SD are shown.

Recombinant soluble CD40L (sCD40L) can also induce activation of CD40$^+$ keratinocytes to cause enhanced production of chemokines.

When CD40$^+$ keratinocytes were cultured for 72 h and culture supernatants were harvested, IL-8, RANTES, and MCP-1 were found to be present in low concentrations. However, when CD40$^+$ keratinocytes were cultured in the presence of increasing concentrations of sCD40L, concentrations of IL-8, RANTES, and MCP-1 in the supernatants were increased in a dose-dependent manner (**Figure 6**). Enhancement in production of MCP-1 was less than that of IL-8 and RANTES but was significant ($p < 0.05$ with all tested concentrations of sCD40L). These results were obtained with different keratinocyte cultures in 2 independent experiments, each in triplicate.

Activation of CD40$^+$ keratinocytes by CD40L transfected J 558 cells or sCD40L does not enhance the production of complement components

Besides cytokines (and chemokines), keratinocytes are known to produced two complement components, C3 and factor B; production of more components is yet to be studied. Production of C3 and factor B is differentially regulated by several cytokines[298;36]. It is not known whether CD40L also acts as one of the signals for enhanced production of these components. To test this, CD40$^+$ keratinocytes were co-cultured with increasing numbers of CD40L transfected or control J558L cells for 72 hours or both cells were co-cultured in 1:1 ratio for different time intervals and the concentrations of C3 and factor B in the supernatants were determined. In some experiments, instead of CD40L transfected or control J558L cells, increasing concentrations of sCD40L were added to CD40$^+$ keratinocytes and cells were cultured for 72 h. The results showed that neither CD40L transfected J558L cells nor sCD40L were able to regulate the production of C3 and factor B (data not shown).

Activation of CD40$^+$ keratinocytes by CD40L transfected J558L cells does not up-regulate the expression of complement regulatory proteins

Some cytokines (*e.g.* TGF-β), known to be present in inflammatory environment, can up-regulate the expression of MCP and CD59 on keratinocytes to protect them from their own complement[37]. Besides, expression of DAF and CD59 is down-regulated in psoriatic epidermis[11]. It is not known whether CD40L, which is transiently expressed on activated CD4$^+$ T cells in inflammatory environment, can interact with CD40 on CD40$^+$ keratinocytes and alter the expression of complement regulatory proteins on keratinocytes. To test this, CD40$^+$ keratinocytes

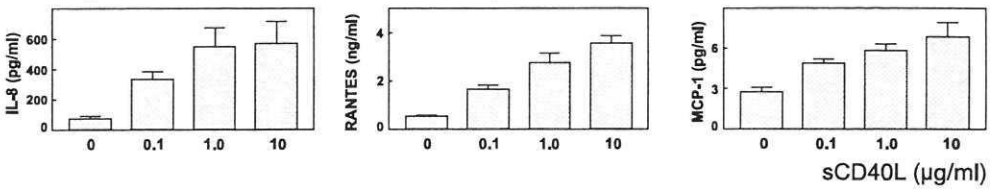

Figure 6. Recombinant soluble CD40L can also induce CD40 activation of CD40⁺ keratinocytes to enhance production of chemokines. Subconfluent CD40⁺ keratinocytes were cultured with increasing doses of sCD40L (0.1 - 10 μg per ml). Supernatants were harvested at 72 h and the concentrations of IL-8, RANTES, and MCP-1 were measured by ELISA. Data represent one of two independent experiments, each in triplicate. Mean values and SD are shown.

were co-cultured with increasing numbers of CD40L transfected or control J558L cells for 72 hours, or in 1:1 ratio for different time intervals, and the expression of MCP, DAF and CD59 on the surface of keratinocytes assessed by flow cytometry. In some experiments instead of increasing numbers of CD40L transfected or control J558L cells, increasing concentrations of sCD40L were incubated with CD40⁺ keratinocytes for 72 h as described in '*Materials and Methods*'. The results showed that ligation of CD40L with CD40 on keratinocytes was not associated with alterations in expression of MCP, DAF, or CD59 (data not shown).

DISCUSSION

In some dermatological conditions, human skin can manifest dramatic inflammation. One such condition is psoriasis in which keratinocytes undergo hyperproliferation and the epidermis is inundated with chemoattractant and proinflammatory cytokines, complement activation products (C3a, C5a, C5b-C9 etc.), and inflammatory cells (including activated T-cells). It is believed that communication between activated T-cells and keratinocytes may initiate hyperproliferation of keratinocytes. This, in turn, may produce excessive amounts of chemokines and complement. It is therefore important to elucidate the interactions involved in communication between activated T-cells and keratinocytes. One such possible interaction is the ligation of CD40 present on keratinocytes in psoriatic epidermis with CD40L transiently expressed on activated T cells. We investigated whether CD40-CD40L ligation on cultured human keratinocytes can activate keratinocytes to produce excessive amounts of chemokines and complement components and alter the expression of complement regulatory proteins *in vitro*, abnormalities which have been seen in psoriatic epidermis *in vivo*[11].

