Detection, modulation and impact of skin pigmentation

Maritza A. Middelkamp Hup

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Aan mijn vader en moeder

Contents

Chapter 1

General introduction and aims of the thesis

List of abbreviations

Adrenocorticotrophic hormone (ACTH)
Allergic contact dermatitis (ACD)
Bisindolylmaleimide (BIS)
Commission Internationale de l'Eclairage (CIE)
Cyclobutane pyrimidine dimers (CPD)
Delayed tanning (DT)
Dihydroxyphenylalanine (DOPA)
Immediate pigment darkening (IPD)
Irritant contact dermatitis (ICD)
Melanocortin receptors (MCR)
Melanocortin 1 receptor (MC1-R)
α-melanocyte-stimulating hormone (α-MSH)
Narrow-band UVB (NB-UVB)
Persistent pigment darkening (PPD)
Polypodium leucotomos (PL)
Proopiomelanocortin (POMC)
Protein kinase A (PKA)
Protein kinase C-β (PKC-β)
Reactive oxygen species (ROS)
Reflectance confocal microscopy (RCM)
Tetrahydrobiopterin ($6BH_4$)
4a-OH-Tetrahydrobiopterin dehydratase (DHase)
Ultraviolet radiation (UVR)

What is pigmentation?

The word "pigmentation" is derived from the Latin word "pigmentum", which is defined as "any normal or abnormal coloring matter of the body" or "a paintlike medicinal preparation to be applied to the skin" (Anderson et al, 2003). Skin color is the combined result of 4 chromophores, namely melanin, carotene, oxyhemoglobin and deoxygenated hemoglobin. Of these four, melanin is the major constituent of skin color (Fitzpartick and Ortonne, 2003), and it is this type of pigmentation that will be the focus of this thesis.

Melanin is produced in highly organized organelles produced by specialised dendritic cells called melanocytes. Melanocytes are derived from the neural crest and enter the epidermis during the 12^{th} and 14^{th} week of embryogenesis (Rawles, 1947). In the epidermis, melanocytes reside in the basal layer, from where they extend their dendrites between basal and spinous keratinocytes. Every melanocyte forms a functional unit with 14-36 keratinocytes (depending on body location), the so called "epidermal melanin unit" (Fitzpatrick and Breathnach, 1963; Frenk and Schellhorn, 1969). The melanocyte can thus distribute the synthesized melanin via its dendrites to these corresponding neighbouring keratinocytes. Melanocytes are also present in hair follicles and are accountable for the color of hair. They are located in the basal layer surrounding the papilla of the hair bulb, were they transfer melanin into the cells derived from the hair matrix.

Besides their presence in the integumentary system, melanocytes can also be found in mucous membranes, the leptomeninges of the brain, the retinal pigment epithelium and uveal tract of the eye, and the stria vascularis and vestibular region of the inner ear. This is why certain pigment disorders concur with defects in some of these organ systems, such as the ocular abnormalities that can be found in albinism or the hearing impairment seen in the Waardenburg syndrome.

The following section will give an overview of melanogenesis, beginning with a description of the regulatory pathways in melanogenesis, to the description of the organelle in which this process takes place, the melanosome.

Melanogenesis

Melanogenesis is a multi-step process that is partly regulated at gene expression level, but it is predominantly controlled by posttranslational

mechanisms involving different enzymes and substrates (rev. in Slominski et al, 2004). The initial step in melanogenesis is the hydroxylation of tyrosine to dihydroxyphenylalanine (DOPA) by tyrosinase, which is considered the key and rate-limiting enzymatic step of melanogenesis.

The produced DOPA also acts as a co-factor for activation of tyrosinase, thus facilitating its own production via hydroxylation of tyrosine to DOPA. This has led to speculations on other mechanisms than the hydroxylation of tyrosine by tyrosinase, through which DOPA ultimately becomes available, as the initiating step in melanogenesis. In view of this, a recent theory proposed by Schallreuter and Wood (1999) states that the pterin-dependent conversion of phenylalanine to tyrosine by phenylalanine hydroxylase may be the most important initiating step in melanogenesis, as the main source of intra-melanocytic tyrosine is thought to be provided by the turnover of phenylalanine to tyrosine by phenylalanine hydroxylase, while direct availability of tyrosine through uptake by melanocytes is thought be of much lesser importance. For conversion of phenylalanine to tyrosine by phenylalanine hydroxylase the cofactor tetrahydrobiopterin ($6BH_4$) is needed. This hypothesis states that further conversion of tyrosine to DOPA would be initially catalysed by tyrosine hydroxylase, thereby supplying the necessary amount of DOPA to subsequently activate tyrosinase for melanogenesis (Marles et al, 2001). However, other studies could not find tyrosine hydroxylase in human melanocytes thereby negating this theory (Kagedal et al, 2004).

Once DOPA is formed, it is further oxidized by tyrosinase to form DOPAquinone. From this point on, the pathway will bifurcate to form either eumelanin, the dark colored brown-black melanin, or pheomelanin, which is the light colored red-yellow melanin (Prota, 1988). To form eumelanin, DOPAquinone is transformed to leukoDOPAchrome that will undergo a series of reactions to ultimately result in eumelanin. Pheomelanin is formed by conjugation of DOPAquinone to cysteine or glutathione that through other reactions will yield pheomelanin. A melanocyte can contain melanosomes that contain eumelanin as well as pheomelanin. However, within a melanosome there is a switch to either one or the other pathway, so both pathways cannot co-exist (Prota, 1988). It is believed that this switch is dependent on the activity of tyrosinase and the subsequent production of DOPAquinone which increases with stimulation of the melanocortin 1 receptor (MC1-R), combined with the availability of enzymes and substrates in the melanosome.

With high DOPAquinone levels, more eumelanin will be produced, while low DOPAquinone levels will lead to increased pheomelanin production (Ito, 1993; Oyehaug et al, 2002).

The regulation of melanogenesis

A major part of the regulation of melanogenesis is via the MC1-R (Mountjoy et al, 1992). The MC1-R is a seven transmembrane G-protein coupled receptor belonging to the group of melanocortin receptors (MCR) that are activated by proopiomelanocortin (POMC)-derived peptides. MC1-R on melanocyte membranes is stimulated by α-melanocyte-stimulating hormone (α-MSH) and adrenocorticotrophic hormone (ACTH), leading to signal transduction through activation of adenylyl cyclase. Besides by the pituitary, POMC is produced locally by skin keratinocytes, and it is this local production that appears to be the most important source of MC1-R stimulation in skin (Thody et al, 1983).

Stimulation of the MC1-R initiates one of the major pathways identified for regulation of melanogenesis. Binding of α-MSH or POMC to the MC1-R leads to activation of adenylyl cyclase that induces an increase of intracellular levels of the second messenger cAMP (rev. in Slominski et al, 2004; Busca and Ballotti, 2000). cAMP activates protein kinase A (PKA), which then phosphorylates regulatory serine residues on melanogenesis related enzymes such as phenylalanine hydroxylase and tyrosine hydroxylase. More importantly, cAMP activated PKA influences transcription of melanogenesis related proteins by upregulation of synthesis of tyrosinase, tyrosinase-related proteins I and II, as well as protein kinase C-β (PKC-β) (Park et al, 2006).

This brings us to a second major melanogenesis pathway, in which PKC-β plays a major role. PKC-β is an isoform of PKC that belongs to the group of classical or conventional PKC. The other two PKC groups of the PKC family are atypical PKC and novel PKC. Grouping of PKC isoforms is performed according to the co-factors that are needed for activation. Within each PKC group different isoforms can be identified, and these are differentially expressed in various tissues. PKC-β expression occurs in tissues such as brain, liver, spleen, heart, and skin among other tissues. In skin, the expression of PKC-β is restricted to melanocytes and has been shown to play a major role in the melanogenesis cascade (rev. in Park and Gilchrest, 1993). In response to extracellular stimuli such as ligand-receptor interaction, phospholipase C

is activated and cleaves inositol phospholipids into diacylglycerol and inositol triphosphate. Due to inositol triphosphate, the concentration of calcium in the cytoplasm is increased by its release from the endoplasmatic reticulum. PKC-β resides in an inactive form in the cytoplasm and is activated when diacylglycerol is released from membrane inositol phospholipids in the presence of elevated intracellular calcium levels and binds to the regulatory domain of PKC-β (Park et al, 1999). Once PKC-β is activated, its most important effect on melanogenesis is the phosphorylation of two serine residues on the cytoplasmic tail of the melanosome bound tyrosinase, causing activation of tyrosinase and hence increasing pigmentation (Park et al, 1999). In fact, topical application of diacylglycerols known to activate PKC have shown to increase skin pigmentation in the guinea pig animal model (Allan et al, 1995). In murine melanoma cells, PKC-β has also shown to upregulate tyrosinase mRNA and protein (Park et al, 1996). The cAMP and PKC-β pathways are intertwined, as cAMP mediates expression of PKC-β in melanocytes (Park et al, 2006) and α-MSH-induced pigmentation known to be effectuated via the cAMP pathway is abolished by depletion of PKC-β (Park et al, 1996). Other melanogenesis pathways such as activation by nitric oxide and the pathway involving phosphatidylinositol 3-kinase have also been described (rev. in Slominski et al, 2004).

Understanding of the melanogenesis pathways leads to interesting possibilities to influence pigmentation. The fact that expression of PKC-β in skin is restricted to melanocytes makes this a promising target for selective pigment modulation, as will be demonstrated in this thesis.

Melanosomes

As previously mentioned, melanosomes are highly specialised organelles in which melanin is formed. A melanosome is a membrane bound organelle formed by outpouching of a smooth membrane from the rough endoplasmatic reticulum at which stage they are called pre-melanosomes (Seiji et al, 1963). The enzymes needed for melanogenesis are then transferred to the melanosome either via delivery by coated vesicles originating from the endoplasmatic reticulum and Golgi complex (Jimbow et al, 2000), or by fusion of the pre-melanosome with late endosomes containing the enzymes (Kushimoto et al, 2001). Melanosomes contain all enzymes and proteins needed for melanogenesis, as well as the necessary elements to provide the adequate

environment for melanogenesis such as the right pH and osmotic pressure and the possibility of transfer of substrate to melanosomes. Four stages of melanosome maturation are identified, during which melanin formation is initialised and completed. In stage I melanosomes (pre-melanosomes) the matrix needed for melanin formation is present but unorganised. In stage II melanosomes, the matrix is structuralized in striations acting as templates for melanin formation, which commences in stage III melanosomes. In stage IV melanosomes, melanin formation and tyrosinase activity is concluded as melanosomes are completely filled with melanin. Melanosomes are relocated from the perinuclear region to the dendritic tips for transfer to keratinocytes by a system of microtubules and protein motors that remain to be further identified (Wu et al, 1998; Hara et al, 2000). The transfer of melanosomes to keratinocytes is also still not completely unravelled. Three possible methods have been proposed that include i) direct fusion of melanocytes and keratinocytes membranes allowing transfer of melanosomes, ii) phagocytosis of the melanocyte dendritic tip by keratinocytes, and iii) secretion of the content of melanosomes into the intercellular space followed by its endocytosis by keratinocytes (Yamamoto and Bhawan, 1994).

Differences in skin color as seen in different ethnic groups has its origin in the number, distribution and size of the melanosome, as there are no differences between number of melanocytes between people of different skin color (Richards et al, 2003). In people with black skin, melanosomes are more numerous, larger in size, and are distributed as single units within keratinocytes. In contrast, in white skinned individuals, melanosomes are less in number, smaller in size and are grouped together in lysosomes. Black skin also contains more fully melanized stage IV melanosomes and melanosomes can be found throughout the layers of the epidermis, while white skin contains more stage II-III melanosomes that are located more in the basal layers of skin (Gates and Zimmermann, 1953).

Ultraviolet radiation-induced pigmentation

Ultraviolet radiation (UVR) is the most important stimulator of melanogenesis under normal conditions. In the continuous spectrum of electromagnetic radiation, UVR is the radiation between wavelengths 100-400 nm. Within

these wavelengths, a distinction has been made into UVC, UVB and UVA. According to the official subdivision of UVR defined by the Commission Internationale de l'Eclairage (CIE) UVA are wavelengths between 315-400 nm, UVB is 280-315 nm, and UVC is 100-280 nm. However, for the purpose of medical photobiology, a more convenient but slightly different subdivision is applied (Kochevar and Taylor, 2003). The upper limit of UVC is set at 290 nm, as wavelengths lower than 290 nm are filtered out by the ozone layer and are therefore not present in terrestrial sunlight. The remaining UVR wavelengths (290-400 nm) comprise about 5% of radiation of terrestrial sunlight. UVB covers wavelengths between 290 and 320 nm and comprises about 2-5% of the UVR in sunlight. UVB wavelengths are the most erythemogenic with a peak around 300 nm (Young et al, 1998), and are mainly accountable for sunburn resulting from sunlight exposure. UVA wavelengths are those between 320-400 nm, and comprise 95-98% of UVR in sunlight (Kochevar and Taylor, 2003).

The principle through which UVR induces biological responses, such as sunburn, pigment induction, skin carcinogenesis, and photoaging is mediated by absorption of UVR photons by skin chromophores. This absorption can lead to the formation of photoproducts, which are cellular structures that undergo a chemical change after direct absorption of the photon by the chromophore. Photoproducts can also result when absorption of the photon by the chromophore leads to interaction between that chromophore and neighbouring cellular structures resulting in new structures (the photoproduct). Another possibility is that the excited chromophores transfer their energy to oxygen molecules to form reactive oxygen species (ROS), which then react with neighbouring structures to become photoproducts.

DNA is one of the most important chromophores for UVR in human epidermis, and UVR exposure of DNA results in the formation of photoproducts (Young, 1997). The most commonly induced photoproducts by direct UVR absorption, comprising about 75%-80% of total DNA damage, are cyclobutane pyrimidine dimers (CPD), particularly thymine dimers. Pyrimidine (6-4) pyrimidone photoproducts are the second most commonly induced photoproducts (Setlow and Carrier, 1966). A photoproduct emerging from reaction with ROS is 8-hydroxydeoxyguanosine, which is considered a marker for oxidative stress (Ichihashi et al, 2003).

UVR-induced photoproducts can initiate biological responses, which broadly can be divided in acute UVR effects such as erythema, tanning and

local immunosuppression, and chronic UVR effects such as carcinogenesis and photo-aging. After exposure to UVR, an inflammatory response of skin becomes visible as erythema or sunburn due to vasodilatation of blood vessels. It has been postulated that DNA is the chromophore for erythema induced by the shorter UVR wavelengths as the action spectra for thymine dimer formation and erythema is similar (Young et al, 1998). The DNA damage of epidermal cells would result in a cascade leading to release of mediators and cytokines activating the erythema response, or the DNA damage would be situated in the endothelial cells themselves resulting in vasodilatation. In the longer UVR wavelengths at least one other, yet unknown, chromophore besides DNA could play a role in the sunburn reaction, which might be mediated via ROS (Young et al, 1998).

Pigmentation (tanning response) is another acute skin response occurring in response to UVR exposure. Immediately after UVR exposure, a darkening of skin is visible due to exposure to UVA. This is called the immediate pigment darkening (IPD) response, which is thought to be the result of photo-oxidation of pre-existing melanin in melanocytes (Beitner and Wennersten, 1985). The persistent pigment darkening (PPD) response is, by definition, the immediate pigment darkening response that is still present 2 hours after exposure, and it is used as a marker for determination of the protective factor of UVA sunscreening chemicals (Moyal et al, 2000). These tanning responses are transitory and gradually disappear within a few hours to days after the exposure, depending on the UVR dose and the person's skin color. The biologic function of this tanning response is not known. IPD and PPD have been shown not to be photoprotective (Black et al, 1985), most likely because no increased melanosome transfer occurs (Honigsmann et al, 1986).

About 3-4 days after the exposure, a longer lasting tanning response emerges, which is the delayed tanning (DT) response. In most individuals DT occurs when a UVR-induced erythema reaction, or sunburn, had been present before, as the action spectrum of erythema induction is broadly similar to that of tanning (Parrish et al, 1982). Persons with dark skin having excellent tanning capabilities can tan with doses that are suberythemogenic. DT is the result of true melanogenesis, coinciding with increases in melanocyte activity (single exposure) and melanocyte numbers (repeated exposures) (Gilchrest et al, 1996). Also an increase in melanocyte dendricity, melanosome number and transfer to epidermal keratinocytes is observed. This response has a peak

at 10 days to 3-4 weeks, depending on the UVR dose and the person's skin color, gradually fading thereafter in the absence of more sunlight exposure. The mechanisms through which UVB and UVA induce tanning are most likely different. When exposed to UVA, DT occurs earlier, sometimes overlapping with the PPD response and usually lasts longer (Gilchrest et al, 1996). The induced pigment is mostly confined to the basal layer for the longer UVA wavelengths, while the shorter UVA wavelengths produce effects that resemble those induced by UVB. Exposures to UVB result in melanin that is also widely distributed in the subrabasal keratinocytes. UVR exposure also results in epidermal hyperplasia and stratum corneum thickening (Lavker et al, 1995).

The mechanisms involved in UVR-induced pigmentation are a matter of much interest as insight in these steps gives opportunities to influence and hence modulate UVR-induced pigmentation. UVR-induced pigmentation is the result of direct effects on melanocytes combined with paracrine secretion of factors by keratinocytes and possibly other skin cells. UVR was shown to increase the cellular content of diacylglycerol in a dose-dependent manner (possibly via changes in the diacylglycerol-kinase, which is an enzyme that converts diacylglycerol in phosphatidic acid) (Punnonen and Yuspa, 1992). It has also been shown that addition of an analogue of diacylglycerol increased basal and UVR-induced melanogenesis in human melanocytes and S91 melanoma cells (Friedmann et al, 1990). As explained above, diacylglycerol is involved in induction of melanogenesis via the PKC-β pathway by activation of tyrosinase. UVR has also been shown to increase the levels PKC-β mRNA within 1 hour after irradiation (Park and Gilchrest, 1996). These data suggest that part of UVR-induced melanogenesis is mediated via the PKC-β pathway. This allows interesting possibilities to modulate UVR-induced pigmentation via the PKC-pathway, as will be demonstrated in this thesis.

DNA, a major UVR chromophore, also plays an important role in UVR-induced pigmentation as has been demonstrated by the group of Gilchrest et al. The hypothesis is based on the SOS response observed in bacteria. When bacteria are exposed to a sublethal dose of UVR, the damaged DNA sequence containing the UVR-induced photoproduct is excised from the DNA strand. This single-stranded DNA photoproduct then interacts with a protease that activates genes involved in DNA repair, replication and cell survival, thereby increasing cell survival and enhancing its protective

capabilities against further UVR damage. The human pigmentation response could be thought of as a similar SOS response of skin. Exposure to UVR leads to DNA photoproducts, which after excision have been hypothesized to increase melanogenesis. As melanin is photoprotective by its ability to absorb UVR and ROS, the pigmentation response also enhances the protective capabilities of skin against further UVR damage. This theory is supported by experiments showing that incubation of S91 murine melanoma cells or human melanocytes with thymidine dinucleotides, the most common UVR-induced DNA photoproduct, increased melanin concentration in a similar way as could be observed after UVR exposure. Also, topical application of thymidine dinucleotides to pigmented guinea pig skin lead to increased skin pigmentation that was photoprotective against subsequent UVR exposure (Eller et al, 1994; rev. in Gilchrest and Eller, 1999; Gilchrest et al, 1996). Apart from being a goal in itself, inhibition of DNA damage may therefore also be targeted for pigment modulation.

Fitzpatrick skin phototypes

The Fitzpatrick skin phototypes are based on an individual's propensity to burn after 30 minutes of unprotected sun exposure in peak season and the ability to develop a tanning response, as the phenotype characteristics such as skin color, hair color and the color of the eyes are unreliable parameters to predict these UVR-induced skin responses (Fitzpatrick, 1975; Fitzpatrick and Ortonne, 2003). Based on these characteristics, 6 skin phototypes are defined, ranging from type I (burns easily and does not tan), to type VI (tans easily and does not burn). Thus, the major determinant of skin phototypes is not determined by the individual's looks, but the burning and tanning response. That is why the spectrum of skin phototypes represents a spectrum of skin pigmentation. The skin phototypes were initially mainly used to estimate doses to be administered in PUVA (psoralens + UVA) photochemotherapy. Nowadays, the skin phototypes are also widely used to categorize subjects or patients in experimental studies.