Cultured keratinocytes express low amounts of CD40. Activation of keratinocytes by

CD40L does not take place in keratinocytes expressing basal levels of CD40 but does take place in IFN-γ treated keratinocytes which express high levels of CD40[90]. Therefore in ligation studies for efficient ligation, CD40 on keratinocytes is first up-regulated by IFN-γ before treatment of keratinocytes with a source of CD40L. In previous studies, the concentrations of IFN-γ used to up-regulate CD40 on keratinocytes were 100 U per ml or more[147;90;349]. Since this concentration of IFN-γ can directly release some inflammatory mediators[298], we investigated if lower concentrations can up-regulate CD40 on keratinocytes. The results showed that 10 U IFN-γ per ml can induce almost as high degree of up-regulation of CD40 as 100-300 U per ml. This low dose of IFN-γ does not induce the release of C components[298]. IFN-γ in this dose primarily appears to act as a priming agent for keratinocytes, placing keratinocytes in a heightened state of readiness for activation through CD40 without major stimulation. Ten U of IFN-γ per ml was therefore selected for pretreatment of keratinocytes to induce optimal expression of CD40 in all subsequent experiments.

For experimental purposes, CD40 activation of $CD40^+$ keratinocytes was achieved in two ways - using CD40L transfected J558L cells or using an agonistic recombinant soluble CD40L (sCD40L). Activation of $CD40^+$ keratinocytes with CD40L of transfected J558L cells induced strong production of IL-8, RANTES and MCP-1. This induction was dependent on the dose of CD40L-transfected J558L cells. It was also time dependent. Experiments in which $CD40^+$ keratinocytes and CD40 transfected J558L cells were co-cultured with and without separation from each other through a porous membrane in a Transwell system showed that contact of CD40L transfected cells with $CD40^+$ keratinocytes was essential for up-regulation of release of chemokines. During cell-cell contact, it was the CD40L molecule on CD40L transfected J558L cells which triggered the CD40 molecule on keratinocytes. This was shown by selectively blocking CD40-CD40L ligation by antibodies against the CD40 molecule. This antibody abolished the induction of IL-8 and MCP-1 production and reduced the induction of RANTES. sCD40L could also induce CD40 activation of keratinocytes to enhance production of chemokines, further confirming the specificity of CD40-CD40L ligation for chemokine production. It also suggests that $CD40^+$ keratinocytes can be activated through CD40 by either cell bound or secreted/shed CD40L to participate in an ongoing inflammatory response by producing chemoattractant cytokines.

The results of this study, using CD40L transfected J558L cells as well as sCD40L, showed that CD40-CD40L ligation on keratinocytes can not induce signalling involved in C3 and factor B up-regulation. This ligation also does not regulate the expression of complement regulatory proteins.

Our results if extrapolated to *in vivo* situation in psoriasis may suggest that the cytokine milieu surrounding the keratinocytes *in vivo* may represent a critical determinant of the cellular

responses mediated through the CD40-CD40L pathway. Of particular relevance would be the tissue concentration of IFN-γ. This cytokine may provide a primary effect of up-regulating CD40 on keratinocytes for CD40 engagement in inflamed tissue and thus can facilitate CD40-CD40L ligation. This ligation may in turn result in amplified production of IL-8, RANTES and MCP-1 and possibly other chemoattractant (and proinflammatory) cytokines. IL-8 may be considered responsible for the infiltration of neutrophils and RANTES and MCP-1 for infiltration of T cells in the psoriatic lesional skin[249;355]. Thus, CD40-CD0L ligation may be important not only in perpetuating and augmenting inflammatory processes in the skin but also in establishing a positive activation loop in regulation of epidermal infiltration. A pathogenic role for CD40-CD40L interaction in skin inflammatory reactions is supported by the fact that functional inhibition of T cell function by cyclosporin A which is known to prevent CD40L expression on activated T cells[356;357], is beneficial in psoriasis[358]. It appears that production of complement[352] and alteration of expression of complement regulatory proteins in psoriasis[11] likely do not involve CD40-CD40L ligation.

In conclusion, CD40-CD40L ligation on keratinocytes can induce keratinocytes to produce enhanced amounts of chemokines but not complement components C3 and factor B. It also does not influence the expression of complement regulatory proteins on keratinocytes. This ligation *in vivo* may play a pivotal role in regulating inflammation in the epidermis.

Acknowledgements

We thank Joost Schuitemaker, Department of Cell Biology, Academic Medical Center, University of Amsterdam, for his technical assistance. We also thank Dr. Jon Laman, Department of Immunology, Erasmus University Rotterdam for helpful discussions.

Chapter Seven

Summary and Discussion

The purpose of the work presented in this thesis was to obtain more information than was available on the role of keratinocytes in skin inflammation. As has been postulated by Barker[1], keratinocytes may be considered initiators of cutaneous inflammatory responses. Keratinocytes, the major cell type in epidermis, may be triggered by diverse stimuli to elaborate specific pro-inflammatory molecules, such as cytokines, chemokines, complement proteins and adhesion molecules. The consequences are activation of dermal microvascular endothelial cells and accumulation of inflammatory cells in the dermis and epidermis. Ultraviolet B (UVB) radiation, contact allergens, and thermal injury are well known exogenous stimuli which activate keratinocytes to synthesize cytokines and thereby initiate inflammation. In many inflammatory diseases of the skin, the nature of initial events leading to inflammation are not known but the endogenous stimuli which influence epidermal keratinocytes may be provided by infiltrating cells, *e.g.*, monocytes/macrophages, neutrophils, and T cells. These cells may do this in two possible ways: (1) by releasing mediators such as cytokines which in turn induce keratinocytes to produce additional mediators such as cytokines, chemokines, and complement components and (2) by interacting with keratinocytes in inflammatory environment through some ligands (*e.g.*, CD40L on T cells) and activating them to produce additional inflammatory mediators. Although some information is available on these two modes of participation of keratinocytes in skin inflammation, it is far from complete. In this thesis we present work focused on the production, expression, and regulation of complement proteins and some chemokines in keratinocytes by some endogenous and exogenous stimuli.

Chapter 1 of this thesis presents a brief introduction into current knowledge on mechanisms leading to inflammatory reactions of the skin. This is followed by a comparatively more detailed description of the complement system and cytokines/chemokines network. The discussion on the complement system includes its two pathways and the regulation of their activation by fluid phase and membrane embedded regulators. The discussion on cytokines includes a short description of a number of well known cytokines which are known to be present amongst the inflammatory mediators of mononuclear cells, namely IL-1α, IL-6, TNF-α, IFN-γ, and TGF-β and whose influences on pro-inflammatory properties of keratinocytes have been

investigated in subsequent chapters of this thesis. A brief overview of the chemokines is given with special reference to the three chemokines, IL-8, RANTES, and MCP-1, the regulation of whose synthesis in keratinocytes is studied in this thesis in view of their chemotactic properties relevant to the pathogenesis of inflammatory diseases of the skin. Since keratinocytes are the most abundant cells in the epidermal compartment of the skin, this chapter also includes an overview of the production, expression, and functional relevance of complement proteins and chemokines in these cells.

In **chapter 2** to **6**, the results of a number of *in vitro* studies on the regulation of synthesis of fluid phase complement proteins and chemokines and the regulation of expression of complement regulatory proteins in keratinocytes are described.

Human keratinocytes have been shown to synthesize two components of complement, namely C3 and factor B[34;35]. Further studies may show that they synthesize other components as well. They may even be found to produce complete classical and alternative pathway cascades as has been shown in other cell types[9;256]. As regards constitutive synthesis of C3 and factor B *in vitro*, it was quite low[34;35]. From this it may be deduced that in normal skin the synthesis of these components may also be low. The ability of keratinocytes and inflammatory cells to produce a number of cytokines under inflammatory conditions raises the prospects that some of the cytokines my regulate the synthesis of C3 and factor B by keratinocytes. We have investigated if cytokines released from activated mononuclear cells, many of them also produced by keratinocytes, are involved in regulation of C3 and factor B synthesis in keratinocytes. Our data presented in **Chapter 2** clearly show that several of these cytokines strongly, but differentially, regulate the synthesis of C3 and factor B from keratinocytes *in vitro*. IFN-γ, and to a lesser extent IL-1α, were found to be inducers for production of both C3 and factor B by keratinocytes, while TNF-α was capable of up-regulating the production of C3 without affecting production of factor B. IL-6 had stimulatory effects on the production of factor B only. The regulatory effects of these cytokines were observed both at protein and mRNA level. Thus, these results clearly demonstrated an important role of cytokines in differentially regulating the complement component production in human keratinocytes.

Complement components produced in high amounts by keratinocytes in response to cytokines, as shown in chapter 2, may potentially damage autologous epidermal cells, since complement activation products, such as C3b, cannot distinguish between invading microbes and self cells. This damage must be prevented by complement regulatory proteins MCP, DAF, and CD59, which inactivate complement activation products deposited on cell membranes. These proteins or most of them should therefore be expressed on the surfaces of keratinocytes, among other cells of the epidermis. Under inflammatory conditions when there is up-regulation of complement synthesis by keratinocytes under the influence of cytokines (see Chapter 2), there should also be up-regulation of the expression of complement regulatory proteins on

keratinocytes to protect them from excessively produced complement. We investigated if cytokines released from activated mononuclear cells, many of them also produced by keratinocytes, are involved in up-regulation of expression of cell surface complement regulatory proteins. **Chapter 3** describes the flow cytometric studies on the effects of mixture of cytokines collectively released from activated mononuclear cells and of individual cytokines known to be produced by inflammatory cells and keratinocytes, on the expression of cell surface complement regulatory proteins in keratinocytes. These experiments showed that supernatants of activated mononuclear cells up-regulated the expression of MCP and CD59 but not DAF. TGF-β isoforms, TGF-β1, TGF-β2, and TGF-β3, also increase the membrane expression of MCP and CD59, without affecting the expression of DAF. None of the other investigated cytokines, IL-1α, IL-2, IL-6, TNF-α, and IFN-γ, was able to induce changes in the expression of the three tested complement regulatory proteins. Experiments with supernatants of activated mononuclear cells using monoclonal antibodies to TGF-β showed that, apart from TGF-β isoforms, another unidentified factor(s) can up-regulate MCP and DAF in keratinocytes. These results supported our hypothesis that protection of keratinocytes against complement should be increased under inflammatory conditions.