Other effects of UVR on skin

Besides pigmentation, UVR induces other effects on skin such as immune suppression, photoaging, and skin cancer. Of these effects, immune suppression and skin cancer are the two acute and long-term effects with

the most impact. The skin immune system and the development of skin cancer are interrelated, as can be inferred from the increase in skin cancer in immunosuppressed individuals. It is well known that exposure of skin to UVR leads to alteration of density and morphology of epidermal Langerhans cells (Seite et al, 2003). One study has shown that after exposure to 2x MED the human epidermis was completely devoided of Langerhans cells 72 hours after exposure (Novakovic et al, 2001). It is believed that Langerhans cell depletion plays a role in the development of skin cancer. Langerhans cells have been shown to be necessary for tumor specific immunity against UVR-induced tumors (Cavanagh et al, 1996) and their presence has shown to be vital for tumor rejection (Grabbe et al, 1991). There are 3 possible mechanisms through which UVR induces immunosuppression of skin. UVR induces DNA damage, isomerization of *trans*-urocanic acid to *cis*-urocanic acid, and cell membrane damage such as lipid peroxidation. These events are thought to lead to a release of diverse immune mediators in skin, which lead to changes in Langerhans cells number and function. This leads to induction of regulatory T cells and a decrease in Th1 cytokine profile, which results in reduced immunity (rev. in Norval, 2006), thereby facilitating UVR-induced carcinogenesis.

The role of oxidative stress in pigmentation

Oxidative stress is omnipresent in the human body and plays an essential role in the control and regulation of several cellular systems. Oxidative stress in skin can also play a role in the induction of pigmentation and most likely also in loss of pigmentation.

Based on the melanogenesis pathways described above, pigmentation may therefore be influenced via manipulation of oxidative stress. Oxidative stress has been shown to directly activate a form of phospholipase C, leading to cleavage of membrane phospholipids to produce increased levels of diacylglycerol (Wang et al, 2001). Besides diacylglycerol and calcium dependent activation of PKC, PKC itself can also be directly activated via oxidative stress (Gopalakrishna and Jaken, 2000). It was shown that under mild oxidative conditions there was a modification of PKC leading to increased activity that was phospholipid and calcium independent (Gopalakrishna and Anderson, 1989). Another study showed that oxidative stress leads to rapid activation of

PKC, resulting in phosphorylation of PKC substrate that was inhibited by a PKC inhibitor (Brawn et al, 1995). Later it was suggested that the activation of PKC by oxidative stress was the result of phosphorylation of tyrosine in the catalytic domain of PKC, possibly through activation of tyrosine kinases by oxidative stress (Konishi et al, 1997). In this regard, it has been shown that exposure to PUVA, a major melanogenesis stimulator, increases the amount of lipid peroxidation and diacylglycerol (Punnonen et al, 1991), suggesting that PUVA-induced pigmentation is at least partly mediated by oxidative stress. PUVA is also known to induce ROS and free radicals, which can also influence pigmentation as explained above.

As stated before, DNA damage plays an important role in pigment induction. Besides DNA damage occurring via direct absorption of UVR photons, DNA photoproducts can also result from oxidative stress (Ichihashi et al, 2003; Ravanat et al, 2001). This type of damage is usually attributed to UVA, as UVA damage is mediated via production of ROS. Among other photoproducts, the typical ROS-induced DNA photoproduct is 8-hydroxydeoxyguanosine. It is not known if ROS-induced photoproducts have an effect on melanogenesis, as described for thymine dimers. However, DNA repair enzymes have been shown to be susceptible to ROS-induced damage (Doshi and Preston, 1990), so manipulation of oxidative stress may indirectly influence pigmentation via this route. Also, it has been shown that UVA can induce pyrimidine dimers in human skin (Freeman et al, 1987). Whether this is via direct absorption or mediated through an endogenous sensitizer is not known (Freeman et al, 1987).

It is also interesting to mention that UVA-induced oxidative stress can be strongly enhanced by pheomelanin and/or intermediates of melanin synthesis, which become photosensitized leading to increased DNA damage (Wenczl et al, 1998). In addition, pheomelanin formation requires the use of cysteine, which plays an important role in protection against oxidative stress (Van Nieuwpoort et al, 2004). Therefore, increased pheomelanin and/or intermediates of melanin synthesis formation can contribute to an increase in oxidative stress. On the other hand, it has been shown that the presence of (eu)melanin decreased the anthralin-induced inflammatory response after topical application, which was believed to be due to the free radical scavenging effect of melanin (Westerhof et al, 1989). Consequently, pigmentation can be influenced by oxidative stress, but oxidative stress can also be influenced by melanin types, and hence possibly by skin color.

Vitiligo vulgaris

A clinical example in which the role of oxidative stress on pigmentation loss has been extensively investigated is the pigmentary disorder vitiligo vulgaris. The etiology of vitiligo is not known, but it is believed to be a cell-mediated auto-immune disease, and/or to result from an increase in oxidative stress in skin. This last hypothesis states that oxidative stress in the form of increased hydrogen peroxide originating from several sources could be the etiopathological mechanism behind the depigmentation observed in vitiligo (rev. in Schallreuter et al, 2001). For conversion of phenylalanine to tyrosine by phenylalanine hydroxylase, the cofactor $6BH_4$ is needed. Patients with vitiligo have an impaired recycle mechanism of $6BH_4$, in which the enzyme 4a-OH-tetrahydrobiopterin dehydratase (DHase) plays a crucial role. In vitiligo, barely detectable levels of DHase have been found. It has been shown that hydrogen peroxide plays an important role in this process, as it can deactivate DHase. To date, there are several possible sources leading to increased hydrogen peroxide levels in vitiligo skin (Hasse et al, 2004), thereby leading to increased oxidative stress in vitiligo. Low activity of DHase can lead to accumulation of an 7-isomer $7BH_4$, which is a potent competitor of $6BH_4$, thereby affecting phenylalanine hydroxylase activities and leading to increased epidermal phenylalanine levels. The increased phenylalanine and $7BH_4$ levels, combined with decreased phenylalanine hydroxylase and DHase activities lead to a shortcut in the $6BH_4$ recycling process to further produce increased levels of hydrogen peroxide. Hydrogen peroxide can oxidize $6BH_4$ and $7BH_4$, resulting in 6-biopterin which is toxic to melanocytes. Restoration of the levels of hydrogen peroxide should therefore restore the level of increased oxidative stress in vitiligo skin. The treatment of vitiligo with a UVB-activated pseudocatalase is based on this hypothesis, as catalase is one of the enzymes responsible for the degradation of hydrogen peroxide (Schallreuter et al, 1999). Several other studies have also shown alterations in oxidative status in vitiligo blood and skin (Koca et al, 2004; Yildrim et al, 2004; Passi et al, 1998; Schallreuter et al, 1991) and increased sensitivity of melanocytes to oxidative stress (Maresca et al, 1997). Manipulation of oxidative stress in vitiligo may therefore lead to possibilities to modulate pigmentation.

Detection of pigmentation by in vivo reflectance confocal microscopy

Background on *in vivo* reflectance confocal microscopy

The concept of confocal microscopy was invented by Marvin Minsky in 1955 while working as a postdoctoral fellow at Harvard University (Minsky, 1988). However, it wasn't until the 1980's that the first publications about this microscope emerged, including several by Brakenhoff et al from the University of Amsterdam (Sheppard and Wilson, 1981; Brakenhoff et al, 1985).

The value of confocal microscopy lies in the possibility to create virtual optical sections of a three dimensional biological specimen, thereby allowing visualisation of one point or plane within a turbid object without physically cutting or destroying the specimen. In confocal microscopy, a light source illuminates a turbid object, by which part of the illuminating light will penetrate the object. Part of these photons will be absorbed by the object, but part of the light will be reflected and will exit the object again. It is on this reflectance that the confocal microscope is based, hence the name reflectance confocal microscopy (RCM). The reflected light is imaged by a detector. However, light will be reflected from different depths within the object. By placing a pinhole in front of the detector one can reject all reflecting light coming from planes that are not of interest, the out-of-focus planes, thereby allowing visualisation of only one point of interest within the specimen. The light source, illuminated spot and detector are all in **con**jugate **focal** planes, and are therefore confocal to each other (Rajadhyaksha et al, 1999b). In order to visualise more than just one spot, but a certain plane within the specimen (similar to a histological section), a scanner rapidly scans the light beam in horizontal and vertical direction, thereby visualising a field of adjacent illuminated spots, creating a horizontal field-of-view (Rajadhyaksha et al, 1999b; Rajadhyaksha et al, 1995). Figure 1 shows a schematic representation of the confocal microscope.

Different light sources have been used for RCM. The tandem scanning confocal microscope uses white light from a mercury lamp (Corcuff and Leveque, 1993). However, near-infrared laser light has the advantage of one specific wavelength, deeper penetration into specimens and better focusing of the light beam (Rajadhyaksha et al, 1999a). In 1995, a confocal scanning laser microscope was developed in the Wellman Laboratories of Photomedicine, Harvard Medical School (Figure 2). This was the first laser RCM to be used to image human

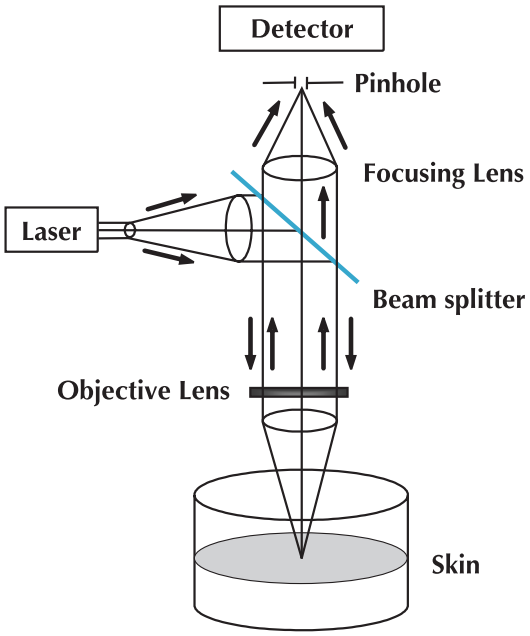


Figure 1. Schematic representation of the confocal microscope.

living skin *in vivo* (Rajadhyaksha et al, 1995). It is this microscope that was the prototype to become commercialised by Lucid, Inc (www.lucid-tech.com) (Figure 3). The commercial RCM uses an 830 nm diode laser of maximal power of 25 mW and a 30x water-immersion objective lens of numerical aperture 0.9. Images have a lateral resolution of 0.5-1 μm and a section thickness of 3-5 μm, similar to conventional histology (Rajadhyaksha et al, 1999a). To image skin, the RCM has a mechanical arm containing the objective lens that is set in a metal holder (Figure 4). This holder with the objective lens can be clicked into a metal ring (with a hole allowing imaging) that is attached to human

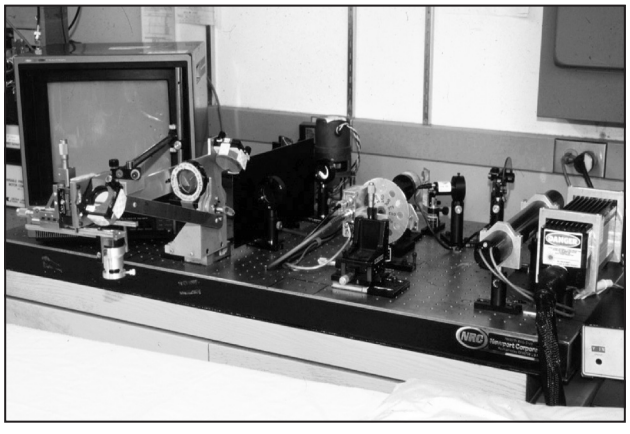


Figure 2. RCM prototype.

skin with double sided tape (Figure 5). With this skin-contact device, lateral movements of skin are prevented to improve image quality. As the laser beam is directed perpendicular to the skin surface (Figure 1), and scanning of the beam in the X and Y direction is also perpendicular to skin, horizontal virtual skin sections are produced. This is contrary to conventional histology, in which vertical skin sections are obtained. By turning a screw mechanism the laser beam can be moved manually in order to allow imaging of the entire part of skin uncovered by the metal ring, which is a circle of approximately 1.5 cm in diameter. The beam can also be moved vertically deeper into skin, thereby producing a continuum of horizontal sections from the stratum

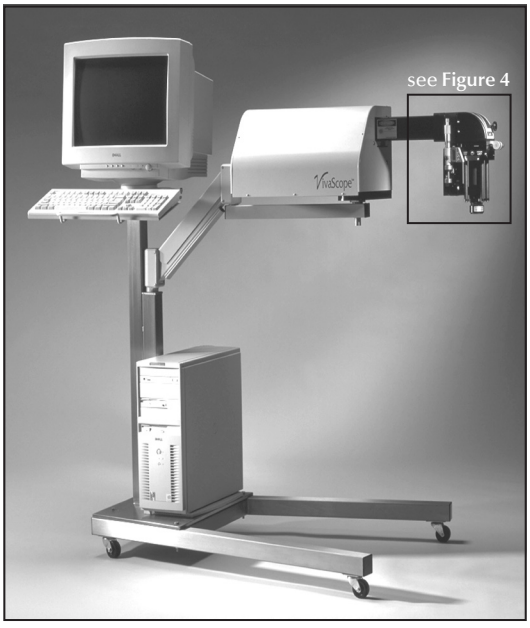


Figure 3. Commercialised RCM, the Vivascope® 1000.

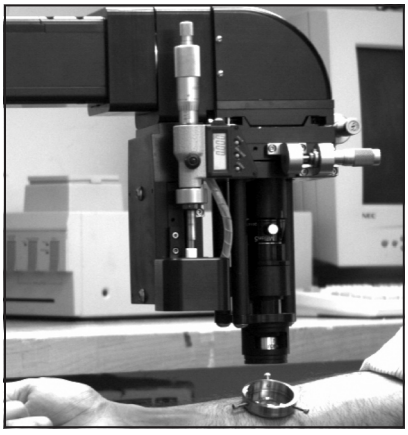
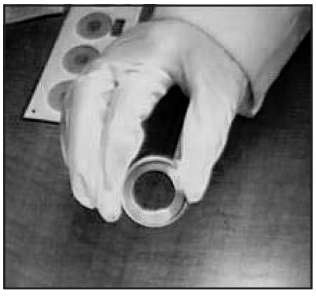


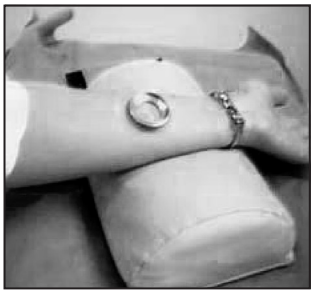
Figure 4. Mechanical arm containing the objective lens in a metal holder.

Figure 5. Attachment of metal ring to skin.



Metal ring…

…put double sided tape on…



…to stick on skin.

corneum to the upper reticular dermis, as the maximum penetration depth of the laser beam is around 200-350 μm, depending partially on the wavelength of the light source. One could achieve imaging of deeper layers in skin, but that would imply increasing the power of the laser with subsequent risk of damaging human skin (Gonzalez et al, 2003). As the objective lens is attached to a micrometer, the length of the vertical movement of the lens between 2 (in focus) points within skin can be determined, thus allowing measurement of several morphometric parameters such as epidermal thickness. Contrast of images results from inherent differences in refractive indices of organelles and microstructures within skin, resulting in black and white images (Rajadhyaksha et al, 1995). When light passes from a structure with low refractivity to one with high refractivity, a strong reflection occurs at the interface. Structures such as a cell nucleus are not very refractile, showing up dark in confocal images, while the organelles in cytoplasm have more refractivity, showing up brighter compared to the nucleus. This makes an epidermal cell visible as a dark circle (nucleus) surrounded by a brighter ring (cytoplasm). Melanin is the best endogenous contrast agent in skin, as its refractive index (n=1.7) is quite different from that from the rest of the epidermis (n=1.34-1.4). This makes melanin and melanin containing cells visible as white and bright structures on RCM images (Rajadhyaksha et al, 1995).

RCM of skin disorders, including skin pigmentation and pigmented skin lesions

In the earlier days, studies with RCM have been focussed on exploration and characterisation of different dermatological conditions, such as psoriasis (Gonzalez et al, 1999a), onychomycosis (Hongcharu et al, 2000), and actinic keratosis (Aghassi et al, 2000a), just to name a few. Due to the possibility to image the same skin lesion repeatedly, it has also been used to characterize dynamic events such as the pathophysiology behind effective treatment of skin disorders with laser therapy (Aghassi et al, 2000b; Aghassi et al, 2000c), as well as leucocyte trafficking in human skin (Gonzalez et al, 2001). Significant work was performed in order to characterize normal skin parameters at different body sites with RCM (Huzaira et al, 2001), and differences between young and older skin (Sauermann et al, 2002).

The past years, research with RCM has been focused more and more on its applicability to diagnose basal cell carcinoma, irritant and allergic

contact dermatitis, and pigmented lesions. The characteristics of basal cell carcinomas were determined by RCM and were compared to characteristics found by routine histology (Gonzalez and Tannous, 2002). Based on these results, 5 criteria that could be easily detected in RCM images by non-dermatopathologists were selected to be tested for their sensitivity and specificity in diagnosing basal cell carcinomas in a retrospective multicenter trial (Nori et al, 2004). This study showed that the presence of 4 or more of these criteria has a specificity of 95.7% and a sensitivity of 82.9% for the diagnosis of basal cell carcinomas. This study also showed that by combining clinical assessment via photographs of skin lesions with RCM images of these lesions, the accuracy to diagnose basal cell carcinoma was significantly improved (Nori et al, 2004). Furthermore, it has been shown that RCM can be used as a non-invasive guidance for Mohs surgery of basal cell carcinomas (Tannous et al, 2003; Rajadhyaksha et al, 2001). These studies are cautious steps towards an implementation of the RCM in the arsenal of diagnostic tools in dermatology. Another area of RCM focus is contact dermatitis. In the first report in 1999 (Gonzalez et al) features of allergic contact dermatitis (ACD) as seen with the RCM were described. The study described in chapter 4 of this thesis describes the use of RCM in investigating the differences in susceptibility between black and white skin to develop irritant contact dermatitis (ICD), and it provided a description of RCM features observed in ICD. Based on these studies, subsequent work was aimed at investigating whether the RCM could be used to discriminate between ACD and ICD. Swindells et al (2004) showed that RCM may differentiate between ACD and ICD, with the main differences being the superficial damage found in ICD versus more vesicle formation observed in ACD. RCM has also been shown to neatly follow dynamics of developing ACD and ICD (Astner et al, 2005a). Besides describing the RCM parameters, this study showed that the evaluated RCM parameters showed higher intensities for ACD as time passed after removal of the patch test chambers, while the intensity of most ICD parameters decreased in time, showing the prolonged activity of ACD reactions after patch test removal. Another study investigating the sensitivity and specificity of RCM parameters in diagnosing ACD demonstrated that the presence of stratum granulosum and spinosum spongiosis showed high sensitivity in diagnosing ACD (Astner et al, 2005b). The latest study by Astner et al (2006) supports the findings reported in chapter 4 of this thesis, as black skin seemed more resistant to

developing ICD compared to white skin as studied with the RCM using a different irritant.

The latest studies with *in vivo* RCM have increasingly focused on studying aspects pertaining skin pigmentation and pigmentary disorders. As stated before, melanin provides the strongest contrast in skin because it is highly refractile, appearing white and bright on RCM images (Rajadhyaksha et al, 1995). This makes RCM an excellent tool to study skin pigmentation. Earlier studies investigated benign and malignant melanocytic lesions and described differences observed between melanocytic nevi, dysplastic nevi and malignant melanomas based on cell morphology, brightness of images, melanocyte dendrites and keratinocytes cell border using RCM (Langley et al, 2001). In another study, pigmented seborrheic keratosis and nevi were imaged, claiming that melanocytes, pigmented keratinocytes and dermal melanophages could be elucidated (Busam et al, 2001a). Case reports have shown the use of RCM in helping to determinate the margin of clinically poorly defined amelanotic malignant melanomas, with the high refractivity of immature melanosomes present in these malignancies as guidance (Busam et al, 2001b) and described the features of lentigo maligna and lentigo maligna melanoma (Tannous et al, 2002). Since 2004, the group of Pellacani has published a series of papers on RCM imaging of different melanocytic lesions. They described the confocal characteristics of Spitz nevi (Pellacani et al, 2004) and benign nevi and melanomas (Pellacani et al, 2005a), also focusing specifically on the pigment network in nevi versus melanomas (Pellacani et al, 2005b). In a recent study (Pellacani et al, 2005c), the specificity and sensitivity of several confocal features of malignant melanomas, Spitz and Reed nevi, and benign nevi were determined, making a cautious step towards using the RCM as an adjunctive diagnostic tool in melanoma detection. It was shown that by creating an algorithm in which 2 points were assigned for major criteria (maximum of 2 criteria) and 1 point for minor criteria (maximum of 4 criteria), a sum score equal or greater than 5 corresponded to a specificity of 96.9% and a sensitivity of 83.8% for detection of malignant melanomas with RCM (Pellacani et al, 2005c).

Pigmentation has also been investigated with RCM in animal models. In the guinea pig animal model, melanocytes can be clearly seen as individual dendritic bright cellular structures. Wang et al (2002) used the RCM to characterize melanocyte features such as cell body size, and dendrite length

and number in the guinea pig animal model, showing excellent correlation with DOPA stained epidermal sheets of skin biopsies. Differences between human and animal skin (mice, guinea pigs and Yacatan micropigs) with regard to melanocytes and keratinocytes supranuclear melanin caps have also been studied with RCM (Yamashita et al, 2005). This study showed that melanocytes cannot be distinguished from surrounding keratinocytes in normal human skin, while they can be seen as dendritic structures in normal animal skin. However, after exposure of human skin to UVR, some activated dendritic melanocytes could be individually seen 8 days after UVR exposure, disappearing gradually thereafter (Yamashita et al, 2005).