It was demonstrated in chapter 2 that several cytokines differentially up-regulate the production of C3 and factor B in keratinocytes. Exogenous stimuli, such as UVB exposure, have been shown to increase the production of several cytokines from keratinocytes. UVB exposure also induces infiltration of inflammatory cells in the skin which also release several cytokines. Cytokines released from these cells may be responsible for initiation of cutaneous inflammation in respose to UVB. We argued that UVB exposure of the skin may also increase the synthesis of complement components by keratinocytes. UVB could do this either directly or indirectly via some cytokines released from above mentioned cells. If synthesis of components of complement is really increased after UVB exposure as envisaged, increased levels of complement can damage keratinocytes. Therefore, a mechanism(s) must exist to protect keratinocytes from excessively produced complement in response to UVB. This mechanism could be the increased expression of complement regulatory proteins on keratinocyte membrane by UVB either directly or indirectly via some cytokines released from above mentioned cells in response to UVB. We investigated whether UVB exposure of cultured human keratinocytes can directly increase (1) the constitutive release of C3 and factor B and, (2) the surface expression of DAF, MCP and CD59. **Chapter 4** describes these *in vitro* studies. It was concluded that UVB can not directly up–regulate the synthesis of C3 and factor B *in vitro*, most likely due to excessive dilution of cytokines released from keratinocytes. The possibility of up-regulation of these components *in vivo* was not ruled out. UVB was shown to up-regulate the expression of complement regulatory proteins MCP, DAF, and CD59. Thus, UVB increases the resistance of keratinocytes against complement attack. This increase in resistance is needed by keratinocytes to protect themselves

from complement produced excessively in response to cytokines of inflammatory cells in UVB induced cutaneous inflammation. UVB exposure *in vivo* has been shown to cause local activation of complement on keratinocytes[359].

Keratinocytes have so far been shown to synthesize two complement components, namely C3 and factor B. Production of C3 and factor B suggests that keratinocytes may also be able to produce factor H and factor I to regulate the activity of these components. We investigated the synthesis of factor H by keratinocytes. The role of factor H in suppression of complement at C3/C5-convertase stage demands that, if produced by keratinocytes, its production be up-regulated when there is up-regulation of C3 and factor B production. C3 and factor B synthesis is up-regulated by some pro-inflammatory cytokines differentially as shown in chapter 2[298]. Thus, if synthesis of factor H occurs in keratinocytes, it should also be enhanced by some pro-inflammatory cytokines to suppress the activation of C3 and factor B and the resulting complement mediated damage of keratinocytes and other epidermal cells. In **Chapter 5** we have described our *in vitro* studies on the synthesis of complement factor H in keratinocytes. Keratinocytes were found to produce both the 45-kD and 155-kD isoforms of this protein. Parallel to our previous findings on the regulation of C3 and factor B (chapter 2), factor H production was also demonstrated to be extremely responsive to IFN-γ. Other cytokines, UVB, and LPS did not have any effect on the production of factor H. This study shows that keratinocytes are able to produce complement proteins other than C3 and factor B. More research is needed to investigate if keratinocytes, like other cell types[9;256], are capable of synthesizing other components of the complement cascade and their fluid phase regulators.

Inflammatory diseases of the skin are characterized not only by the presence of cytokines but also by the presence of inflammatory cells (*e.g.*, lymphocytes and neutrophils) in the skin.

Some of these cells bear ligands which can interact with their receptors on keratinocytes and activate them to produce inflammatory mediators. Not much is known about these ligations. One such ligation is the interaction of CD40 with its ligand, CD40L. Keratinocytes are known to express CD40 and activated T cells transiently express CD40L. CD40 activation of keratinocytes by CD40L is known to release IL-8 and up-regulate the pro-inflammatory molecule ICAM-1[147;350]. We investigated whether CD40 activation of keratinocytes by CD40L can release other chemokines (IL-8, RANTES, and MCP-1) and complement components (C3 and factor B) and alter the expression of complement regulatory proteins (MCP, DAF, and CD59) on keratinocytes. These studies are described in **Chapter 6.** Using two *in vitro* models of CD40 activation of human keratinocytes we were able to demonstrate that the production of IL-8 and RANTES was strongly up-regulated, whereas the production of MCP-1 was moderately increased. We also showed that CD40 activation of keratinocytes through CD40-CD40L ligation does not affect the release of C3 and factor B nor the expression of complement regulatory proteins MCP, DAF, and

CD59. We therefore conclude that strongly increased chemokine production in some inflammatory conditions may be regulated, in part, by interaction of keratinocytes with T cells through CD40 activation. Factors other than CD40 activation may be responsible for increased production of complement components and decreased expression of MCP and CD59 observed in psoriasis.

In summary, keratinocytes produce a number of inflammatory mediators including chemokines and complement proteins. Their production is tightly and differentially regulated by cytokines, UVB, and direct interaction of keratinocytes with inflammatory cells.

Chapter Eight

Samenvatting

Het in dit proefschrift beschreven onderzoek is verricht met het doel meer inzicht te krijgen in de rol die de keratinocyt speelt in ontstekingsreacties van de huid. Barker heeft gepostuleerd dat ontstekingsreacties in de huid geïnitieerd kunnen worden door keratinocyten[1]. De keratinocyt, het belangrijkste celtype in de epidermis, zou door diverse stimuli aangezet worden om specifieke pro-inflammatoire moleculen, zoals cytokinen, chemokinen, complement eiwitten en adhesie-moleculen te maken. Gevolg hiervan is activatie van dermale microvasculaire endotheliale cellen leidend tot een accumulatie van ontstekingscellen in de dermis en epidermis. Ultraviolet B (UVB) straling, contact allergenen, en thermale beschadiging zijn bekende exogene stimuli die een dergelijke ontstekingsgreactie kunnen veroorzaken door keratinocyten aan te zetten tot synthese van cytokinen. In veel ontstekingsziekten van de huid is de aard van de stimulus die tot ontsteking leidt onbekend, maar infiltrerende cellen, zoals monocyten/macrofagen, neutrofiele leukocyten en T cellen zouden epidermale keratinocyten kunnen beïnvloeden door endogene stimuli. Infiltrerende cellen zouden dit op twee manieren kunnen doen: (1) door afgifte van mediatoren, zoals cytokinen, die op hun beurt keratinocyten stimuleren om meer en andere mediatoren te gaan produceren zoals cytokinen, chemokinen en complement componenten en (2) door cel-cel interactie met keratinocyten via enkele liganden (bijvoorbeeld via CD40L op T cellen) en zo hen te activeren om meer ontstekingsmediatoren te gaan maken. Er is enige kennis beschikbaar over deze twee modellen met betrekking tot deelname van keratinocyten aan ontstekingsreacties in de huid, maar deze is nog allesbehalve volledig. De onderzoeken die we dit proefschrift presenteren zijn gericht op het verhelderen van de rol die gespeeld wordt door een aantal endogene en exogene stimuli op de productie, expressie en regulatie van complement eiwitten en een aantal chemokinen door keratinocyten.

Hoofdstuk 1 van dit proefschrift omvat een beknopte inleiding in de huidige kennis van de mechanismen die leiden tot ontstekingsreacties in de huid, gevolgd door een meer diepgaande beschrijving van het complement systeem en cytokine/chemokine netwerk. De beschrijving van het complement systeem omvat zijn twee activatie-routes en hoe deze activatie gereguleerd wordt door oplosbare en celmembraan-gebonden regulerende eiwitten. De beschrijving van cytokinen

omvat een korte introductie van een aantal daarvan, namelijk IL-1α, IL-6, TNF-α, IFN-γ, en TGF-β. Van deze cytokinen is bekend dat ze door mononucleaire cellen geproduceerd worden. Hun effecten op pro-inflammatoire eigenschappen van keratinocyten zijn beschreven in achtereenvolgende hoofdstukken van dit proefschrift. Een korte beschrijving van chemokinen is gegeven, waarbij speciale aandacht is besteed aan IL-8, RANTES, en MCP-1. In dit proefschrift is de regulatie van de synthese door keratinocyten van deze drie chemokinen onderzocht, omdat hun chemotactische eigenschappen relevant lijken voor de pathogenese van ontstekingsziekten van de huid. Dit hoofdstuk omvat ook een overzicht over de productie, expressie en functionele relevantie van complement eiwitten en chemokinen in keratinocyten, omdat veruit de meeste cellen in het epidermale compartiment van de huid keratinocyten zijn.

In **hoofdstuk 2** tot **6** beschrijven wij de resultaten van een aantal *in vitro* onderzoeken naar de regulatie van synthese door keratinocyten van oplosbare complement eiwitten en chemokinen en over de regulatie van de expressie van complement regulerende eiwitten op deze cellen.

Onderzoek tot nu toe heeft aangetoond dat humane keratinocyten twee complement componenten synthetiseren, namelijk C3 en factor B[34;35]. Aanvullend onderzoek zou kunnen aantonen dat zij ook andere componenten synthetiseren en het is zelfs denkbaar dat ze in staat blijken te zijn alle componenten van de klassieke en alternatieve route te produceren, zoals ook voor andere celtypes is aangetoond[9;256]. De synthese van C3 en factor B *in vitro* is onder niet-gestimuleerde omstandigheden tamelijk laag[34;35], wat suggereert dat ook in de normale huid de basale synthese van deze componenten laag zou kunnen zijn. Het vermogen van keratinocyten en inflammatoire cellen om in geval van ontsteking een breed scala aan cytokinen te produceren maakt het mogelijk dat een of meer van deze cytokinen een regulerende rol speelt in de synthese van C3 en factor B door keratinocyten. We hebben onderzocht of cytokinen, die geproduceerd kunnen worden door geactiveerde mononucleaire cellen en ten dele ook door keratinocyten, betrokken zijn in regulatie van de synthese van C3 en factor B door keratinocyten. De gegevens die wij presenteren in **hoofdstuk 2** laten duidelijk zien dat een aantal van deze cytokinen een sterke, maar gedifferentieerde, rol speelt in de regulatie van de synthese van C3 en factor B door keratinocyten *in vitro*. IFN-γ, en in mindere mate IL-1α, bleken in staat te zijn de productie van zowel C3 als factor B door keratinocyten te stimuleren, terwijl TNF-α de productie van C3 stimuleerde zonder de productie van factor B te veranderen. IL-6 stimuleerde uitsluitend de productie van factor B. De regulerende effecten van deze cytokinen zijn gevonden op zowel eiwit- als op mRNA-niveau. Al met al tonen deze resultaten duidelijk aan dat cytokinen een belangrijke rol spelen in het op een gedifferentieerde manier reguleren van de productie van complement eiwitten door humane keratinocyten.