Aims of the thesis

The overall aim of the studies presented in this thesis was to enlarge our knowledge on pigmentation, in particular on how to optimize the detection of pigmentation and to study methods to safely influence and modulate pigmentation, especially when it concerns UVR-induced pigmentation. In the first 2 chapters we used the pigmented guinea pig animal model to study aspects on pigment detection and modulation. This animal model is frequently employed to study the effects of UVR on skin and to study modulation of UVR-induced pigmentation, because their skin tans comparably to human skin after exposure to UVR. In **chapter 2**, we used the *in vivo* RCM to investigate whether pigmentation induced by UVR can be detected non-invasively in this animal model. We especially looked to see if detection with RCM would precede the clinical detection of pigmentation. In **chapter 3** we used the same animal model to study pigment modulation using a pigment modulating agent called bisindolylmaleimide (BIS), a selective PKC inhibitor with a higher selectivity for PKC-β. We studied the effect of topically applied BIS on constitutive and UVR-induced pigmentation on guinea pig skin, also using the *in vivo* RCM. In addition, we looked at the effect of BIS on the tyrosinase activity of cultured human melanocytes *in vitro* and on hair color of black mice. In **chapter 4**, we describe the use of the RCM to study the susceptibility to develop ICD in humans with different skin color, as it is believed that black skin may be more resistant to develop ICD than white skin. This chapter also gives extra insight in RCM as a valid detection method of histopathological changes in human skin.

In the following 3 chapters, we investigated the effect of an extract of *Polypodium leucotomos* (PL) in modulating UVR-induced effects such as pigmentation in humans. PL is an extract from a fern plant endowed with anti-oxidant and immunomodulating properties. In **chapter 5** we describe the specific clinical and histological effects that orally administered PL exerts on human skin when exposed to UVR from a solar simulator under carefully controlled laboratory conditions. In **chapter 6**, we investigated the photoprotective effect of oral PL on PUVA-induced phototoxicity and the subsequent pigmentary response in healthy human skin on a clinical and histological level. In **chapter 7**, we investigated whether oral PL could be of aid in the treatment of the depigmentation disorder vitiligo vulgaris. The etiology of vitiligo is unknown, but it is believed to be a cell-mediated auto-immune disease, and/or to result from an increase in oxidative stress in human skin. Because of this last hypothesis, we investigated whether the anti-oxidative properties of orally administered PL could be employed to increase repigmentation induced by narrow-band UVB (NB-UVB) phototherapy.

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Chapter 2

Detection of UV-induced pigmentary and epidermal changes over time using *in vivo* reflectance confocal microscopy

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Abstract

In vivo reflectance confocal microscopy (RCM) provides high resolution optical sections of skin in its native state, without needing to fix or section the tissue. Melanin provides the excellent contrast for RCM, giving a bright signal in confocal images. The pigmented guinea pig is a common animal model to study human pigment induction and modulation, as their tanning response is comparable to human tanning after exposure to ultraviolet radiation (UVR). We investigated the applicability of RCM to detect UVR-induced pigmentary changes in this model. Animals were exposed to solar simulator radiation for 7 days. RCM was performed during the irradiation and follow-up period. Compared to non-irradiated skin, an increase in melanocyte size, dendricity and number, as well as increased pigment in keratinocytes were seen in irradiated epidermis. Interestingly, these changes could be detected even before a tanning response was clinically visible. UVR-induced epidermal hyperplasia could also be detected and quantified. In conclusion, *in vivo* RCM is a sensitive non-invasive imaging technique that can repeatedly measure epidermal pigmentation and thickness, as demonstrated in the guinea pig model. This technique should greatly enhance our appreciation of dynamic pigmentary changes in human or animal skin over time and in response to specific stimuli.

Introduction

In vivo reflectance confocal microscopy (RCM) is a non-invasive imaging tool allowing visualization of skin without tissue alteration by placement of a microscope directly on living skin (Rajadhyaksha et al, 1995, Rajadhyaksha et al, 1999a). One obtains a continuum of *en face* images with a lateral resolution of 0.5-1 μm and a section thickness of 3-5 μm, similar to conventional histology (Rajadhyaksha et al, 1999a). Because skin can be visualised as many times as desired without alteration of the tissue, and melanin is the best endogenous contrast agent of skin appearing bright and white on RCM images, this technique is ideal to follow dynamic events pertaining pigmentation.

The pigmented guinea pig is a well established animal model for human pigmentation as their skin contains active interfollicular epidermal melanocytes located in the basal layer in a similar pattern to human skin (Bolognia et al, 1990), and tans comparably to humans after exposure to ultraviolet radiation (UVR) (Imokawa et al, 1986). This animal model is therefore frequently used to study effects of oral and topical pigment modulating agents interfering at different points in the UVR pigmentation cascade, providing useful information on pathogenesis and therapeutic options for pigmentary skin disorders (Yamakoshi et al, 2003, Park et al 2004, Allan et al, 1997, Imokawa et al, 1986, Yoshida et al, 2002). The disadvantage of conventional evaluation is that with clinical evaluation subtle changes can be missed, while histological evaluation alters the tissue, removing the sampled area, preventing serial examination of a single area.

This study investigates the ability of RCM to detect pigmentary changes occurring in guinea pig skin during and after a one week exposure to UVR. We conclude that RCM is a sensitive method to detect UVR-induced pigmentary changes in melanocytes and keratinocytes, even when invisible to the naked eye, as well as UVR-induced hyperplasia.

Materials and methods

Animals

Outbred pigmented guinea pigs, American Shorthair X Abyssinian (Kuiper Rabbit Ranch, Chicago, IL), aged 12 to 20 weeks at the beginning of the study

were selected. Animals had free access to chow and chlorinated water. Animals were shaved with an electric clipper (Oster, number 40 blade, Niles, IL) to remove long hair. Remaining stubble was removed with the commercially available depilatory Nair (Carter-Wallace, New York, NY), which was repeated at least once weekly.

Irradiation procedure

A 1000 watt xenon arc lamp (Oriel Corp, Stratford, CT) emitting a collimated beam, equipped with a WG 305 cut-on filter (Schott Glas, Mainz, Germany) and a first surface mirror (Edmund Scientific, Barrington, NJ) was used as a solar simulator. The irradiance as measured by an IL 1700 research radiometer (International Light, Newburyport, MA), fitted with a UVB probe (SED 240, Serial number 2093, SCS 280 UVB 1#5440, W#3790) was 0.17 mW/cm^2. All animals were irradiated once a day for 7 days with 150 mJ/cm^2 through an adhesive template stuck on the hairless back exposing a square of 3.2 cm^2, resulting in dark tanning by the end of the exposure period (Park et al, 2004). An adjacent covered (non-irradiated) skin site at a distance of 2-3 cm served as control. A 0.3cc solution of 0.25cc Ketamine (50mg/ml, Bedford Laboratories, Bedford, OH) and 0.05cc Xylazine (100mg/ml, Lloyd Laboratories, Shenandoah, IA) was used to anaesthetize the animals before irradiation and RCM imaging. Animal protocols were approved by the Institutional Review Board and met all guidelines of the Institutional Animal Care and Use Committee at Massachusetts General Hospital Use and Care of Animals policy.

***In vivo* RCM**

We used a commercially available RCM (Vivascope 1000, Lucid Inc, Henrietta, NY) equipped with a 830 nm diode laser of maximal power of 25 mW and a 30x water-immersion objective lens of numerical aperture 0.9. After anaesthetizing the animals, irradiated and non-irradiated sites were imaged with RCM during a 10 to 15 minutes procedure as described previously (Rajadhyaksha et al, 1999a). *In vivo* RCM imaging was first performed in week 1 after 4 days of irradiation, and was repeated once weekly during the follow-up of the study until week 4.

The imaging procedure was video-taped, and video-clips of non-irradiated skin, skin irradiated for 4 days but showing no clinical tanning, and skin

3 weeks after irradiation with visible tanning have been compiled, and are provided as Supplementary Material.

Quantification of epidermal thickness

As the objective lens is attached to a micrometer, movement of the lens between 2 (in focus) points within skin can be measured, thus allowing measurement of epidermal thickness. The epidermal thickness was defined as the distance between the bottom of the stratum corneum and the top of the suprapapillary plate (SPP), which is defined as the basal layer located above a dermal papilla (Huzaira et al, 2001). During each imaging procedure, the epidermal thickness above at least 4 SPP´s was measured in irradiated and non-irradiated skin. The mean value of these measurements was calculated and represented the suprapapillary epidermal thickness.

Histochemical procedure

Punch biopsies (4mm) were obtained in week 4 at the end of the study from irradiated and non-irradiated skin. Each biopsy was placed epidermal side down onto a coverslip covered with a thin layer of permount. The biopsy with coverslip was emerged in 2M sodium bromide solution for 2 hours at 37°C, after which the dermis was separated from the epidermis (Staricco and Pinkus, 1957). This method was chosen by reason of our ample experience with it (Wang et al, 2002). The coverslip with the epidermal sheet was rinsed three times with 0.1M sodium phosphate buffer (pH 7.4), and was then immersed in 0.1% deoxyphenylalanine (DOPA) solution at 37°C for 4-6 hours, while changing the solution after the first 30 minutes and then every 2 hours. The epidermis was checked under the microscope every hour until a dark brown color developed in at least one of the paired sections. All paired specimens were then fixed in 10% formalin. Epidermal sheets were evaluated and correlated with RCM images obtained at the level of the SPP at week 4.

Statistical analysis

Epidermal thickness in irradiated and non-irradiated skin was compared using the repeated measures Manova with one within factor and one between factor. Data analyses was conducted SPSS software (SPSS Inc., Chicago, IL, www.spss.com). Differences were considered statistically significant when $p<0.05$.

Results

Melanocytes and epidermis of non-irradiated skin

A video of the real-time RCM imaging of non-irradiated skin is provided online (see Video S1 "Non-irradiated skin"). Highly refractile bright dendritic cells are seen in the epidermal basal layer at an approximate depth of 50 μm and identified as melanocytes based on their morphology and correlation with DOPA-stained epidermal sheets (Figure 1A). Vertical movement of the laser beam farther down into skin revealed dermal papillae surrounded by these bright dendritic cells, consistent with the known localization of melanocytes in the basal layer above dermal papillae (suprapapillary plate; SPP). Inside dermal papillae, erythrocytes flowing through dermal capillaries are appreciated during real-time imaging. Melanocytes are well demarcated and individually distinguishable from one another, showing long, slender dendrites (Figure 1A and 2). A faint dark round nucleus can be seen within the cell body (Figure 1A). A composite image of 16 RCM images obtained at the level of the SPP clearly illustrates the typical distribution of melanocytes in alternating rows of greater and lesser cell density in the interfollicular epidermis, with the adjacent follicular ostia, as previously described in this animal model (Allan et al, 1995) (Figure 3). Next to the melanocytes and above the rete pegs, small polygonal cells with dark nuclei and relatively brighter cytoplasm are appreciated, representing cells of the stratum spinosum, that is, basal keratinocytes (Figure 1A). Above this layer, a delimited stratum spinosum is absent and there is a rapid transition to bigger polygonal to round cells of the stratum granulosum (Figure 1D). The stratum corneum is the final most superficial image, visible as a highly refractile bright layer with dark lines, representing grouped corneocytes separated by skin folds.

Melanocytes and epidermis of irradiated skin

After 4 days of irradiation, when only faint erythema and no clinical tanning was yet visible (3 out of 4 animals, data not shown), RCM could already detect distinct pigmentary changes (Figure 1B and E, see also Video S2 "4 days after irradiation, no visible tanning"). Imaging at a level above the SPP revealed a delimited layer of small cells as in the stratum spinosum of non-irradiated skin (Figure 1A), representing a well-defined stratum spinosum. In this layer, keratinocytes contained highly refractile material, indicating melanin transfer

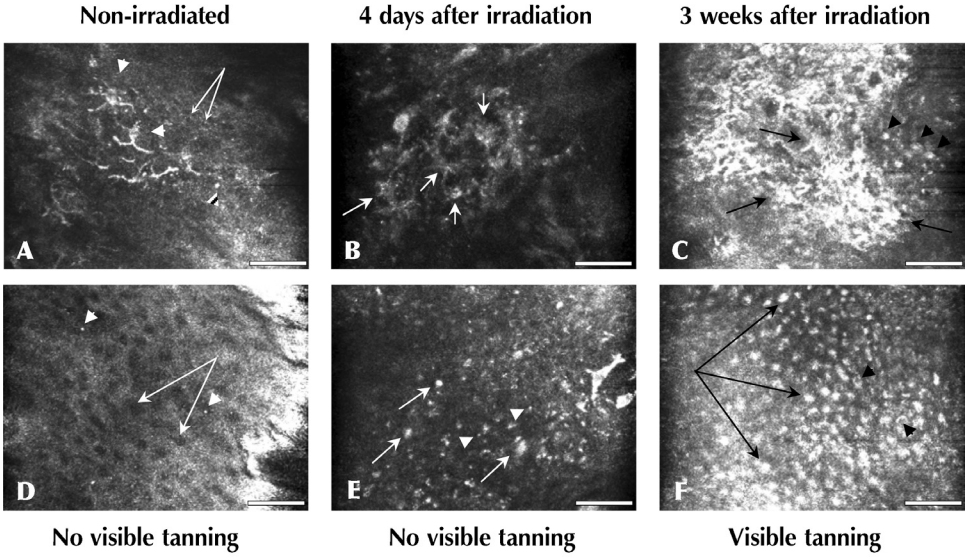

Figure 1. RCM visualization of the development of pigment induction in UVR-exposed guinea pig skin.
(A and D) RCM images of non-irradiated (control) skin. (A) Suprapapillary plate with melanocytes visible as bright dendritic cells (white arrowheads). The stratum spinosum, composed of small polygonal cells with a dark nucleus and a brighter ring of cytoplasm (arrows), is seen adjacent to melanocytes. Some dendrites viewed in cross-section are seen as bright "dots" (striped arrowhead). (D) Round bigger cells of the stratum granulosum (arrows), containing a dark center (nucleus) with a ring of brighter cytoplasm. Some dendrites viewed in cross-section are seen as bright "dots" (arrowheads).
(B and E) RCM images 4 days after starting irradiation; skin shows no clinically visible tanning (3 out of 4 animals). (B) Melanocytes have heavier cell bodies (arrows). Image brightness is lower due to epidermal hyperplasia. (E) A defined cellular layer emerged showing keratinocytes in different degrees of melanization (arrows). Note that cell size corresponds to cell size of spinous cells in fig 1A, revealing cells to belong to the stratum spinosum. Many dendrites viewed in cross-section appeared as small bright "dots" (arrowheads), showing increased dendricity of melanocytes.
(C and F): RCM images 3 weeks after starting irradiation; skin has clinically visible tanning.
(C) Suprapapillary plate: melanocytes (arrows) are increased in size and number, and pigmented keratinocytes of the stratum spinosum can be seen next to them (arrowheads). (F) Stratum spinosum, consisting out of pigment-loaded keratinocytes (arrows). In some cells, the nucleus can still be seen as a dark round center (arrowheads). Scale bars: 25 μm.

to keratinocytes (Figure 1E). These pigmented keratinocytes were found either adjacent to or just above melanocytes. Between these keratinocytes, many small bright "dots" appeared (Figure 1E), that during real time imaging could be followed to a melanocyte cell body, revealing them as dendrites in cross sections. This indicates the increased dendricity of melanocytes in irradiated compared to non-irradiated skin. In the basal layer, melanocytes have larger cell bodies (Figure 1B). The overall brightness of the SPP images was lower than in non-irradiated skin (Figure 1B *vs*. 1A). However, melanocytes remain recognizable by their suprapapillary location and dendritic shape.

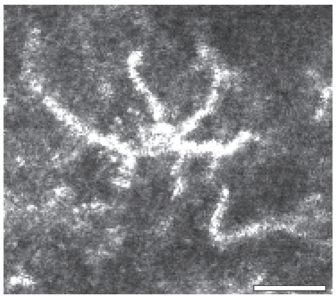

Figure 2. Magnification (3x) of a melanocyte in non-irradiated guinea pig skin. Scale bar: 75 μm.

Images obtained in week 2, 3 and 4 show dramatic changes in melanocyte aspect and keratinocyte pigmentation, coinciding with the presence of a clinically visible tanning response (Figure 1C and F; see also Video S3 "3 weeks after irradiation, with visible tanning"). The spinous layer is dominated by bright round structures, representing melanin-loaded keratinocytes (Figure 1F). At the level of the SPP, melanocytes are increased in size, dendricity and seemingly also in number, making it hard to distinguish individual cells from one another (Figure 1C). These pigmentary changes persist relatively unchanged until the end of the experiment (week 4), when biopsies were taken.

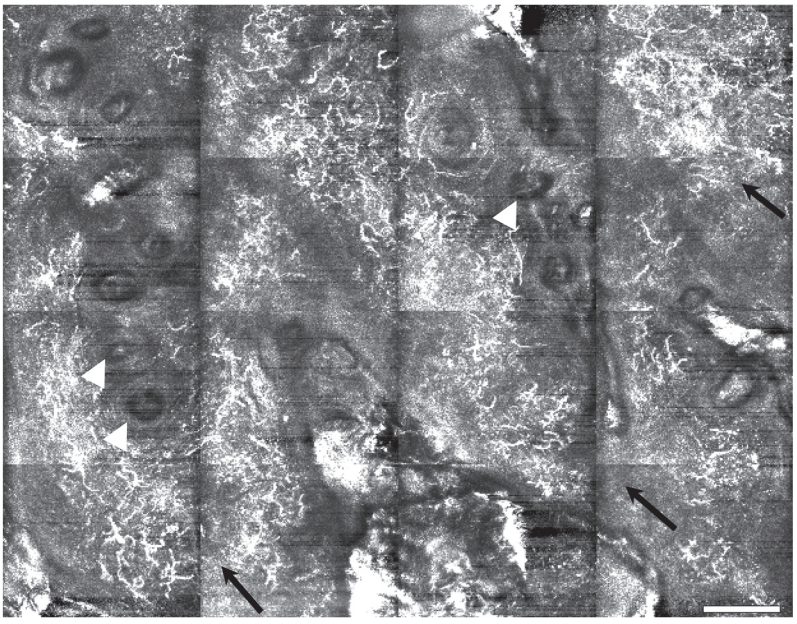

Figure 3. Composite of 16 RCM images shows typical striated anatomy of guinea pig skin.
Note 3 bands of interfollicular skin containing melanocytes (arrows) with follicular ostia (arrowheads) in between. Scale bar = 100 μm.

Correlation of RCM images with DOPA stained epidermal sheets

By comparing RCM images of week 4 to DOPA stained epidermal sheets from biopsies taken in week 4, RCM images showed an excellent correlation with DOPA stained epidermal sheets, confirming the interpretation of RCM images. DOPA-stained non-irradiated skin showed melanocytes that were slender and individually perceptible (Figure 4B), as seen in the RCM images from these skin sites (Figure 4A). In contrast, the *en face* sections of DOPA stained irradiated epidermis showed larger melanocyte cell bodies, increased dendricity and seemingly an increase in melanocyte number (Figure 4D), in agreement with the RCM images (Figure 4C).

Pigmentary changes in non-irradiated skin during follow-up

During the post-irradiation follow-up period, non-irradiated skin developed changes suggestive of a slight pigmentation response. Keratinocytes located close to melanocytes showed an increasingly refractile perinuclear cytoplasmic ring, suggesting increased melanization (Figure 5). Melanocytes also seemed to have an increase in brightness and dendricity, as seen by an

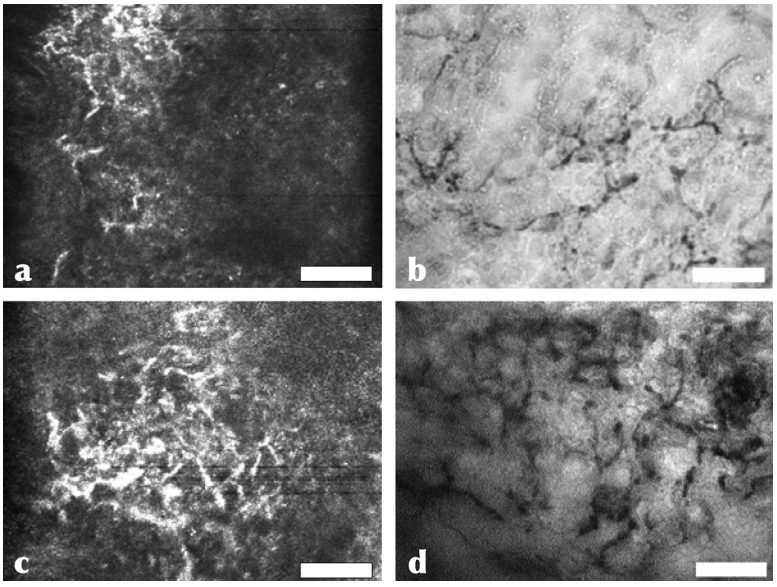

Figure 4. Images at the suprapapillary plate correlate well with DOPA-stained epidermal sheets.
(a) RCM images of non-irradiated skin show few melanocytes that are individually distinguishable, similar to (b) melanocytes in DOPA-stained epidermal sheets.
(c) Irradiated skin shows increased number and dendricity of melanocytes seen with RCM, which corresponds to (d) the appearance of melanocytes in DOPA-stained epidermal sheets of irradiated skin. Note the parallel between increased brightness in RCM images and increased darkness in DOPA sheets, both indicating increased melanization. Scale bar: 25 μm.