Complement activatie producten, zoals C3b, kunnen geen verschil maken tussen

binnendringende microben en lichaamseigen cellen, zodat autologe epidermale cellen mogelijk beschadigd kunnen worden door de grote hoeveelheden complement componenten die cytokine-gestimuleerde keratinocyten produceren, zoals beschreven in hoofdstuk 2. Beschadiging van lichaamseigen cellen door geactiveerd complement wordt verhinderd door complement regulerende eiwitten, zoals MCP, DAF en CD59. Deze membraan gebonden eiwitten inactiveren complement activatie producten die op de celmembraan terecht komen. Ook keratinocyten en andere epidermale cellen moeten hen op hun oppervlak tot expressie brengen. In omstandigheden waarin sprake is van ontsteking van de huid en waar een toename is van complement synthese door keratinocyten onder invloed van cytokinen (zie hoofdstuk 2), zou ook een toename in expressie van complement regulerende eiwitten op keratinocyten verwacht kunnen worden teneinde hen te beschermen tegen overmatig geproduceerd complement. Wij hebben onderzocht of cytokinen die afgegeven worden door geactiveerde mononucleaire cellen, en veelal ook door keratinocyten, de expressie van membraan gebonden complement regulerende eiwitten doen toenemen. **Hoofdstuk 3** beschrijft flow cytometry experimenten met de expressie van membraan gebonden complement regulerende eiwitten op keratinocyten en de effecten van cytokinen op deze expressie. Onderzocht zijn een mengsel van cytokinen, zoals dat wordt afgegeven door geactiveerde mononucleaire cellen, en een aantal individuele cytokinen waarvan bekend is dat ze geproduceerd worden door ontstekingscellen en keratinocyten. Deze experimenten lieten een toename zijn in de expressie van MCP en CD59 door supernatant van geactiveerde mononucleaire cellen, zonder dat de expressie van DAF veranderde. Ook onder invloed van de TGF-β isovormen TGF-β1, TGF-β2, en TGF-β3 nam de expressie van MCP en CD59 toe, zonder dat de expressie van DAF veranderde. Geen van de andere onderzochte cytokinen, IL-1α, IL-2, IL-6, TNF-α, en IFN-γ, was in staat om de expressie van de drie onderzochte complement regulerende eiwitten te veranderen. Proeven met supernatanten van geactiveerde mononucleaire cellen waarbij monoklonale antilichamen tegen TGF-β gebruikt zijn toonden dat, naast TGF-β isovormen, ook een of meer ander niet geïdentificeerde factoren in staat zijn de expressie van MCP en DAF op keratinocyten te verhogen. Deze resultaten ondersteunden onze hypothese dat de weerstand van keratinocyten tegen complement onder inflammatoire omstandigheden versterkt zal zijn.

In hoofdstuk 2 zagen we een door cytokine geïndiceerde gedifferentieerde toename van productie van C3 en factor B door keratinocyten. Ook exogene stimuli, zoals blootstelling aan UVB, hebben een toename in de productie van een aantal cytokinen door keratinocyten laten zien. Blootstelling aan UVB induceert ook een infiltratie van eveneens cytokine producerende ontstekingscellen naar de huid. Deze cytokine afgifte zou verantwoordelijk kunnen zijn voor door UVB geïnduceerde ontstekingsreactie van de huid. We veronderstelden dat blootstelling van de huid aan UVB ook een toename zou kunnen veroorzaken van de productie van complement

componenten door keratinocyten. Blootstelling aan UVB zou dit zowel rechtstreeks als indirect kunnen doen, indirect via cytokinen uitgescheiden door de bovengenoemde ontstekingscellen. Indien, zoals we vermoedden, de synthese van complement componenten inderdaad toeneemt na blootstelling aan UVB, dan kunnen hogere spiegels van complement ook hier leiden tot schade aan de keratinocyten. Om die reden moet er een mechanisme bestaan dat keratinocyten beschermt tegen hoge spiegels van complement dat aanwezig is na blootstelling van de huid aan UVB. Dit mechanisme zou een door UVB veroorzaakte toename in expressie van complement regulerende eiwitten op de celmembraan van keratinocyten kunnen zijn. Blootstelling aan UVB zou dit zowel rechtstreeks als indirect kunnen doen parallel aan de eerder genoemde toename in productie van complement componenten. Indirect zou UVB dit kunnen doen via cytokinen uitgescheiden door de bovengenoemde ontstekingscellen. Wij onderzochten of blootstelling van gekweekte humane keratinocyten aan UVB leidt tot een rechtstreekse toename van (1) de basale afgifte van C3 en factor B en (2) de expressie van DAF, MCP en CD59 op het celoppervlak. **Hoofdstuk 4** beschrijft deze *in vitro* onderzoeken. We concluderen dat UVB niet leidt tot een rechtstreekse toename van de synthese van C3 en factor B *in vitro*, waarschijnlijk door de zeer sterke verdunning van de (stimulerende) cytokinen die UVB gestimuleerde keratinocyten produceren in het kweekmedium. Het blijft wel mogelijk dat blootstelling van keratinocyten aan UVB *in vivo* leidt tot een toename van de synthese van C3 en factor B. Blootstelling aan UVB leidde wel rechtstreeks tot een toename van de expressie van complement regulerende eiwitten MCP, DAF, en CD59 en dus tot een hogere weerstand van keratinocyten tegen aantasting door complement. Deze toename in weerstand is noodzakelijk om keratinocyten te beschermen tegen complement dat ze in grote hoeveelheden gemaakt hebben in reactie op door ontstekingscellen afgegeven cytokinen in een door UVB geïnduceerde onstekingsreactie van de huid. Blootstelling van de huid aan UVB *in vivo* heeft laten zien dat er lokale complementactivatie op de keratinocyten optreedt[359].

Tot nu toe is aangetoond dat keratinocyten twee complementeiwitten produceren, namelijk C3 en factor B. Productie van C3 en factor B suggereert dat keratinocyten mogelijk ook in staat zijn om factor H en factor I te maken om de activiteit van C3 en factor B te reguleren. We onderzochten of keratinocyten factor H synthetiseren. Als factor H inderdaad door keratinocyten gemaakt wordt, dan is het door de rol die factor H speelt in onderdrukking van complement op niveau van C3/C5-convertase noodzakelijk dat de productie toeneemt bij een toename in productie van C3 en factor B. Zoals hoofdstuk 2 laat zien neemt de synthese van C3 en factor B toe door een aantal pro-inflammatoire cytokinen[298]. Als keratinocyten factor H maken, dan zou de synthese van factor H ook moeten toenemen door een aantal pro-inflammatoire cytokinen. Dit om het risico op schade aan keratinocyten en andere epidermale cellen voortvloeiend uit toegenomen activatie van C3 en factor B te voorkomen. In **hoofdstuk 5** worden *in vitro*

onderzoeken beschreven met betrekking tot de synthese van complement factor H door keratinocyten. Keratinocyten bleken zowel de 45-kD als de 155-kD isovorm van dit eiwit te produceren. Parallel aan onze voorgaande bevindingen over de regulatie van C3 en factor B (hoofdstuk 2), bleek ook de productie van factor H productie zeer gevoelig voor stimulatie met IFN-γ. Andere cytokinen, UVB en LPS hadden geen enkele invloed op de productie van factor H. Dit onderzoek liet zien dat keratinocyten in staat zijn om meer complement eiwitten dan alleen C3 en factor B te maken. Er is meer onderzoek nodig om na te gaan of keratinocyten, net als een aantal andere celtypen[9;256], in staat zijn om nog meer componenten van de complement cascade en oplosbare regulator eiwitten te synthetiseren.

Ontstekingsziekten van de huid worden niet alleen gekenmerkt door de aanwezigheid van cytokinen, maar ook door de aanwezigheid van ontstekingscellen (zoals lymfocyten en neutrofiele leukocyten) in de huid. Een aantal van deze cellen heeft liganden die inwerken op hun receptoren op keratinocyten en hen zo aanzetten om ontstekingsmediatoren te maken. Er is weinig bekend over deze ligaties. Een voorbeeld van de ligaties die kunnen optreden is de interactie van CD40 met zijn ligand, CD40L. Van keratinocyten is bekend dat zij CD40 tot expressie brengen en van geactiveerde T cellen weten we dat ze kortdurend CD40L tot expressie brengen. Ook is bekend dat CD40 activatie van keratinocyten door CD40L leidt tot afgifte van IL-8 en een hogere expressie van het pro-inflammatoire molecuul ICAM-1[147;350]. Wij hebben onderzocht of CD40 activatie van keratinocyten door CD40L leidt tot afgifte van andere chemokinen (IL-8, RANTES, en MCP-1) en complement componenten (C3 en factor B) door keratinocyten en een verandering in hun expressie van complement regulerende eiwitten (MCP, DAF, en CD59). Deze onderzoeken zijn beschreven in **hoofdstuk 6.** Met behulp van twee *in vitro* modellen van CD40 activatie van humane keratinocyten waren we in staat om aan te tonen dat de productie van IL-8 en RANTES sterk toenam, terwijl de productie van MCP-1 slechts matig werd verhoogd. Ook hebben we laten zien dat CD40 activatie van keratinocyten door CD40-CD40L ligatie geen verandering veroorzaakt in de afgifte van C3 en factor B en de expressie van complement regulerende eiwitten MCP, DAF, en CD59. We concluderen daarom dat de sterk toegenomen productie van chemokinen in een aantal ontstekingsreacties, ten dele, gereguleerd zou kunnen zijn door interactie van keratinocyten met T cellen via CD40 activatie. Andere factoren dan CD40 activatie zouden verantwoordelijk kunnen zijn voor de toegenomen productie van complement componenten en de afgenomen expressie van MCP en CD59 zoals gezien worden in de ontstekingsreactie zoals bijvoorbeeld bij psoriasis.

Samenvattend, keratinocyten produceren een aantal ontstekingsmediatoren, waaronder chemokinen en complement eiwitten. De productie hiervan wordt strak en gedifferentieerd gereguleerd door cytokinen, UVB en directe interactie van keratinocyten met ontstekingscellen.

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Dankwoord

Bij deze bedank ik iedereen die direct of indirect heeft bijgedragen aan de totstandkoming van dit proefschrift. Meer in het bijzonder wil ik bedanken:

Dr. S.S. Asghar, mijn co-promotor, dank ik voor zijn vertrouwen en soepele begeleiding. Met zijn kennis, inzicht en vasthoudendheid heeft hij er voor gezorgd dat de ingeslagen weg niet werd verlaten. Mijn promotor, Prof. dr. J.D. Bos, wist met zijn overzicht over de gehele immunologie van de huid het onderzoek binnen een dermatologisch relevante context te houden.