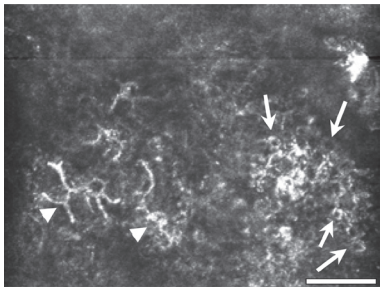

Figure 5. Non-irradiated skin shows mild pigmentary changes in weeks 2-4.
Melanocytes (arrowheads) show increased dendricity compared to week 1 (Fig 1A). Note that the adjacent spinous cells (arrows) developed a bright cytoplasm compared to week 1, indicating melanization. The dark round nucleus can be appreciated. Scale bar: 25 μm.

increase in white "dots" in the epidermis. Clinically, no pigmentary changes could be seen in the guinea pig skin. These subtle changes are reminiscent of the previously reported increased pigmentation in the non-irradiated control ear of mice subjected to repeated UV exposure that was statistically above basal levels, although only a fraction of the increase observed in the irradiated contralateral ear, and interpreted to reflect release of a systemic and melanization signal (Rosdahl and Szabo, 1978)

Quantification of increase in suprapapillary epidermal thickness

Suprapapillary epidermal thickness was measured in both irradiated and non-irradiated skin during RCM imaging. After 4 days, epidermal thickness doubled in irradiated skin compared to non-irradiated (control) skin, and stayed higher at all time-points of RCM imaging ($p<0.05$) (Figure 6), although it diminished slightly by week 4.

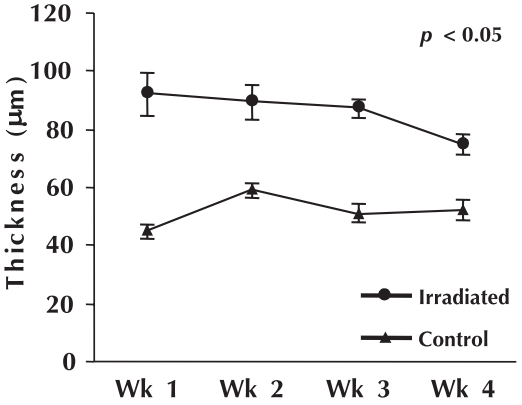

Figure 6. Epidermal thickness is significantly greater in irradiated *vs.* non-irradiated (control) skin ($p < 0.05$), indicating quantification of ultraviolet radiation-induced hyperplasia by RCM.
The epidermal thickness, defined as the distance between stratum corneum and the top of the suprapapillary plate, was quantified in week (Wk) 1 to Wk 4 (Y-axis: mean ± SEM, n = 18-25).

Epidermal thickness in non-irradiated skin increased slightly after day 4, and remained slightly higher through week 4, possibly in parallel to the increase in melanization described above.

Discussion

RCM is a non-invasive imaging tool based on inherent differences in refractive indices of cellular structures in skin (Rajadhyaksha et al, 1995, Rajadhyaksha et al, 1999b). The high refractivity of melanin makes it the best endogenous contrast agent in skin (Rajadhyaksha et al, 1995), causing melanocytes and other melanin-containing cells to be visible as bright structures on RCM images compared to their surroundings. Compared to other non-invasive imaging techniques such as high-frequency ultrasound and optical coherence tomography, RCM offers the highest resolution and provides images comparable to routine histology (Gonzalez et al, 2003). Hence, it is an ideal method to study pigmentation non-invasively. Conversely, it is the optical sectioning of confocal microscopy that allows cells lying beneath the surface of human tissue to be imaged without removing or cutting it. In contrast, conventional transmission microscopes don´t perform optical sectioning, tissue must be biopsied from a patient and thin physical sections of tissue must be cut from the biopsy specimen with a microtome to be prepared and stained before viewing.

The interpretation of RCM images is based on pattern recognition that with training can be easily mastered. Based on histological knowledge of skin anatomy, the shape and relative brightness of a structure combined with its location in the epidermis give the clue to the interpretation of the observed structures. Interpreting still RCM images however, is more difficult than real-time images because the advantage of the three-dimensional view of structures located in relation to one another is lost. Comparison of the real-time imaging procedures (see the videoclips) with the corresponding still images from Figure 1, reinforces this conclusion.

Different animals and guinea pig strains have been reported to be used for pigmentation detection with RCM (Yamashita et al, 2005). Wang et al assessed melanocyte characteristics such as number, cell body size, and dendricity in non-irradiated guinea pig skin with RCM (Wang et al, 2002). In this study

we assessed the time course of UVR-induced pigmentation and epidermal hyperplasia in the guinea pig animal model by *in vivo* RCM. RCM could clearly distinguish 3 major changes in irradiated versus non-irradiated skin: i) an increase in melanocyte size, dendricity and apparent number, ii) the increase of pigment within keratinocytes, and iii) an increase in epidermal thickness. Irradiated skin showed more and larger melanocytes with increased in dendricity compared to non irradiated skin (Figure 1A *vs.* C), known responses of both guinea pig skin (Bolognia et al, 1990) and human skin (Gilchrest et al, 1996) to UVR. Our observations were confirmed by correlating RCM images with *en face* sections of DOPA-stained epidermal sheets obtained at the end of the study (Figure 4). In the suprabasal compartment, irradiated skin showed an abundance of round highly refractile cells in the spinous layer, indicating the known increased melanization of keratinocytes after UVR exposure (Bessou et al, 1995), while these pigmented keratinocytes could not be found in the epidermis at the start of the study (Figure 1D *vs.* F). These RCM observations are consistent with the appearance of a clinically visible tan that was first detected approximately 6 days after beginning UVR exposures, similar to the time course of delayed tanning seen in human skin (Gilchrest et al, 1996).

However, RCM detected pigmentary changes in irradiated skin in 3 of 4 guinea pigs studied before they developed a clinically visible tan (Figure 1B and E). Most striking was the appearance of bright round structures located beside and above melanocytes in the subrabasal layer (Figure 1E), similar to the pigmented keratinocytes seen in tanned skin (Figure 1F). We also detected an increase in dendrites as small bright "dots" in the suprabasal layer (Figure 1E). Images of the basal layer in week 1 yielded images with decreased brightness, which we concluded to be a result from UVR induced hyperplasia allowing less penetration and reflection of laser light to visualize the SPP. Therefore we could not employ a constant laser power, but instead adjusted the power according to the need per imaging site in order to obtain sufficient power to illuminate and visualize the foci of our interest. Laser powers usually varied roughly between 15-25 mW. Still, melanocytes could be detected, based on recognition of their shape, location and relative brightness.

RCM also detected increased melanocyte dendricity and pigmentation of spinous cells in non-irradiated skin during the course of the study, while no pigmentary changes were appreciated clinically (Figure 5). Although

unexpected at first, these findings support observations that pigment induction may occur indirectly in non-irradiated skin of both mice (Rosdahl and Szabo, 1978) and humans (Stierner et al, 1989) presumably via local or systemic release of cytokines by irradiated keratinocytes and fibroblasts (Gilchrest et al, 1996). Alternatively or in addition, in the present experiment the repetitive irritation from the depilation process resulted in a sub-clinical post-inflammatory hyperpigmentation response (Bolognia et al, 1990). Regardless, these observations establish that RCM is sensitive enough to detect subtle pigmentary changes in melanocytes and keratinocytes, even in clinically normal skin.

We did not quantify the changes occurring in melanocytes and keratinocytes, because individual discernment of these cells in irradiated skin was difficult due to their abundance. A computer-assisted analysis system could be employed to quantify changes in brightness, representing changes in melanization of skin (Gambichler et al, 2004). However, as stated before, brightness of images is influenced by epidermal thickness posing a complication to this technique. We quantified epidermal thickness and found a significant increase in irradiated skin during the entire period of 4 weeks (Figure 6). RCM imaging revealed that this increase was partly due to the development of spinous layers in irradiated skin (Figure 1E and F). During weeks 2, 3 and 4 the image brightness increased again, which can be explained by the combination of increased melanin production by melanocytes and decreased epidermal hyperplasia (Figure 6), allowing a better reflection of light. The thickness of the stratum corneum was not quantified separately, because we considered this parameter unreliable due to repetitive removal through chemical depilation during the study. Guinea pig skin thus has a proliferative response of viable epidermis to UVR similar to human skin (Walker et al, 2003), making it a useful model to study UVR-induced epidermal hyperplasia as well.

In conclusion, RCM is a sensitive method to detect pigmentary changes in melanocytes and keratinocytes, even when not visible to the naked eye, as well as in clinically tanned guinea pig skin. Changes in melanocytes and keratinocytes could be perceived separately. Epidermal hyperplasia occurring in a similar way as in human skin was quantified, making this a useful model for UVR-induced epidermal hyperplasia. Due to its non-invasive, painless character, this technique is ideal to follow UVR-induced skin changes occurring over time. This opens new possibilities to non-invasively study

effects of pigment induction and modulation in this model. Moreover, RCM may provide a novel approach to examine the changes in skin pigmentation non-invasively in human subjects.

Supplementary material

Video S1. Non-irradiated skin.

Video S2. 4 days after irradiation, no visible tanning.

Video S3. 3 weeks after irradiation, with visible tanning.

Each video is composed out of a number of clips. A timer is seen in the right corner, and explanation of each clip is given with the help of this timer. For each clip, a cartoon representing skin is given at the end showing the level at which imaging was performed. The videos are composed out of imaging from different guinea pigs.

Videos can be seen at www.nature.com/jid/archive/index.html

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Chapter 3

Topical application of a protein kinase C inhibitor reduces skin and hair pigmentation

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Abstract

Protein Kinase C-β (PKC-β) activates tyrosinase, the rate-limiting enzyme in melanogenesis, by phosphorylating serines at amino acid (aa) positions 505 and 509. To determine whether inhibition of PKC-β activity decreases pigmentation, paired cultures of primary human melanocytes were first pre-treated with bisindolylmaleimide (Bis), a selective PKC inhibitor, or vehicle alone for 30 min, then treated with 12-0-tetradecanoyl-phorbol-13 acetate (TPA) for an additional 90 min to activate PKC in the presence of Bis. Bis blocked the expected induction of tyrosinase activity by activation of PKC. Addition of a peptide corresponding to aa 501-511 of tyrosinase and containing its PKC-β phosphorylation site, a presumptive PKC-β pseudosubstrate, gave similar results, consistent with the established role of PKC-β in stimulating melanogenesis. To determine whether topical application of Bis reduces pigmentation *in vivo*, the backs of four shaved and depilated pigmented guinea pigs were ultraviolet light (UV) irradiated daily with a solar simulator for 2 weeks excluding weekends. Compared to vehicle alone, Bis (300 μM), applied twice daily to paired sites for various periods encompassing the irradiation period, decreased tanning in the irradiated skin. Total epidermal melanin, assessed by Fontana-Masson staining and reflectance-mode confocal microscopy, as well as tyrosinase activity *in vivo* as determined by split-dopa assay, were significantly reduced in Bis-treated sites compared to vehicle-treated sites. Bis also, although less strikingly, reduced basal epidermal melanin when topically applied twice daily, 5 days/week, for three weeks to shaved and depilated un-irradiated skin. Moreover, topical application of Bis (100 μM) once daily for 9 days to the freshly depilated backs of 8 week old mice markedly lightened the color of regrowing hair. Cross-sections of treated skin revealed no cytological damage, atrophy, irritation or inflammation after topical application of Bis. These results demonstrate that inhibiting PKC activity *in vivo* selectively blocks tanning and reduces basal pigmentation in the epidermis and in anagen hair shafts. In combination with previous *in vitro* studies, they suggest that inhibiting PKC-β specifically may offer a safe and effective means of reducing unwanted pigmentation in human skin.

Introduction

Pigmentation due to synthesis and dispersion of melanin protects the skin from harmful effects of sunlight (Quevedo and Holstein, 1998), but unwanted hyperpigmentation can also produce a significant psychological stress. Development of effective therapeutics to modulate skin pigmentation has been slow due to the complexity of molecular mechanisms regulating pigmentation. Our laboratory has determined that protein kinase C-β (PKC-β) activates tyrosinase (Park et al, 1993), the key and the rate-limiting enzyme in pigmentation (Pawelek and Chakraborty, 1998), by phosphorylating serine residues at amino acid positions 505 and 509 (Park et al, 1999). Loss of PKC-β prevents melanogenesis in cultured pigment cells (Yamanishi et al, 1991; Powell et al, 1993; Yamanishi et al, 1994; Park and Gilchrest, 1996; Park et al, 1999), suggesting that inhibition of this isoform specifically or as part of pan-PKC inhibition might lead to skin and hair lightening *in vivo*.

PKC is a family of serine/threonine kinases with at least eleven isoforms (Dekker and Parker, 1994), categorized as classical PKC (cPKC), novel PKC (nPKC) and atypical PKC (aPKC). PKC-β belongs to the category of cPKC, which also includes PKC-α and PKC-γ (Nishizuka, 1992). PKC-α is ubiquitously expressed (Nishizuka, 1988) while the expression of PKC-γ is restricted to brain (Nishizuka, 1984), and PKC-β is expressed in many tissues, including brain, endocrine tissues, liver, spleen, lung, heart and testis (Nishizuka, 1988). In skin, PKC-β expression appears to be restricted to melanocytes (Park et al, 1991), while PKC-α is expressed in melanocytes, keratinocytes, and fibroblasts (Park et al, 1991; Reynolds et al, 1994; Racchi et al, 1994). In general, PKC resides in the cytoplasm in an inactive form and is activated when diacylglycerol (DAG) is generated from the membrane when cell surface receptors interact with their specific ligands (Nishizuka, 1992; Nishizuka, 1984). Activation of cPKC requires the presence of both membrane-generated DAG and elevated levels of intracellular Ca^{++} (Dekker et al, 1995; Newton, 1995; Nishizuka, 1985; Jaken, 1996), whereas DAG alone is sufficient to activate both nPKC and aPKC (Dekker et al, 1995; Newton, 1995; Nishizuka, 1985; Jaken, 1996). Activated PKC then translocates to the plasma membrane or to another particular site within the cell (Clemens et al, 1992; Dempsey et al, 2000; Leach and Raben, 1993; Lehel 1995a; Lehel, 1995b) where it phosphorylates its substrate protein(s).

The role of PKC in pigmentation was first suggested by the observation that addition of DAG increased the melanin level in cultured human melanocytes and the PKC inhibitors H-7 and sphingosine blocked DAG-induced increases in the level of melanin in these cells (Gordon and Gilchrest, 1989). Topical application of DAG induced skin pigmentation in guinea pigs in a reversible fashion, whereas a PKC-inactive analogue of DAG failed to induce skin pigmentation (Allan et al, 1995), indicating that the activation of PKC increases pigmentation *in vitro* and *in vivo*. Conversely, depletion of PKC by chronic treatment of cells with phorbol esters markedly reduces the basal melanin level and tyrosinase activity in human melanocytes (Park et al, 1993) and murine S91 melanoma cells (Park et al, 1996). The PKC inhibitors sphingosine and calphostine also block α-melanocyte stimulating hormone-induced pigmentation (Park et al, 1996), suggesting that the cAMP-dependent protein kinase A pathway known to mediate α-melanocyte stimulating hormone effects ultimately interacts with the PKC pathway.

Bisindolylmaleimide GF 109203X (Bis), a selective inhibitor for cPKC (Toullec et al, 1991), is a synthetic derivative (MW 0.4 Kd) of the microbial product staurosporine that exerts its effects at least part by competing with ATP (Tamaoki et al, 1986). Bis was shown to have higher selectivity for PKC-β than for PKC-α and -γ, with an IC_{50} of 16 nM versus an IC_{50} of 20 nM (Toullec et al, 1991; Davis et al, 1992), and a 10-100 fold higher IC_{50} for other isoforms of PKC and for other serine/threonine and tyrosinase kinases (Martiny-Baron et al, 1983; Toullec et al, 1991). In the present study, Bis reduced pigmentation in cultured human melanocytes and reduced basal pigmentation and prevented UV-induced increased melanogenesis (tanning) via topical application in guinea pigs. In mice, topical application of Bis to depilated skin lightened the color of regrowing hairs. In the context of prior studies, these findings demonstrate that inhibition of PKC-β activity reduces pigmentation *in vivo* and *in vitro*.

Materials and methods

Cell culture

Primary human melanocytes were cultured from neonatal foreskins as previously described (Park et al, 1999). In brief, the epidermis was separated from the

dermis after overnight incubation in 0.25% trypsin at 4°C. Primary cultures of melanocytes were established by seeding 0.5-1 x 10^6 total epidermal cells per 100 mm dish in Medium 199 supplemented with 10 μg/ml insulin, 10^{-9}M triiodothyronine, 10 μg/ml transferrin, 1.4 x 10^{-6} hydrocortisone, 80 μM dbcAMP, bovine pituitary extract (35 μg/ml), bFGF (10 ng/ml), epidermal growth factor (100 ng/ml), and 1- 2% fetal calf serum. All post primary cultures were maintained in a low calcium (0.03 mM) version of this medium known to selectively support melanocyte growth (Naeyaert et al, 1991). Cells at first to third passage were used for all experiments.

UV irradiation

The solar simulator consisted of a 1000 Watt Xenon arc lamp (Oriel, Stratford, CT), equipped with a WG 305 cut-on filter (Schott, Germany). The irradiance as measured by an IL 1700 research radiometer (International Light, Newburyport, MA), fitted with a UVB probe (SED 240, Serial number 2093, SCS 280 UVB 1#5440, W#3790) was 0.17 mW/cm^2 (or 1.7x10^{-4}W). The animals were anaesthetized with 0.3cc of a mixture of 0.75cc Ketamine (50mg/ml, Bedford Laboratories, Bedford, OH) and 0.15cc Xylazine (100mg/ml, Lloyd Laboratories, Shenandoah, IA) and irradiated through an adhesive template exposing three paired 3.2 cm^2 skin sites daily during 7 days for 14 min and 42 seconds (150 mJ/cm^2), a protocol previously shown to cause dark tanning (Allan et al, 1995).

Tyrosinase activity

Tyrosinase activity was measured according to Pomerantz, et al (1964). In brief, 5 x 10^5 cells were briefly sonicated in 80 mM PO_4 (pH 6.8) containing 1% Triton-X-100, and tyrosinase was extracted for 60 min at 4°C. 10 - 50 μg of cellular protein was incubated with 250 nM L-tyrosine, 25 nM L-dihydroxyphenylalanine, 12.5 μg chloramphenicol and 5 μCi of L-[3,5 -^{3}H] tyrosine (40-60 Ci/mmol) for 30 - 60 min at 37°C. The reaction was stopped by addition of 500 μl of 10% TCA containing 0.2% bovine serum albumin. TCA soluble material was reacted with Norit A and released ^3H_2O was measured using a scintillation counter. The activity was expressed as counts per minute of ^3H_2O released/μg protein/hr minus the non-specific incorporation of radioactivity, determined by using lysate boiled for 30 min (background, generally less than 5 - 10% of the sample).

Phosphorylation of tyrosinase

Phosphorylation of tyrosinase in cultured melanocytes was performed as previously described (Park et al, 1999). Cultured melanocytes were pre-incubated overnight with serum free DMEM devoid of phosphate, containing 50 μM sodium vanadate, and [^{32}P]-orthophosphate (8,500-9,120 Ci/mmol). Then cells were treated with 12-0-tetradecanoyl-phorbol-13 acetate (TPA) (10^{-7}M) for 90 min to activate PKC and lysed in a buffer containing 20 mM Tris (pH 7.5), 200 mM NaCl, 1% Triton X-100, 2 mM EDTA, 2 mM EGTA, 0.5 μg/ml aprotinin and 1 mM PMSF. The lysate was clarified by spinning at 800 x g for 15 min, and tyrosinase was immunoprecipitated from the supernatant using polyclonal antibody specific for tyrosinase (Jimenez et al, 1991). Immunoprecipitated proteins were separated using 7.5% sodium-dodecyl sulfate gel electrophoresis, dried and exposed to Kodak X-OMAT film.