De meeste experimenten zijn verricht in het Neurozintuig Laboratorium en later in het daar deel van uitmakende Laboratorium voor Experimentele Dermatologie. Ik wil het hoofd van het Neurozintuig Laboratorium, dr. F. Baas, bedanken voor de prettige samenwerking. Mia van Meegen wil ik bedanken voor het geduld waarmee ze inging op mijn vragen, ook al was ze druk met de vele andere taken van hoofd-analiste. Het PCR-werk dat is verricht, is met name te danken aan de volharding van Norbert van den Bosch. In de twee jaar dat hij als analist met mij samenwerkte heeft hij werk tot een goed einde weten te brengen dat door velen onmogelijk werd geacht. Abdel Ararou heeft als HLO-stagiair op een enthousiaste manier meegewerkt aan het werk dat is gepresenteerd in hoofdstuk 5. Ook aan de samenwerking met de andere medewerkers van het "Dermat-lab" heb ik goede herinneringen. Regien, Cock, Susan en Evert bedankt voor jullie hulp. De wetenschappelijke en niet-wetenschappelijke discussies die ik heb mogen voeren met Marcel Teunissen en Sergio di Nuzzo hebben mijn tijd op K2 veraangenaamd. Mijn kamergenoot Arthur noem ik vanwege zijn geduld en begrip als ik weer eens onaangekondigd een hele middag achter onze computer moest zitten.

Verder bedank ik de mensen van de afdelingen celbiologie, experimentele hematologie, experimentele inwendige geneeskunde en het SKILL. Jan, René, Ludo, Johan, Frank, Rob, Fannie en al die anderen, bedankt voor het meedenken, de goede adviezen en bereidheid om te helpen met reagentia. Joost Schuitemaker, jouw inzet wil ik hier roemen omdat je altijd weer op tijd J558 cellen voor mijn experimenten klaar had staan.

Ook mensen van buiten het AMC hebben een bijdrage geleverd aan mijn werk. De grote kennis van het complementsysteem en het 'Fingerspitzengefühl' van Prof. dr. M.R. Daha (LUMC) hebben ertoe geleid dat op de onvermijdelijke momenten van stagnatie de juiste keuzes zijn gemaakt. Hoewel de interessante resultaten van de samenwerking met dr. Jon Laman en Marjan van Meurs van de Afdeling Immunologie van het Dijkzigt Ziekenhuis in Rotterdam nog niet gepresenteerd kunnen worden in dit proefschrift, bedank ik hen toch voor de verhelderende discussies.

Tenslotte bedank ik Helle, Eline en Jeroen. Helle, bedankt dat je altijd achter mijn keuzes bent blijven staan. Eline en Jeroen, bedankt voor de paar nachten dat jullie hebben doorgeslapen en mij zo weer voldoende energie gegeven hebben om dit boekje af te ronden.

Curriculum vitae

Marcel Pasch werd geboren op 24 december 1966 te Solingen, Duitsland. Een jaar later verhuisde het gezin Pasch naar Nederland. Marcel haalde in 1985 zijn VWO diploma aan het Isala College in Silvolde. In dat jaar begon hij met de studie Geneeskunde aan de Katholieke Universiteit Nijmegen. Na het behalen van het doctoraal-examen in 1989 verrichtte hij korte tijd onderzoek op de afdeling Cardiologie van het Canisius-Wilhelmina Ziekenhuis in Nijmegen. Op deze afdeling vond hij ook zijn eerste baan, na het behalen van het arts-examen in februari 1992. Een half jaar later kreeg hij de gelegenheid over te stappen naar het Catharina Ziekenhuis in Eindhoven om als arts-assistent te werken in een cardiologische opleidingskliniek (opleider: Dr. M.I.H. El Gamal). De mogelijkheid werd geboden om de specialisatie tot cardioloog te volgen, maar voor de aanvang van zijn vooropleiding interne geneeskunde besloot hij de cardiologische richting niet te vervolgen; het zou niet bij hem passen. Hierop volgde een baan als bedrijfsarts bij de Arbo Unie West-Brabant, een functie waarin onvoldoende uitdagingen aanwezig bleken. Het besluit om terug te keren naar de klinische geneeskunde en met name naar de dermatologie lag voor de hand, omdat dit specialisme door het grote aantal ziektebeelden altijd uitdagingen zal blijven bieden. Voordat hij met zijn opleiding tot dermatoloog kon beginnen zocht hij zijn uitdagingen in het wetenschappelijk onderzoek, waarvan de resultaten zijn gepresenteerd in dit proefschrift. Na een inwerkperiode van drie maanden in het Laboratorium voor Experimentele Endocrinologie van het Academisch Ziekenhuis Nijmegen (destijds onder leiding van Prof. dr. Th.J. Benraad) vertrok hij voor zes maanden naar de Verenigde Staten. In het laboratorium van dr. P.J. Jensen, op de afdeling dermatologie van de University of Pennsylvania in Philadelphia, deed hij onderzoek naar de plasminogeen cascade. De vaardigheden opgedaan in Nijmegen en Philadelphia bleken dermate goed aan te sluiten bij de vereiste vaardigheden voor het hier gepresenteerde onderzoek dat zijn huidige co-promotor, Dr. S.S. Asghar, hem een onderzoeksbaan aanbood in het Laboratorium voor Experimentele Dermatologie (onderdeel van het Neurozintuigen Laboratorium) in het Academisch Medisch Centrum. Op dit moment is hij werkzaam op de afdeling Dermatologie van dit ziekenhuis (hoofd: Prof. dr. J.D. Bos) waar hij per 1 oktober 1998 begonnen is aan zijn opleiding tot dermatoloog.