Animals

Outbred pigmented guinea pigs, American Shorthair X Abyssinian (Kuiper Rabbit Ranch, Chicago, IL), aged 12 to 20 weeks at the beginning of the study, were used. The animals had free access to guinea pig chow and chlorinated water and were housed in individual cages. Prior to topical applications, each animal was shaved with an electric clipper (Oster, number 40 blade) to remove the long hair. The remaining stubble was removed with the commercially available depilatory Nair (Carter-Wallace).

Experimental protocol

All protocols were approved by the Institutional Animal Care and Use Committee at Boston University School of Medicine. Three separate protocols were employed to study different aspects of pigmentation. To study effects of Bis on UV-induced pigmentation, three pairs of treatment sites (3.2 cm^2) were chosen in areas of comparable baseline pigmentation on the left and right sides of the back, equidistant from the dorsal midline, for subsequent daily applications and UV exposure. The left sites of each pair was treated with vehicle alone (25% DMSO : 75% propylene glycol) and the right sites with 300 μm Bis twice a day. The Site 1 pair was treated for one week before UV irradiation, two weeks during irradiation and two weeks after irradiation. The Site 2 pair was treated twice daily during two weeks of UV irradiation and two weeks after irradiation. The Site 3 pair was treated only during two

weeks of irradiation. Weekends were excluded from applications. These three protocols were included to account for the possibilities that some time may be required for Bis to penetrate stratum corneum and reach melanocytes, Bis might be expected to be most effective during the UV irradiation period when PKC is known to be induced (Rubeiz et al, 2002; Park and Gilchrest, 1999) and some time may be required for PKC levels to return to their basal level after irradiation. Photographs were taken and *in vivo* reflectance confocal laser microscopy was performed during and after the period of application. A higher concentration of Bis (300 μM) was used for *in vivo* then for studies *in vitro* in light of the fact that the compound needs first to penetrate the stratum corneum before reaching the target melanocytes.

The possibility that Bis might act as a sunscreen was excluded by metering UV transmittance through the 300 μM Bis preparation versus vehicle alone, applied as an even film to tissue culture plastic. There was no significant difference in transmittance: 0.15mW *vs* 0.14mW in the UVB range and 30mW *vs* 28mW in the UVA range for vehicle versus Bis preparations, respectively.

To study the effects of Bis on basal pigmentation, backs of three guinea pigs were shaved and depilated using Nair as described above. One pair of treatment sites (3.2 cm^2) in the mid-back was treated with either vehicle or Bis (300 μM) twice daily 5 days/week for three weeks.

To study the effect of Bis on hair pigmentation, 7-8 week old black female mice (C57BL/6) were anesthetized with ketamine/xylosine mixture (80 mg/kg and 16 mg/kg of body weight respectively) to depilate the entire back using a wax-rosin mixture as previously described (Stenn et al, 1993). Bis (100 μM) was applied on the cephalad half of the back and vehicle alone was applied to the caudal half of the back beginning three days after depilation once daily for 9 days. The 100 μM Bis concentration was employed because skin barrier function is known to be less efficient in mice than in guinea pigs. Photographs were taken to document the color of regrowing hair 24 hours after the last application.

In vivo reflectance confocal laser microscopy

A commercially available reflectance-mode confocal microscope (Vivascope 1000, Lucid, Henrietta, NY) equipped with a 830 nm diode laser with a maximal power of 25 mW and a 30x objective lens of numerical aperture 0.9 was used for imaging skin of the anesthetized guinea pigs. Technical details of

the microscope and the imaging procedure have been previously described (Rajadhyaksha et al, 1995 and 1999). Briefly, the skin is immobilized against the microscope by a ring-template fixture, so the laser beam is directed perpendicular to the skin. Horizontal movement of the beam allows screening of the skin site, while axial movement of the beam results in a continuum of *en face* images from stratum corneum to upper papillary dermis. The images, equivalent to optical sections, have a lateral resolution of 0.5-1 μm and a section thickness (axial resolution) of 3-5 μm, similar to conventional histology. The immersion medium used for imaging was water, as the refractive index of water is closest to that of skin, thereby reducing artefacts during imaging. Animals were anesthetized during confocal microscopy to minimize motion during imaging which was performed once a week. All imaging procedures were video-taped and transferred to a computer. Still images were grabbed at the level of the suprabasal layer to visualize spinous keratinocytes and at the level of the basal layer to visualize basal keratinocytes and melanocytes. The VivaScope user interface is driven by a Windows NT-based personal computer with menu-driven software for initiating and controlling the scanners, laser illumination power, z-depth focusing and image capture.

Histological analysis

Punch biopsies (4mm) were taken from vehicle-treated and Bis-treated skin 24 hours after the last application. Specimens were fixed in 10% neutral-buffered formalin for 24 hours, dehydrated, and embedded according to standard histologic protocols. Vertical cross sections, stained with both hematoxylin and eosin and Fontana-Masson silver nitrate were examined under 10X and 40X magnification using an Olympus BH-2 microscope as previously described (Allan et al, 1995). The amount of interfollicular epidermis occupied by melanin in serial sections was quantitated by computer-image analysis as previous described (Bhawan et al, 1991).

***In vivo* split-dopa staining**

Separate punch biopsies (4mm) were taken simultaneously from vehicle-treated skin and Bis-treated skin to determine whether tyrosinase activity is reduced by application of Bis *in vivo*. Each biopsy was placed onto a coverslip, epidermal side down in a thin layer of permount. The biopsy was then covered with 2M sodium bromide solution for 2 hours at 37°C. Dermis was

then removed from the epidermis, and the coverslip was rinsed with 0.1M sodium phosphate buffer (pH 7.4) three times. The entire coverslip with the epidermal sheet was immersed in 0.1% deoxyphenylalanine solution at 37°C for 4-6 hours, changing solution after the first 30 minutes and then every 2 hours. The epidermis was checked under the microscope every hour until dark brown color developed in at least one of the paired sections. The stained epidermis of all paired specimens was then fixed in 10% formalin.

Results

Competitive inhibition of PKC-β activity reduces tyrosinase activity *in vitro*

To test the hypothesis that inhibition of PKC-β activity would decrease phosphorylation of tyrosinase, and subsequently decrease tyrosinase activity, a tyrosinase mimetic peptide (TMP) was employed. TMP is composed of 11 amino acids whose sequence is identical to amino acids 501-511 of tyrosinase (Kwon et al., 1987) (Figure 1). A control peptide had identical sequence except for alanines in place of serines, the known phosphorylation sites for PKC-β (Park et al., 1999). Paired cultures of subconfluent human melanocytes were treated with 3 μM of TMP or control peptide for 16 hours. To enhance their delivery, TMP and control peptides were first treated with Lipofectamine for 30 minutes. Cells were then treated with 10^{-7}M TPA for 90 minutes to activate PKC, harvested and analyzed for tyrosinase activity. Compared to the

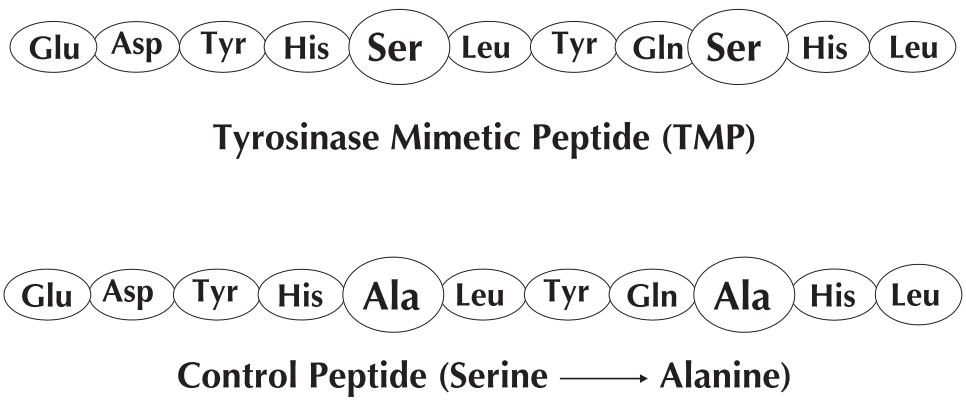

Figure 1. Amino acid sequences of the tyrosinase mimetic peptide (TMP) and control peptide. Serine residues, the site of PKC-β-mediated phosphorylation in TMP are highlighted. In the control peptide, serine is replaced by alanine, which cannot be phosphorylated.

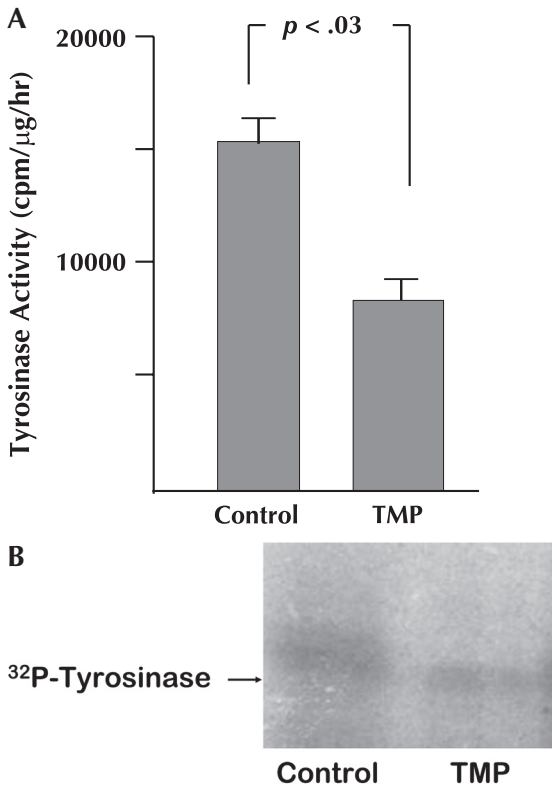

Figure 2. TMP inhibits tyrosinase activity by blocking phosphorylation of tyrosinase by PKC-β. (A) Paired cultures of subconfluent melanocytes were treated with 3μm of TMP or control peptide for 16 hours, then with 10^{-7}M TPA for 90 minutes to activate PKC, and then harvested. Tyrosinase activity was measured as previously described (Pomerantz, 1964). Student paired t-test was employed for statistical analysis.
(B) Paired cultures of melanocytes were first treated with 3μm TMP or control peptide for 16 hours, then treated with 10^{-7}M TPA in DMEM without phosphate in the presence of ^{32}P-ortho-phosphate for 90 minutes. Cells were harvested and tyrosinase was immunoprecipitated, using a specific polyclonal antibody against tyrosinase. Immunoprecipitated proteins were separated by subjecting them to 7.5% SDS-PAGE. The gel was dried and exposed to Kodak X-OMAT film to demonstrate ^{32}p-tyrosinase. A representative result from two independent experiments is presented.

cells treated with control peptide, TMP-treated cells had significantly lower tyrosinase activity (Figure 2A).

To confirm that TMP inhibits phosphorylation of tyrosinase by PKC-β, paired plates were treated with 3μM control peptide or TMP for 16-18 hours and subsequently treated with 10^{-7}M TPA for 90 minutes in the presence of ^{32}P-ortho-phosphate to activate PKC. Then tyrosinase was immunoprecipitated using a polyclonal antibody specific for tyrosinase (Jimenez et al, 1991). Incorporation of ^{32}P-ortho-phosphate into tyrosinase was significantly less in TMP-treated melanocytes than in control peptide-treated melanocytes (Figure 2B). These results are consistent with previous reports (Park et al, 1993; Park et al, 1999) that tyrosinase activity is modulated by PKC-β mediated phosphorylation and, in combination with the earlier reports, demonstrate that PKC-β inhibition decreases this rate-limiting melanogenic activity.

Inhibition of PKC activity with Bis decreases tyrosinase activity *in vitro*

To further demonstrate that inhibition of PKC-β activity would reduce pigmentation, Bis, a commercially available selective inhibitor for cPKC with

minimal effect on other serine/threonine kinases including nPKC and aPKC or on tyrosine kinases (Toullec et al, 1991), was employed. Paired subconfluent melanocyte cultures were first treated with 50μM Bis for 30 minutes, then treated with 10^{-7}M TPA for an additional 90 minutes. Preliminary dose-response studies using Bis concentrations of 1 μM to 100 μM indicated that 50 μM was the optimal concentration to inhibit TPA-induced increases in tyrosinase activity without any visible effects on cell survival or morphology. Cells were then harvested and tyrosinase activity was measured. The TPA-induced increase in tyrosinase activity was blocked by Bis treatment (Figure 3), further confirming that PKC-β activity is necessary for tyrosinase activity, at least *in vitro*.

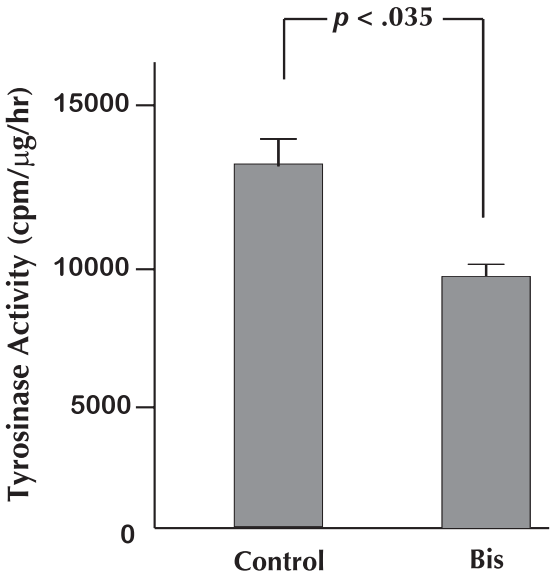

Figure 3. Bis inhibits tyrosinase activity in cultured melanocytes. Paired cultures of subconfluent melanocytes were first pretreated with 50μM Bis or vehicle for 30 minutes. Then the cells were treated with 10^{-7}M TPA for an additional 90 minutes in the presence of Bis. Cells were then harvested and tyrosinase activity was measured. A representative result from three independent experiments is presented and Student Paired T-test was used for statistical analysis.

Effects of Bis on UV-induced skin pigmentation *in vivo*

To test whether inhibition of PKC-β activity *in vivo* reduces skin pigmentation, pigmented guinea pigs were employed as described in Materials and Methods. These animals have melanocytes in the interfollicular epidermis and are known to tan in response to UV irradiation (Allan et al, 1995). Biopsies of the UV irradiated sites taken 24 hours after the last exposure showed the expected increase in epidermal melanin, as well as epidermal thickening (Figure 4A). In all four animals, topical application of Bis decreased skin pigmentation in all application sites (Figure 5). There was no erythema or other evidence of

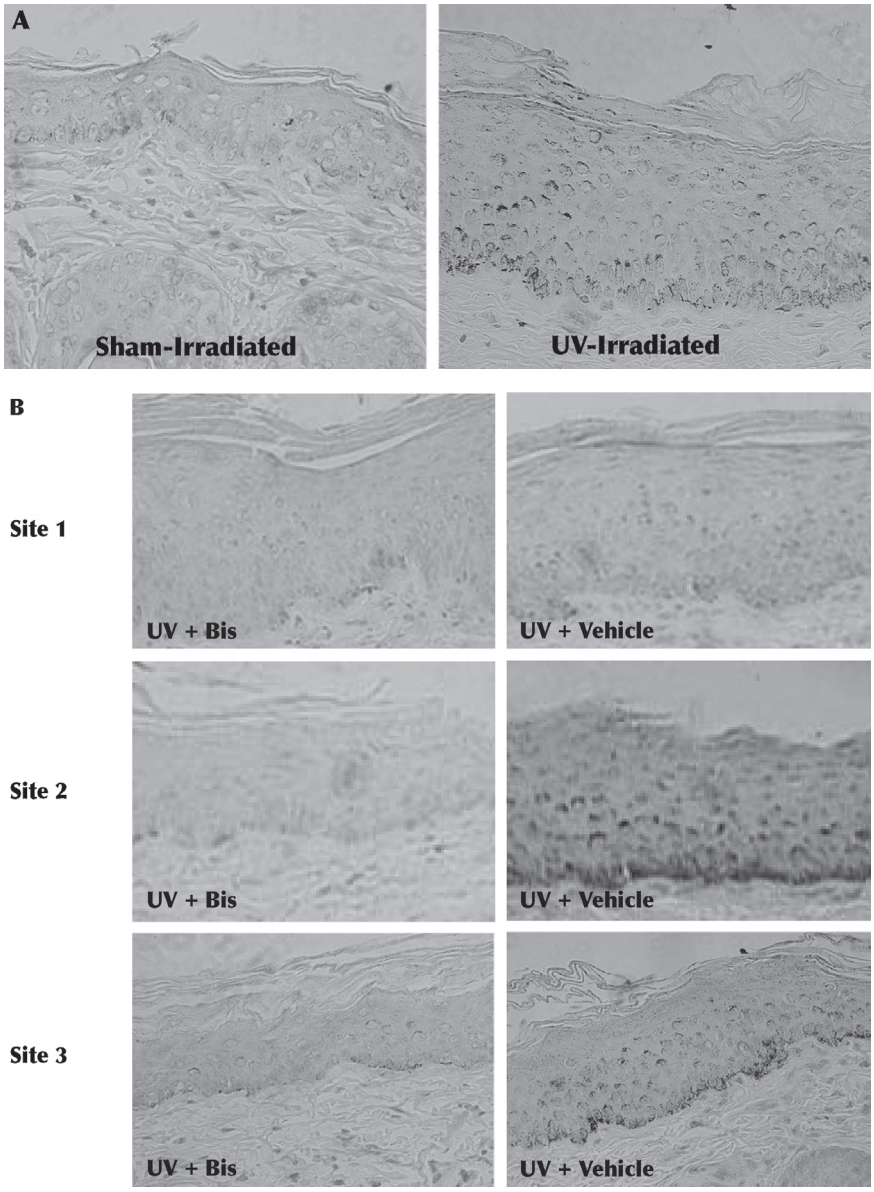

Figure 4. Bis blocks UV- or sham-induced increases in epidermal melanin. Guinea pigs were treated and irradiated over a two-week period, as described. Biopsies taken 24 hours after the last irradiation were stained with Fontana-Masson to visualize total epidermal melanin. (A) UV or sham irradiation only, no topical treatment. (B) UV irradiation plus daily treatment with Bis or vehicle alone. Representative sections from each site are shown. (☞ page)

cutaneous irritation during or after the application. Interestingly, the period of Bis application (before, during and after UV irradiation; during and after only; or during only) did not affect the degree of skin lightening. However, the effect of Bis on skin lightening was consistently less on Site 1, located closest

to the head, compared to Sites 2 and 3 which displayed both darker tanning and a more striking reduction in pigmentation by Bis. This is consistent with our previous observation that the cephalad part of the guinea pig back does not pigment as darkly in response to UV irradiation or other stimuli (Allan et al, 1995), and our reasoning that the degree of observed pigment reduction resulting from PKC-β inhibition depends on the basal or stimulated level of PKC-β activity.

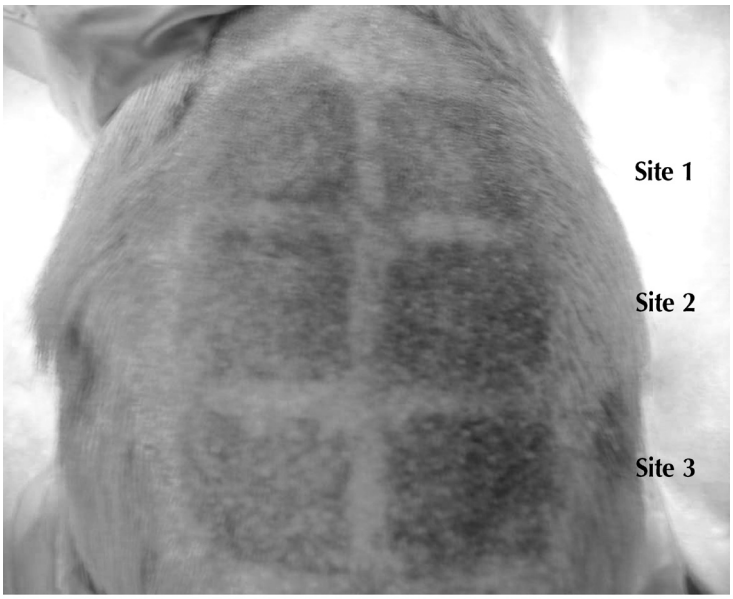

Figure 5. Bis reduces skin pigmentation of guinea pigs. Four pigmented guinea pigs were treated with Bis or vehicle as described in the Methods section. After the last application, the hair was re-shaved and photographs were taken to demonstrate the effect of Bis on pigmentation. (☞ page)

Bis decreased total epidermal melanin and tyrosinase activity

To assess the total amount of epidermal melanin in each treatment site, the paraffin-embedded biopsies were sectioned and stained with Fontana-Masson (silver nitrate). There was a consistent decrease in total epidermal melanin in Bis-treated areas compared to vehicle-treated areas (Figure 4B). Consistent with these results, split-dopa staining of *en face* sections was far less in Bis-treated sites than vehicle-treated sites (Figure 6).

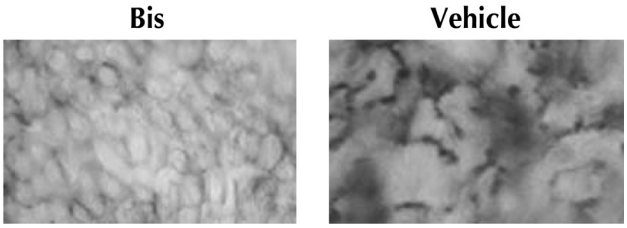

Figure 6. Tyrosinase activity in vehicle and Bis-treated skin. Guinea pigs were treated and irradiated over a two-week period, as described. Biopsies taken 24 hours after the last application were processed for *in vivo* split-dopa assay as described under Methods. Brown staining indicates melanin in melanocyte dendrites. Bis-treated and vehicle-treated skin from Site 2 is shown. Sites 1 and 3 showed comparable differences. (☞ page)

In vivo reflectance confocal microscopy showed decreased melanin in Bis-treated sites

In reflectance-mode confocal imaging of skin, melanin is the best endogenous contrast agent and melanin-containing cells are highly refractile (Rajadhyaksha et al, 1995). When imaging pigmented guinea pig skin, melanocytes are easily identifiable by their white color and dendritic morphology (Wang et al, 2002). Animals were anesthetized and reflectance confocal microscopy was performed once a week. As shown in Figure 7, the UV-induced increase in melanin was clearly visible after 9 days. In non-irradiated skin, keratinocytes can be visualized in the suprabasal layer as polygonal to round structures with a dark nucleus surrounded by a brighter cytoplasm, while in irradiated skin keratinocytes contain far more melanin, seen as bright round spots (Figure 7). After one week and more striking after 2 weeks, the overall brightness of keratinocytes was clearly less in Bis-treated skin than in skin treated with vehicle alone, consistent with the reduction of cutaneous pigmentation observed clinically at the end of the irradiation period and with the histologic determinations. Melanocytes could be easily recognized at the basal layer level by their brightness and dendricity, both in non-irradiated and irradiated skin. However, as expected, melanocytes in irradiated skin were increased in number and size compared to those in non-irradiated skin. Again, this difference was less pronounced in Bis-treated skin than in vehicle-treated skin. These results further confirm that inhibition of PKC-β reduces pigmentation *in vivo* and demonstrate the feasibility of performing detailed non-invasive time course studies through serial examination of single animal or human subject.

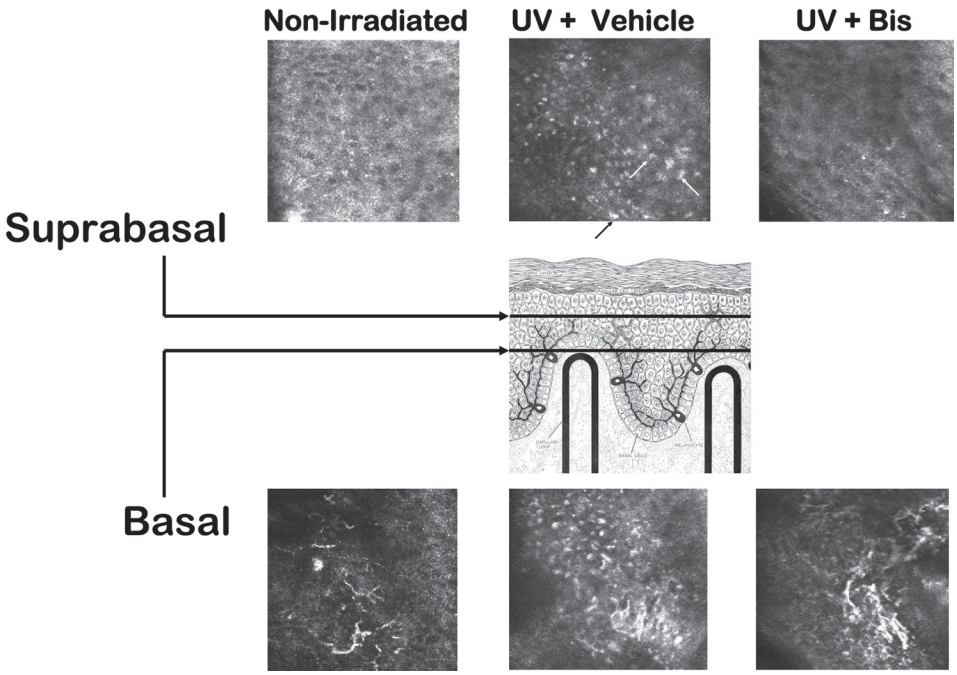

Figure 7. Reflectance–mode confocal microscopy of non-irradiated skin and vehicle and Bis-treated irradiated skin. Images from the suprabasal and basal layer of skin show an overall decrease in brightness (autofluorescence due to melanin present in keratinocytes and melanocytes) of Bis-treated irradiated skin compared to vehicle-treated irradiated skin, corresponding to a decrease in pigmentation. As seen most easily in non-irradiated skin, the suprabasal layer shows the characteristic honey-combed pattern created by keratinocytes with central nuclei and relatively little cytoplasmic melanin, while the image obtained at the basal layer shows melanocytes that are easily identified by their dendritic morphology. In irradiated vehicle-treated (tanned) skin, both the suprabasal and basal layers of the epidermis contain far more melanin (detected as bright areas) that obscure these patterns. The irradiated Bis-treated skin is intermediate in melanin content but more closely resembles the non-irradiated central images. (In vivo confocal images, 30X water immersion objective lens, image width corresponds to 250 microns.) The cartoon is modified from Daniels et al (1968).

Bis decreases basal skin pigmentation *in vivo*

In order to determine whether topical application of Bis would also reduce basal pigmentation, the backs of three guinea pigs were shaved and naired. Bis (300μM) or vehicle alone was topically applied as described above and biopsies were obtained 24 hours after the last applications. Clinically, there was only subtle lightening in the Bis-treated skin but Fontana-Masson stained sections from the Bis-treated areas showed a significant reduction ($p<0.03$) in epidermal melanin compared to vehicle-treated areas as assessed by image analysis (Figure 8A and B). Therefore, the effect of Bis is not limited to UV-induced pigmentation. Rather, as anticipated on the basis of the *in vitro* studies, Bis inhibits basal pigmentation as well.

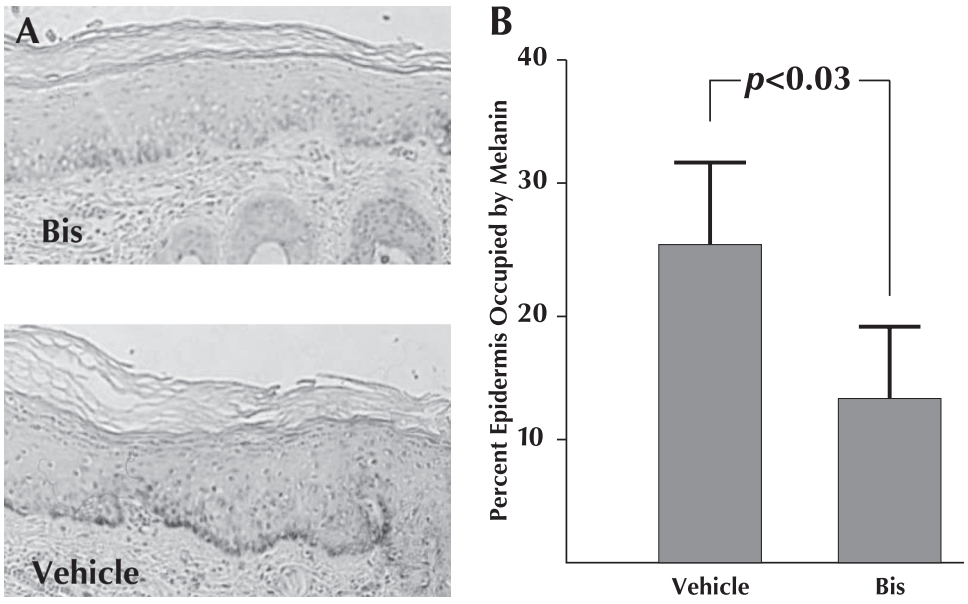

Figure 8. Bis reduces basal pigmentation. Guinea pigs were topically treated with Bis or vehicle alone for 3 weeks, as described. Biopsies taken 24 hours after the last application were stained with Fontana-Masson to visualize total epidermal melanin. (A) Representative sections from Bis and vehicle- treated areas. (B) Percent epidermis occupied by melanin on serial sections from Bis and vehicle-treated areas determined using computer-image analysis as previously described (Bhawan et al, 1991). For statistical analysis, Student t-test was performed. (☞ page)

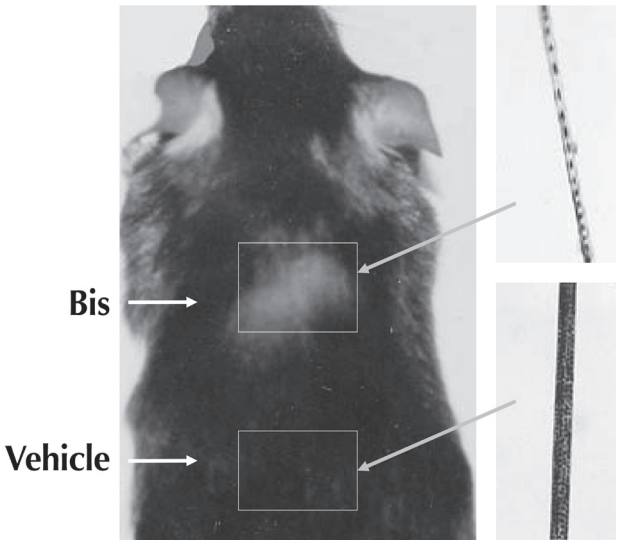

Figure 9. Mouse coat color is lightened by Bis treatment. Nine week-old black mice were depilated to synchronize the hair cycle and initiate a new anagen growth phase. Beginning 3 days later, either 100 μM of Bis (top) or vehicle alone (bottom) was topically applied once a day for 9 days and mice were photographed on day 13. Inset: Hair from Bis or vehicle-treated areas were plucked on day 13 and photographed under a dissecting microscope. Representative hairs (midshaft, approximately 0.3 mm in length) from 50 hairs plucked from each area are shown. (☞ page)

Effect of Bis treatment on murine coat color

To determine whether topical application of Bis would also lighten hair color, Bis (100 μM) was applied once daily to freshly depilated mice for 9 days, beginning on day 3 after depilation. As the coat regrew, hair color was strikingly lighter in Bis-treated but not in vehicle-treated areas (Figure 9). The striking loss of coat pigmentation on casual inspection was confirmed by examining individual hairs plucked from vehicle versus Bis treated areas under the dissecting microscope (Figure 9, inset).

Discussion

In combination with previous studies (Park et al, 1993; Park et al, 1999), the present results demonstrate that inhibition of PKC-β activity reduces pigmentation both *in vivo* and *in vitro* by preventing phosphorylation of tyrosinase. Although Bis, the inhibitor employed for the *in vivo* studies, is not expected to be completely selective for the β isoform of PKC (Toullec et al, 1991), the only detected effects of Bis treatment were those attributable to PKC-β inhibition on the basis of prior *in vitro* studies. Cultured human melanocytes treated with the PKC inhibitor Bis did not display any morphologic changes other than decreased melanin content, and topical application of Bis twice daily for up to five weeks did not produce any clinically or histologically detectable changes in guinea pig skin other than decreased pigmentation. Skin biopsy cross sections obtained at the end of the experiment, 24 hours after the last application, confirmed that Bis caused no cytologic damage, atrophy, irritation or inflammation. Visual inspection, histologic analysis, and skin imaging by *in vivo* reflectance confocal microscopy all demonstrated pigmentation reduction by topical application of Bis with no other detectable effect of the treatment. These findings suggest that Bis minimally affects cell behaviors other than melanogenesis, perhaps because of the relative selectivity of Bis for PKC-β (Toullec et al, 1991), and isoform that appears to be exclusively expressed in melanocytes in skin (Park et al, 1991) and appears to act only by stimulating melanogenesis (Park et al, 1993; Park et al, 1996).

Our results further indicate that restricting Bis application to the two-week UV irradiation period is sufficient to block tanning. Additional applications, for one week before irradiation or two weeks after irradiation, did not further

decrease pigmentation. These results are consistent with the expectation that PKC-β is most abundant and active in the period immediately following UV irradiation, given that UV irradiation up-regulates the expression of PKC-β mRNA within 1 hour (Park and Gilchrest, 1999; Rubeiz et al, 2002) and UV-irradiation also rapidly increases the intracellular level of DAG (Punnonen and Yuspa, 1992). Generation of DAG would expect to activate PKC-β immediately, leading to the activation of tyrosinase. Our findings are also consistent with those of Hallahan et al (1991) that UV up-regulates the proto-oncogenes c-jun and c-fos, known to be regulated through the PKC-dependent pathway, and that these inductions are inhibited by PKC inhibitors. The lower activity of PKC-β in resting, unirradiated melanocytes should result in Bis having less effect, as observed.

Topical application of Bis also reduced basal pigmentation. However, despite a striking difference in melanin content determined by Fontana-Masson staining of the sections obtained from vehicle versus Bis treated areas, there was only a subtle difference in pigmentation clinically. This probably reflects the fact that melanin was located primarily in the basal layer (Figure 8A), rather than also substantially in the suprabasular layers, as in UV-irradiated skin, given that melanin near the skin surface is perceived as darker than melanin restricted to the basal layer (Quevedo and Holstein, 1998).

Cultured human melanocytes express the -α, -β, -δ, -ε and -ζ isoforms of PKC (Oka et al, 1995). Among these isoforms, only PKC-β has a well-defined role in the biology of melanocytes. The phorbol ester TPA was found to support long-term growth of melanocytes (Eisinger and Marko, 1982; Eisinger et al, 1983) via down-regulation, not activation of PKC, as was later shown (Brooks et al, 1991), although growth rate over a two-week period is not accelerated relative to that of melanocytes cultured in an otherwise optimized medium lacking pharmacologic modulators of PKC levels (Gilchrest and Friedmann, 1987). Down-regulation of PKC-α, -δ, -ε and -ζ has been associated with growth of melanocytic cells, but a causal relationship was not established (Brooks et al, 1993; Oka et al, 1995).

PKC-α and -β, classical PKCs, have similar activation requirements (Nishizuka, 1992) and both phosphorylate substrate sequences of XRXXSXRX, where X is any amino acid (Kemp and Pearsson, 1990). The amino acid sequence of TMP deviates considerably from this sequence, and it remains to be determined

whether TMP is a pseudo-substrate only for PKC-β. Unique substrate sequences have not yet been identified for any PKC isoform.

Ideally, the present studies would have been performed with an agent known to be completely specific inhibitors of PKC-β, but no such agent now exists. The TMP peptide has the same sequence as the portion of tyrosinase shown to be phosphorylated exclusively by PKC-β in melanocytes, but it remains unclear whether this specificity is a property of the amino acid sequence or selective localization of PKC-β to the melanosome membrane, adjacent to tyrosinase (Park et al, 2000). Hence, TMP introduced into melanocytes with Lipofectamine may serve as a pseudo-substrate for other PKC isoforms as well. Bis is described as a selective PKC-β inhibitor (Toullec et al, 1991) but at the concentrations employed in our experiments might easily have also inhibited PKC-α, the other cPKC isoforms expressed in both melanocytes and other cell types in skin, notably keratinocytes. Although the exact mechanism of action has not been elucidated, PKC-α has been implicated in the proliferation and differentiation of cultured keratinocytes (Gherzi et al, 1992; Lee et al, 1998; Stanwell et al, 1996). Therefore, topical application of Bis might influence keratinocyte behavior as well as reduce pigmentation through its effects on melanocytes. Despite this ambiguity, however, the present data demonstrate that PKC inhibition either in isolated melanocytes or in intact skin reduces pigmentation without having other detectable effects after up to 5 weeks of application.

In addition to blocking UV-induced pigmentation and reducing basal pigmentation in the epidermis inhibition of PKC-β activity also lightened mouse coat color, indicating that Bis not only penetrates into the viable epidermis but also into the follicular root sheath. One application per day almost totally blocks melanogenesis during the anagen phase of the hair cycle.

Although not studied in the present experiments, it is likely that the effects of Bis on pigmentation are reversible. The half-life and stability of Bis in tissues have not been determined, but the effects of Bis on PKC activity appear to be transient (Toullec et al., 1991), implying that its effect is reversible. Supporting this idea, the activation of PKC by DAG, known to have a short half-life (Nishizuka, 1984), increases pigmentation in guinea pig skin in a reversible fashion (Allan et al, 1995).

In summary, we have demonstrated that topical application of a PKC inhibitor reduces skin pigmentation *in vivo*, an effect attributable specifically to PKC-β inhibition. Topical application of such inhibitors may prove beneficial in

treating unwanted hyperpigmentation of human skin and/or in lightening hair color.

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Chapter 4

Confocal histopathology of irritant contact dermatitis *in vivo* and the impact of skin color (black vs white)

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Abstract

The pathogenesis of irritant contact dermatitis and its modulation according to skin color is not well understood. Reflectance confocal microscopy (RCM) enables high resolution real-time *in vivo* imaging of human skin. The goal of our study was to utilize RCM to determine whether susceptibility to irritant contact dermatitis differs between black and white skin. Participants were placed in groups based on skin color and their volar forearms exposed to 1% and 4% sodium lauryl sulfate (SLS) using Finn Chambers. They were evaluated at 6, 24 and 48 hours by RCM, transepidermal water loss, laser Doppler velocimetry and routine histology. Participants with white skin had more severe clinical reactions than participants with black skin. RCM revealed microscopic changes even without clinical evidence of irritation. Confocal features included parakeratosis, spongiosis, perivascular inflammatory infiltrate, and microvesicle formation, and these features were confirmed by routine histology. Also, white participants had greater mean increases in transepidermal water loss after exposure to 4% SLS than black participants. In conclusion, *in vivo* RCM can track early pathophysiologic events revealing differences between black and white skin during the development of irritant contact dermatitis, and may support the theory that black participants are more resistant to irritants.

Introduction

Irritant contact dermatitis (ICD) is the most common form of dermatitis and is defined as non-specific damage to the skin after exposure to an irritant. Clinical manifestations are influenced by several factors including the concentration of the chemical and duration of exposure as well as temperature, humidity and anatomic location. Additionally, these manifestations can be influenced by individual characteristics such as age, sex, pre-existing skin disease and ethnic origin.[1,2]

Our work investigates further the influence of skin color (black versus white skin). Several reports demonstrate that black individuals may be more resistant to skin irritants than white individuals.[3,4] Tape stripping eliminates this difference suggesting that susceptibility to irritants relates to stratum corneum function.[4]. Structural studies support the theory that the stratum corneum of black people provides a more resistant barrier due to an increased intercellular cohesiveness of the stratum corneum, possibly related to a more dense, compact stratum corneum.[5,6] Also, Reinertson and Wheatley[7] demonstrated a higher lipid content in the stratum corneum of black individuals and this may provide better barrier function. A more recent study also found a greater lipid content in black than white skin and a more compact but equally thick stratum corneum.[8] However, other authors have shown conflicting data on the relative susceptibility to irritants in black and white skin.[9,10,11,12]. These differences probably reflect the variable response of human skin to irritants in general, making it difficult to draw definite conclusions about a specific subpopulation.

Reflectance confocal microscopy (RCM) has been used to non-invasively image human skin, providing a virtual window into tissues *in vivo* without obvious artifacts or destruction.[13,14] The device operates by tightly focusing a low-power, near-infrared laser beam on a specific point in the skin, and then detecting only the light reflected from the focal point through a pinhole-sized spatial filter.[15] The beam is then scanned horizontally over a two-dimensional grid to yield a horizontal section. The measured lateral resolution of 0.5-1μm and measured axial resolution (section thickness) of 2-5μm yield images comparable to routine histology.[16] Penetration depth is sufficient for imaging the epidermis, as well as the papillary and upper reticular dermis.

The goals of this investigation were to define the major histologic features of ICD in real time by *in vivo* RCM, and to determine whether racial or ethnic differences exist in the susceptibility to the early development of ICD by comparing two subject groups: blacks and whites. Transepidermal water loss (TEWL) measurement and laser Doppler velocimetry (LDV), standard non-invasive research techniques for ICD evaluation, were also utilized as additional means of study.

Materials and methods

Participants

Fourteen healthy volunteers between the ages of 18 and 40 were recruited to participate in this study which was approved by the Institutional Review Board. There were 8 individuals with white skin (6 with Fitzpatrick skin phototype II and 2 with Fitzpatrick skin phototype III), and 6 individuals with black skin (5 with Fitzpatrick skin phototype V and 1 with Fitzpatrick skin phototype VI). Exclusion criteria included age greater than 40 years and a positive history of atopy.

Exposure to irritant

The volar forearm skin was exposed to 35 μl of 4% and 1% sodium lauryl sulphate (SLS), and 10X phosphate buffered saline (PBS) using a strip of four 10mm Finn Chambers (Allerderm Laboratories, Inc., Petaluma, CA). Chamber #1 contained 4% SLS and was removed after 6 hours of exposure. Chambers #2-4, containing 4% and 1% SLS, and PBS, respectively, were removed 24 hours after application.

Evaluation

Participants returned for evaluation 6, 24, and 48 hours after initial application of the patch. Prior to all evaluations, 15 to 30 minutes were allowed for the skin to recover from chamber occlusion. During the 6-hour visit, site #1 and an adjacent normal skin site were evaluated. The remaining sites #2-4 were assessed at both the 24 and 48 hour follow-ups.

At each follow-up session, each site was evaluated using several techniques.

Clinical evaluation: Sites were clinically graded using a visual scoring scale (Table I) and digital photographs were obtained.

Transepidermal water loss: TEWL measurements were performed using the Dermalab device (Cortex Technologies, cyberDERM, Inc, Media, PA). One probe was applied to the skin surface of the exposure site with appropriate technique and environmental controls;[17,18] TEWL measurements were obtained during a continuous 60 second period, and expressed in units of $g/m^2/h$.

Laser doppler velocimetry: LDV was performed using a laser Doppler flowmeter, model ALF 21 (Transonic Systems Inc, Ithaca, NY). A low-power infrared diode laser was applied via a small probe constantly maintained at a fixed distance (8-10 mm) from the skin surface. The technique measures cutaneous microcirculatory flow and is based upon the Doppler broadening of laser light scattered by moving particles (erythrocytes). This broadened signal is detected and analyzed to give information about moving particle density and flux (used to express blood volume flow).[19] These measurements are expressed in units of ml/min/100g.

Table I. Visual Scoring Scale used to assess clinical development of irritant contact dermatitis after exposure to SLS

Score	Term	Meaning
0	Negative	No response
1	Minimal	Slight erythema
2	Mild	Mild erythema
3	Moderate	Moderate erythema + edema
4	Severe	Severe erythema + edema + vesicles
5	Necrosis	

In vivo reflectance confocal microscopy: *In vivo* RCM was accomplished with a commercially available device (Vivascope 1000, Lucid Inc, Henrietta, NY) using an 830nm diode laser with a maximum power of 15mW. A 30x water immersion objective lens of numerical aperture 0.95 was used. The skin-contact device consists of a housing that encloses the objective lens, and a ring and template which was affixed to the skin. While moving the objective lens relative to the skin with X, Y, and Z - plane micrometer screws, images captured were in axial sections beginning with the stratum corneum, through the epidermis, and into the upper reticular dermis.

As previously reported,[14] images of optimal quality are obtained by matching the refractive index (n) of immersion medium with that of the epidermis (n = 1.34). The immersion medium that we employed for this study was water

(n = 1.33). A coverslip was used because it produced a more stable image for reliably measuring stratum corneum thickness.

In vivo RCM was used to measure thickness of the stratum corneum (SC), and depth of the suprapapillary epidermal plate (SPP) and rete pegs in real time. SPP depth was defined as the distance between the top of the SC and the bottom of the cells in the uppermost portion of the stratum basale.[20] We also grabbed sequential images of blood vessels to record the intraluminal flow. Papillary dermis and upper reticular dermis depths were noted. At each evaluation site, a minimum of 6 images of each of the aforementioned skin layers were captured.

Histology

Up to two confirmational skin biopsies (3 mm punch) were obtained from each individual after local intradermal anesthesia (2% lidocaine with epinephrine). Biopsies were obtained from the site exposed to PBS and the site that demonstrated maximal evidence of ICD. The biopsies were fixed in buffered 10% formalin, processed and embedded in paraffin. Each sample was cut in half and sectioned both horizontally and vertically for hematoxylin and eosin staining, and for Fontana-Masson staining.

Statistical Analysis

Mean and standard deviation (SD) values were calculated. The Student's two-tailed t-test was used to determine whether there was a significant difference ($p<0.05$) between the black and white groups for the following parameters: a) Thickness of the SC and depth of the SPP measured by *in vivo* RCM for each site at each evaluation timepoint; b)TEWL measurements; and c) LDV measurements. Also, Pearson r correlation was calculated to determine correlation between SC thickness and TEWL for each site at all evaluation timepoints.

Results

Clinical Evaluation of irritant contact dermatitis

The average clinical scores tended to be lower in black than white individuals, at most time points assessed, for various concentrations of SLS, though this difference was not statistically significant (Table II). Although none of the

Table II. Average clinical scores for black and white individuals at different timepoints and with different concentrations of SLS

SLS concentration and timepoint		Black skin	White skin
1% SLS	24 h	0.42	0.88
	48 h	0.83	1.19
4% SLS	6 h	1.08	1.06
	24 h	1	1.44
	48 h	1	1.69

SLS: Sodium lauryl sulfate

individuals had severe reactions to this concentration of SLS, white individuals showed more mild-moderate reactions and less negative-minimal reactions to the SLS, while black individuals consistently showed more negative-minimal reactions.

Confocal images of irritant contact dermatitis

Descriptive analysis: Differences between normal skin and skin exposed to SLS were easily appreciated using *in vivo* RCM, both in the black and white groups, since several key histopathologic features characteristic of ICD could be seen with *in vivo* RCM (Figure 1). Even skin exposed to lower SLS concentrations (1%) revealed live microscopic changes with RCM, frequently without clinical evidence of ICD. One early change seen with RCM was disruption of the stratum corneum, revealing characteristic "breaks" and gray, punched-out areas in the normally homogeneous highly-refractile pattern (Figure 1a). This finding was seen in all individuals. Focal parakeratosis, visible as retained nuclei in the stratum corneum, and spongiosis (seen as intense intercellular brightness) were further recognized as early changes with *in vivo* RCM (Figure 1b & 1c). Other microscopic changes such as exocytosis and acantholytic keratinocytes (1d), microvesicles containing inflammatory cells and detached keratinocytes (1e), and perivascular inflammatory infiltrate were also seen (1f). An interesting finding was an increase in brightness of basal keratinocytes in ICD, probably due to increased melanin content (the best endogenous contrast agent in RCM[13]). This finding was supported by routine histology using the Fontana-Masson stain (Figure 2) This was typically visible at 48 hours after application of 4% SLS and was more prominent in the black population.

Quantitative analysis: The black and white individuals did not show a significant difference between the SC thickness of control sites (Table III). However, the

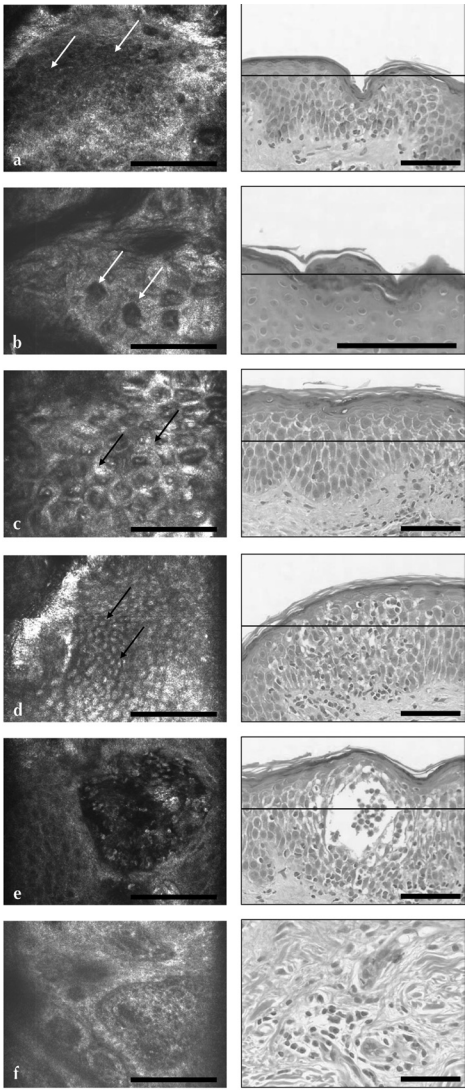

Figure 1. Histopathologic correlation: CM images (left panel) and routine H&E sections (right panel). Images are representative images from different patients. (a) SC disruption (arrows); (b) Parakeratosis, arrows show retention of nuclei in SC; (c) Spongiosis, arrows highlight increased intercellular brightness, edema; (d) Exocytosis and acantholysis, confluence of dyskeratotic keratinocytes, examples shown with arrows; (e) Microvesicle, containing inflammatory cells and dyskeratotic keratinocytes; (f) Perivascular infiltrate, arrows to individual inflammatory cells. Scale bars represent 100μm. Horizontal lines on H&E sections show corresponding levels of *en face* images. (☞ page)

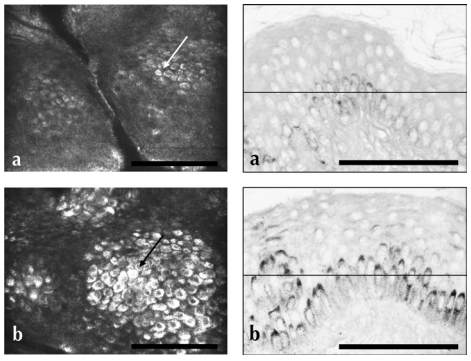

Figure 2. Histopathologic correlation: CM images (left panel) and routine H&E sections (right panel). Control: highly-refractile basal keratinocytes seen at dermal-epidermal junction (a, left panel) and Fontana-Masson stain showing melanin in basal keratinocytes (a, right panel). Site exposed to *4% SLS* in same individual, showing brighter basal keratinocytes after 48 hours (b, left panel) and Fontana-Masson demonstrating increased melanin content (b, right panel). Scale bars represent 100μm. Horizontal lines on H&E sections show corresponding levels of *en face* CM images. (☞ page)

Table III. Comparison of SC thickness and SPP thickness between the black and white groups after SLS application at various time-points using Student t-test

Site	Time	Histologic parameter	Mean depth (μm) for groups		t-value	α
			Black skin	White skin		
Control	0h	SC	13.4 ± 2.7	13.3 ± 4.0	0.05	NS
4%	48h	SC	10.4 ± 4.7	12.4 ± 4.6	2.35	0.05
Control*	0h	SPP	53.3 ± 12.1	53.0 ± 7.8	0.10	NS
4%*	24h	SPP	53.2 ± 10.5	61.0 ± 17.7	2.40	0.05
4%*	48h	SPP	51.3 ± 5.5	57.8 ± 10.3	3.13	0.01
1%*	48h	SPP	50.5 ± 7.5	56.6 ± 9.9	2.82	0.01

NS: non significant, SC: stratum corneum thickness, SPP: suprapapillary plate thickness

SC thickness was significantly less in the black group compared to the white group after exposure to 4% SLS at 48 hours ($p<0.05$). No other sites revealed any additional significant differences in SC thickness at the time-points assessed. Similarly, SPP depths of the control sites showed no significant difference between the two groups. Assessment of sites exposed to 4% SLS revealed that the SPP depth was significantly greater in white than in blacks after 24 hours ($p<0.05$), and after 48 hours of exposure ($p<0.01$). This was also observed in sites exposed to 1% SLS at the 48-hour evaluation ($p<0.01$). Other sites demonstrated no additional significant differences in SPP depth at other time-points.

At all timepoints assessed, white individuals had a trend towards greater mean increases in TEWL after exposure to SLS than did black individuals, although the differences were not statistically significant (Figure 3). There was no statistically significant difference in LDV measurements between the black and white individuals. Changes in both the TEWL and in SC thickness between

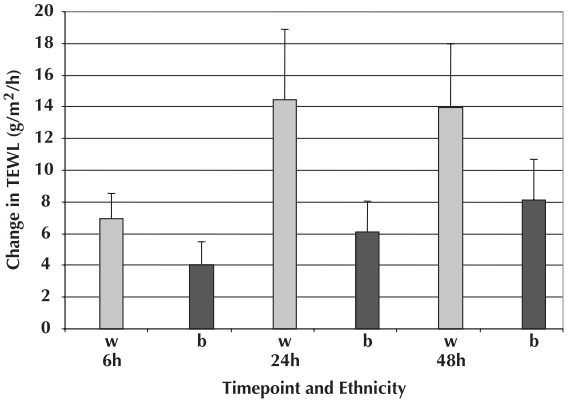

Figure 3. Change in TEWL with irritant exposure for white and black individuals.
The difference in TEWL between control sites, and sites at various timepoints after application of 4% SLS, is shown for white (n=7) and black (n=6) individuals. Data points shown are mean ±SEM. White skin = gray bars, black skin = solid bars.

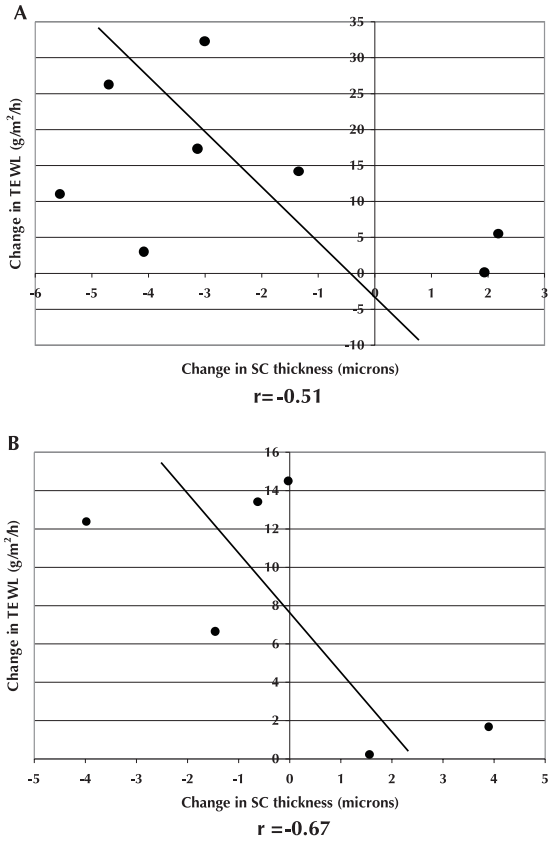

Figure 4. Change in SC thickness *vs* change in TEWL at 48 hours after application of 4% SLS. (A) white individuals (n = 8), (B) black individuals (n=6). The difference between SC thickness in a site exposed to 4% SLS and a control site, at 48 hours, was calculated and compared with the difference in TEWL for the same irritant concentration and time point.

the control site and the site exposed to 4% SLS at 48 hours were negatively correlated, as shown in figure 4, for both black and white individuals. Such a clear pattern was not seen at 6 or 24 hours, or with lower concentrations of SLS. White individuals, however, had greater mean increases in TEWL after exposure to 4% SLS than did black individuals, and this was seen at all time points assessed. (Figure 4).

Discussion

Racial or ethnic differences in skin reactivity to cutaneous irritants is an important area of research. The majority of the literature examining differences between black and white individuals points towards a reduced susceptibility to ICD among black individuals, and relates this to better barrier (stratum corneum) function.[3,5,6,12]

We found that white individuals did tend to have more intense clinical reactions to SLS than did black individuals. This could be related to the pathophysiological epidermal events like spongiosis, evidenced by significantly increased SPP depth measured by *in vivo* RCM in whites. Skin color differences may also play a factor as darker skin tones of the black individuals may have prevented accurate assessment of clinical erythematous reaction, although edema and papule formation was easily appreciated in all individuals.

In vivo near infrared RCM is a novel imaging tool that permits real-time qualitative and quantitative study of healthy [20] and diseased [21,22] human skin. It also enables monitoring of dynamic events.[23] It uses a near infrared laser beam which is focused into the skin and detects backscattered light that passes through a pinhole. This enables "virtual sectioning" of live tissue and yields high resolution *en face* images comparable to routine histology.[16] In this study we have described the common histopathologic features of ICD using this technique, including stratum corneum disruption, parakeratosis, spongiosis, acantholysis, microvesicle formation, and perivascular inflammatory infiltrate. These features have previously been demonstrated in allergic contact dermatitis using RCM.[21] We also described a marked increase in pigmented basal keratinocytes. These features were confirmed by routine histology.

There was no difference between the black and white groups for the SC thickness at the control site. This supports a previous finding by Weigand and colleagues[5] who reported that although there were more layers in the stratum corneum of blacks, it was more compact and the same thickness as that of whites. After exposure to 4% SLS, both groups experienced a relative "thinning" of the SC as compared to control by 48 hours. Moreover, there was a significant difference between the two groups, with the black individuals having a thinner SC than the white group. One possible explanation is that the SC is simply thinner in the black group because it experienced more erosion by the irritant than the white group. This explanation does not support the aforementioned findings of Weigand et al[5] because if the SC of blacks were more densely layered, one would expect that their skin would be more impenetrable to SLS.

The suprapapillary epidermal plate of the white group was significantly deeper than that of the black group exposed to 4% SLS, when assessed at 24 and 48 hours, and also to 1% SLS, assessed at 48 hours. This increased SPP depth could possibly be attributed to more severe spongiosis and vesicle

formation within the epidermis, as stated previously,[21] and is consistent with white individuals having a more severe clinical reaction. This appears contrary to the finding that SC thinning is greater in black individuals and the reason for this is incompletely understood.

The negative correlation between SC thickness and TEWL is intuitive and supports the current understanding of TEWL as a measurement of SC barrier function.[24] It also supports the legitimacy of *in vivo* RCM as a method for evaluating SC integrity. TEWL additionally reflects damage of the SC in proportion to SC thickness in both the black and white groups. The fact that this correlation is seen at 48 hours is interesting because it suggests that although the irritant is removed at 24 hours, the damage persists and perhaps even progresses for at least 24 more hours. The mean increases in TEWL of the white group were consistently greater than those of the black group. This was not statistically significant, probably due to small sample size. This finding may support the concept that the SC barrier function is more durable in blacks than in whites.

LDV was another non-invasive method used as an adjunct to *in vivo* RCM. There was no significant difference in the mean values of the black and white groups for any of the sites or time-points tested. We found that problems arose during administration of the flowmeter to the individual, however. As previously reported in the literature,[19] any minute change in position or orientation of the probe can cause significant changes in estimation of blood flow. Furthermore, two measurements from different sites (or measurements from what is believed to be the same site at different points in time) cannot be directly compared, minimizing the usefulness of this technique within a person over time.[19] Thus, we are not convinced of its reliability as a method for the evaluation of cutaneous blood flow for various sites at different time-points.

To summarize, we found that there was a difference in the reactivity to SLS of black and white individuals as assessed by clinical reaction, morphometric epidermal changes as measured by *in vivo* RCM, and TEWL measurement. Our data may support the theory that black individuals are more resistant to the development of acute ICD induced by surfactants such as SLS. Similar studies with larger sample sizes should be structured with extended evaluation timepoints to follow the reparative process of the SC and other epidermal layers.

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Chapter 5

Oral *Polypodium leucotomos* extract decreases ultraviolet-induced damage of human skin

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Abstract

Ultraviolet radiation (UVR) induces damage to human skin. Protection of skin by an oral photoprotective agent would have substantial benefits. We investigated the photoprotective effect of oral administration of an extract of the natural anti-oxidant *Polypodium leucotomos* (PL). Nine healthy subjects of skin types II–III were exposed to varying doses of artificial UVR without and after oral administration of PL (7.5 mg/kg). Twenty-four hours after exposure the erythema reaction was assessed and paired biopsies were obtained from PL-treated and untreated skin. A significant decrease in erythema was found in PL-treated skin ($p<0.01$). Histologically, PL-treated biopsies showed less sunburn cells ($p<0.05$), cyclobutane pyrimidine dimers ($p<0.001$), proliferating epidermal cells ($p<0.001$), and dermal mast cell infiltration ($p<0.05$). A trend towards Langerhans cell preservation was seen. In conclusion, oral administration of PL is an effective systemic chemophotoprotective agent leading to significant protection of skin against UVR.

Introduction

Exposure of human skin to sunlight, containing ultraviolet (UV) radiation A and B, leads to deleterious effects on skin such as sunburn, immune suppression, pigmentary changes, photo-aging and skin cancer.[1] The mechanism of such cutaneous damage induction is complex, but can be broadly divided in direct oxygen-independent damage through absorption of photons, and in oxidative damage, caused by formation of free radicals and reactive oxygen species.[2] This is why anti-oxidants have been increasingly studied as inhibitors or quenchers of UV-induced cutaneous damage. Currently the most widely used method of protection against UV-induced damage is the use of topical sunscreens enriched with UV absorbing chemicals. A systemic photoprotective agent would obviously have an advantage over topical protection as this would provide a uniform, total body surface protection without the variance in protection commonly observed with topical sunscreens.[3] Attempts have been made to investigate the photoprotective effects of systemic anti-oxidants. However, oral anti-oxidants such as tocopherol, ascorbate or carotenoids have shown varying effects as some studies show a slight to moderate increase in the minimal erythema dose (MED) in humans,[4-9] while others did not find an increased MED,[10-12] reduction of sunburn cells,[10,13] or protection against DNA damage.[11] When a photoprotective effect was observed, this was usually after prolonged administration of the anti-oxidants.[4,5,8] Green tea also seems a promising anti-oxidant, but its protective effect after oral administration has so far not been evaluated in controlled human studies.[14]

In this study we investigated the photoprotective effect of orally administered *Polypodium leucotomos* (PL) against acute UV-induced damage to human skin using a solar simulator. PL is an extract from a fern plant grown in Central America.[15] *In vitro* studies have shown that PL acts as an effective anti-oxidant by quenching superoxide anion, singlet oxygen, lipid peroxides and the hydroxyl radical.[16,17] Previous studies showed that topical and oral PL decreased acute sunburn response and resulted in Langerhans cell preservation of human skin when exposed to sunlight[18] and PUVA.[19] The goal of this study is to further analyze the ability of orally administered PL to decrease UV- induced erythema and the resulting histological skin damage under carefully controlled laboratory conditions.

Subjects and Methods

Subject selection

We included 9 healthy subjects with skin phototype II or III in this open-label study after they read and signed a written informed consent form and protocol approved by an Institutional Review Board of Massachusetts General Hospital. The study was conducted over a period of one year in our laboratories. The group consisted of 5 men and 4 women with ages ranging from 25 to 46 years old. Subjects were excluded when having a personal or family history of skin cancer, a history of abnormal photosensitivity, or taking any drug that might alter the response of skin to UV radiation (UVR). We used the skin of the back of each subject for these studies, which had to be free of any blemishes and not exposed to sunlight or artificial UV radiation sources (tanning booth) for at least 8 weeks prior to the study.

Radiation source and exposure conditions

A solar simulator consisting of a 1000 watt high-pressure Xenon arc lamp (Oriel Corp., Stratford, CT) emitting a collimated beam and equipped with a 2 mm WG-305 filter (Schott Glas, Mainz, Germany) and a first surface mirror (Edmund Scientific, Barrington, NJ) was used as radiation source (305-400 nm). A high velocity fan eliminated any impact of heat from the infrared radiation of the lamp on the exposed skin of the subject. The emitted UVR intensity of the source was measured with a calibrated radiometer (International Light, Newburyport, MA) before each experiment when the lamp had warmed up for 30 minutes, and after each experimental protocol to ensure stability of the UVR output, which was usually around 0.2 mW/cm^2. The skin was irradiated through adhesive UVR-reflecting aluminum stickers containing 6 or 7 exposure windows each 3.3 cm^2 in size (DV Die Cutting Inc., Danvers, MA). One photo-test consisted of exposure of each skin site to UVR doses with a relative increase of 6-33% to obtain an exposure range with regular incremental UVR doses. A dose between 2 to 3 times the MED was always included when a biopsy was obtained. The MED was defined as the minimal dose of UVR inducing confluent erythema at 20-24 hours (hrs) with 4 sharp borders of the exposed skin site. From the 7 exposed skin sites, either the 1st, 2nd, 3rd or 4th site was exposed to the MED dose, and either the 5th or 7th site was exposed to the 2-3x MED. During exposures the rest of the skin of

the back of the subject was covered by an opaque UV-protective cloth. The distance between the exposed skin and the solar simulator was kept constant by stabilizing the back skin against an aluminum template located at a fixed distance from the exit port of the lamp.

Study design

Prior to the start of the study each subject's MED was assessed. Once the MED was known, a set of 6 or 7 skin sites was exposed to UVR without PL. The erythema reaction of all subjects was visually graded 24 hrs later. A 3 mm punch biopsy was taken from 5 subjects after 24 hrs and from 2 subjects 72 hrs after exposure.

After completion of this initial part of the protocol, the subjects received the first dose of oral PL the evening before the second exposure. The next day each subject received the second dose of oral PL, after which they were exposed to the same set of fluences given in the first part of the protocol, but at 5 different time-points, i.e. after 30 minutes, 1 hr, 1 hr and 30 minutes, 2 hrs, and 3 hrs of ingestion of oral PL. Skin erythema was again evaluated after 24 hrs, and a second skin biopsy was taken from the same subjects: 5 after 24 hrs, and 2 after 72 hrs. Specifics about the biopsies are given further on in the "Histology" section.

The capsules containing PL (180 mg each) were supplied by I.F.Cantabria, S.A. (Madrid, Spain) and were administered orally in a dose of 7.5 mg/kg body weight.

Clinical evaluation

Clinical evaluation of the erythema response of every exposed skin site was performed by at least 2 experienced investigators using a scoring system for the intensity of erythema and edema ranging from grades 0 to 4 (0= no erythema; 1= trace erythema; 2= visible, not confluent erythema, no sharp borders; 3= confluent erythema with 4 sharp borders and no edema (MED); 4= intense erythema with edema). The mean of the grades of each skin site was calculated for all subjects at every time-point of exposure.

Histology

Biopsies taken during the first part of the protocol were obtained from the skin site exposed to the 2-3x MED. Likewise, the biopsy obtained in the second

part of the protocol was taken from the skin site exposed to the same fluence, from the photo-test at the time-point showing maximal photo-protection. All skin specimens were cut in half. One half was fixed in 10% buffered formalin and embedded in paraffin for sectioning and microscopic evaluation. Three µm sections were routinely stained with hematoxylin and eosin for histologic gross evaluation and quantitative assessment of sunburn cells. The other half was imbedded in OCT compound (Miles Inc. Diagnostic Division, Elkhart, IN, USA) and stored at –70ºC.

Immunohistochemistry

Five µm sections were used for immunohistochemical analysis. They were deparaffinized with xylene and rehydrated with graded ethanols. Endogenous peroxidase was blocked with hydrogen peroxide, and the samples were then rinsed in phosphate-buffered saline. To achieve adequate intensity signals with the respective antibodies, heat-induced antigen retrieval was carried out by microwave pretreatment in citric acid buffer (10 mM; pH 6.0) for 20 minutes. For reduction of background labeling, the sections were blocked for 30 minutes in normal horse serum. The sections were later incubated overnight at 4°C with the commercially available antibodies: i) prediluted anti-CD1a for Langerhans cells (Immunotech, clone O10, cat. No. 1590, Marseille, France)[20]; ii) 1:150 diluted anti-Ki67 for proliferating keratinocytes (DAKO, clone MIB-1, cat. No. M72470, Glostrup, Denmark)[21]; iii) 1:100 diluted anti-tryptase for mast cells (DAKO, clone AA1, cat. No. M7057)[22]; and iv) 1:10 diluted anti-CD31 for endothelial cells (DAKO, clone JC/70A, cat. No. M0823).[23] After washing with phosphate-buffered saline, sections were incubated with biotinylated secondary antibody, avidin-biotin-peroxidase complex (ABC Elite; Vector Laboratories, Burlingame, CA), and then 3,3'-diaminobenzidine (DAB). Each section was counterstained with hematoxylin, dehydrated, and covered with a coverslip.

For detection of UV-induced cyclobutane pyrimidine dimers (CPDs) we fixed 5 µm sections of frozen tissue (from 3 subjects 24 hours after UV irradiation) on slides with ice-cold methanol-acetone (1:1) for 10 minutes at –20°C then air-dried. For immunohistological detection of CPDs, we incubated acetone-fixed sections with CPD-specific antibodies (kind gift from Drs. Toshio Mori and Nobuhiko Kobayashi) overnight, then used the AEC mouse tissue detection system (LabVision, Fremont, California) as described in the manufacturer's

protocol. Positive and negative controls were always included in each staining run.

Quantitative histological analysis

All parameters listed below, except for CPDs, were quantified in a blinded fashion.

Sunburn cells: Sunburn cells were defined as cells with a hypereosinophilic cytoplasm and a dense, small, dark, and irregularly formed nucleus in comparison to neighboring cells, and located in the epidermis away from areas of blistering or crush artifacts.[24] Sunburn cells were quantified in 4 entire sections per specimen (10-11 fields per section) using a light microscope at a magnification of 40x. Using an eyepiece micrometer, the average number of sunburn cells per mm epidermal length was calculated.

CPDs: The number of CPD positive cells was counted in approximately 5 fields-of-view in 6 paired biopsies at a magnification of 20x. CPD positive cells were defined as bright red stained cells. The number of CPD positive cells was calculated per mm epidermis.

Proliferating cells: Ki67 immunoreactivity (proliferating cells) was quantified with an image analysis system (Visilog, Noesis, France). The equipment used included a microscope (Elipse E400, Nikon, Tokyo, Japan) with a 20x objective lens, a digital color camera (Polaroid Corporation, Waltham, MA), and image-processing and analysis software (version 5.2, Visilog). This program is able to differentiate and count hematoxylin-stained objects and Lag-red/brown (DAB-stained) objects. The percentage of proliferating epidermal cells was calculated by dividing the number of immunoreactive nuclei by the total number of epidermal nuclei.

Langerhans cells and mast cells: CD1a positive cells (Langerhans cells) and tryptase positive cells (mast cells) were quantified by light microscopy using a 40x objective lens and an eyepiece micrometer. A cell with a nucleus and clear immunoreactivity was considered a positive cell. In each biopsy the number of positive cells was counted in 8-10 consecutive fields, and then calculated per mm^2 of epidermal or upper dermal surface, respectively. To determine the number of mast cells, the micrometer and grid were aligned on one edge along the epidermal dermal junction.

Microvessels: Using a 40x objective lens, 8-10 consecutive fields were counted. CD31 positive cell (endothelial cell) clusters consisting of more than two cells

as well as large microvessels were included in the microvessel count.[25] The number of microvessels per mm^2 dermis was counted, and vasodilatation assessed as a percentage of the total surface area occupied by vessels divided by the total dermal surface area.

Statistical analysis

Clinically scored erythema grades were analyzed using ANOVA test. Mann-Whitney tests were performed to compare the number of sunburn cells, proliferating cells and microvessels as well as vasodilation in PL-treated and PL-untreated skin specimens. Repeated measurement MANOVA test was performed to compare the number of CPD-positive cells, Langerhans cells and mast cells. Analyses were performed on data obtained either at 24 hr, 72 hr or combined paired data. A *p*-value of 0.05 or smaller was considered statistically significant.

Results

Clinical results

PL-treated skin showed clearly less acute erythema reaction compared to PL-untreated skin when evaluated at 24 hrs after exposure (Figure 1). The difference in erythema was quantified by comparing the means of the

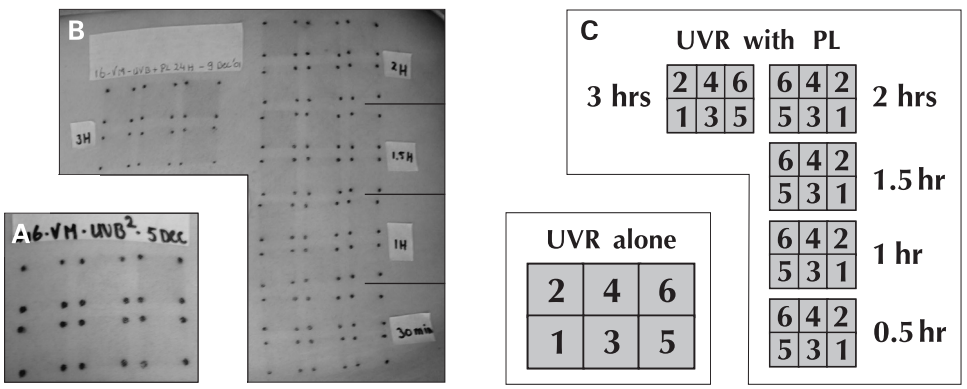

Figure 1. Pictures of 1 subject. (A) Erythema reaction 24 hrs after exposure to UVR alone. (B) Erythema reaction 24 hrs after exposure to UVR with PL. (C) Schematic illustration of UVR intensities of each site skin shown in pictures, indicated by numbers in squares (1 = lowest UVR intensity, 6 = highest UVR intensity). Some sites are mirrored. (☞ page)

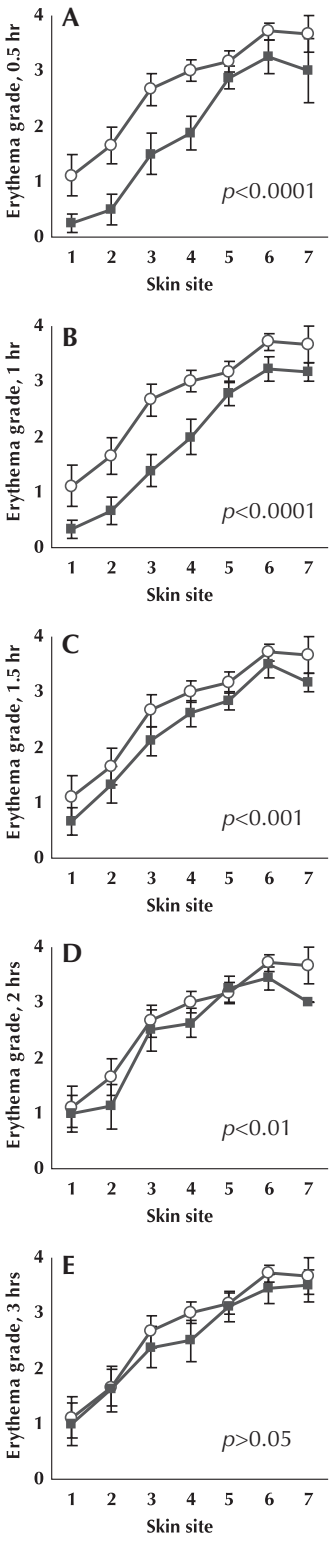

Figure 2. Open circles represent mean grades of skin sites exposed to UVR without PL, and closed squares to UVR with PL after: (A) 30 minutes; (B) 1 hr; (C) 1 hr and 30 minutes; (D) 2 hrs; and (E) 3 hrs. Values are the mean of the erythema grades at 24 hrs (*y*-axis) of each exposed skin site (*x*-axis) ± standard error of the mean (SEM). PL-treated skin has statistically significant lower erythema grades up until 2 hrs after administration (N=9 patients).

erythema grading between PL-treated and PL-untreated skin (Figure 2). The difference between the means of erythema grading of PL-treated and PL-untreated skin was statistically significant ($p<0.01$) up to 2 hours after administration of PL. After 3 hrs of PL administration, there was no significant difference anymore between the means of erythema of PL-treated and untreated skin ($p>0.05$).

Histological results

Hematoxylin and eosin stained sections from PL-treated skin clearly showed gross morphological differences when compared to PL-untreated skin at 24 hrs. PL-treated skin showed less UV-induced epidermal damage such as maturation disarray, microvesiculation, and vacuolization of keratinocytes, whereas this was more noticeable in PL-untreated skin (Figure 3a). The number of sunburn cells/mm epidermis was significantly lower in PL-treated skin when compared to PL-untreated skin at 24 hrs ($p=0.03$) (Figure 3a). The amount of CPDs was significantly lower in PL-treated skin compared to PL-untreated skin at 24 hrs ($p<0.001$) (Figure 3b). There was a significant difference in epidermal proliferation seen by Ki67 immunoreactivity in PL-treated *vs.* untreated skin at 72 hrs ($p<0.001$) (Figure 3c). PL administration also resulted in a significant reduction of tryptase

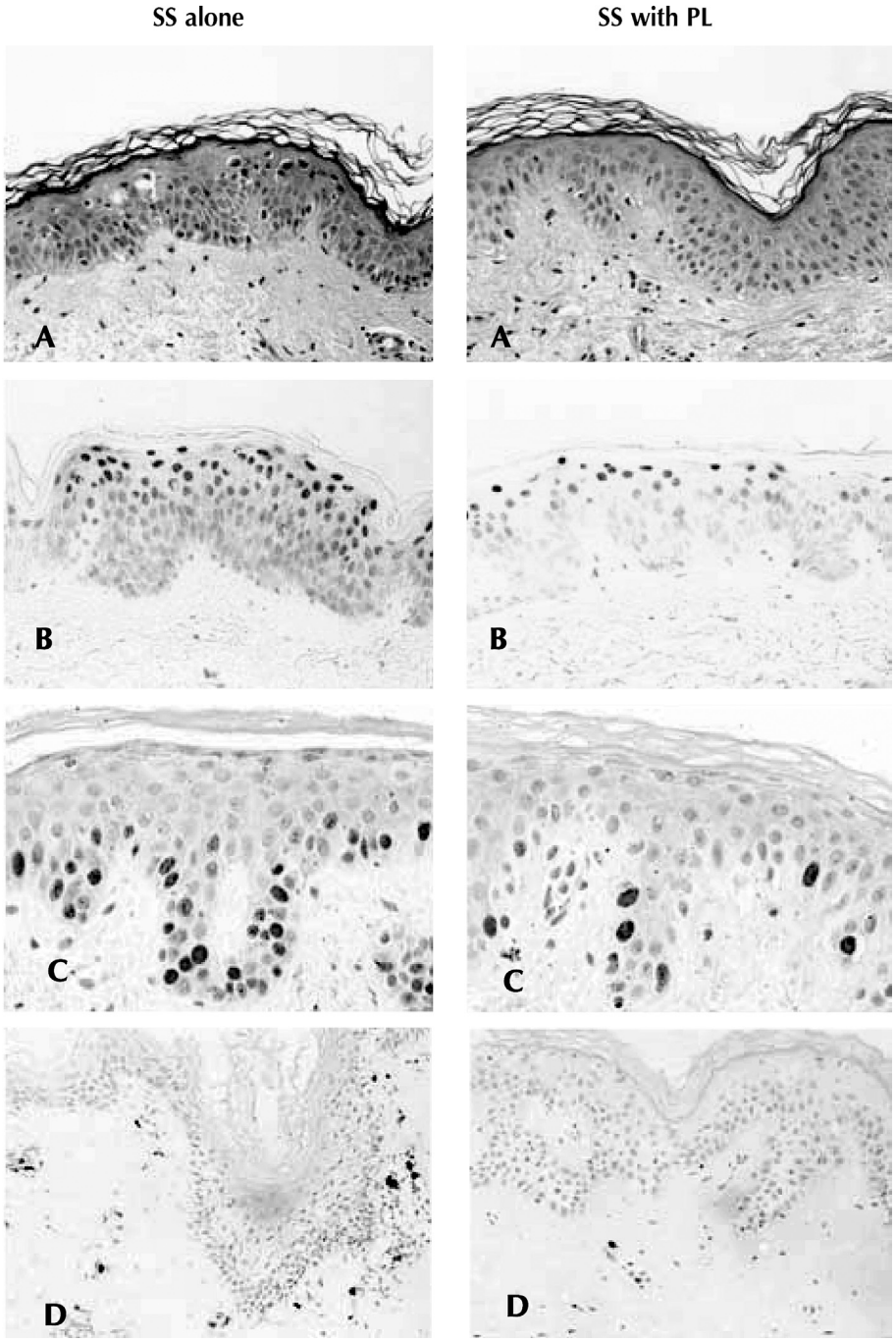

Figure 3. Histology from paired biopsies of skin treated with ultraviolet radiation alone (left columns) and with *Polypodium leucotomos* (PL) (right columns). PL-treated skin shows: (A) less sunburn cells, maturation disarray, microvesiculation, and vacuolization; (B) less cyclobutane pyrimidine dimers; (C) less epidermal proliferation; and (D) less dermal mast cells infiltration.
(☞ page)

Table I. Quantitative overview of histologic parameters and *p*-values between skin treated with UVR alone *vs*. UVR with PL

Histologic parameter	UVR alone Mean (SEM)	UVR with PL Mean (SEM)
Sunburn cells/mm epidermis*	22.4 (2.03)	16.3 (2.9)[a]
CPD positive cells/mm epidermis*	74.7 (4.58)	43.7 (4.03)[b]
Mast cells/mm^2 upper dermis**	173.76 (19.36)	126.4 (14.72)[a]
Vasodilatation (% vessel surface area) **	2.38 (0.21)	1.94 (0.18)
% Ki 67 positive epidermal cells***	38.85 (2.15)	25.94 (1.9)[b]
Langerhans cells/mm^2 epidermis***	18.56 (5.76)	24.8 (6.08)

For each slide the number of CPD positive cells, Langerhans cells and mast cells were counted in at least 10 view fields (ocular grid, 0.0625 mm^2; x400). Data from biopsies obtained at 24 hrs (*), at 24 and 72 hrs (**), and at 72 hrs (***). a = p<0.05; b = p<0.001.

positive mast cells in the upper dermis compared to PL-untreated skin at 24 hrs and 72 hrs ($p \leq 0.05$) (Figure 3d). Although not statistically significant, PL-treated skin showed preservation of Langerhans cells/mm^2 epidermis in response to UVR when compared to PL-untreated skin at 72 hrs. In addition, Langerhans cells in PL-untreated skin were increased in size and showed a loss of dendritic morphology, while in PL-treated skin these cells preserved their size and dendritic appearance. And finally, there was a decrease in the surface area occupied by microvessels in PL-treated *vs.* untreated skin 24 hrs and 72 hrs, although the difference was not statistically significant. Table I gives an overview of all histologic parameter counts and statistical results.

Discussion

In this paper we demonstrate that oral administration of only two doses of PL, a naturally occurring agent endowed with anti-oxidant properties, was able to lead to a significant decrease in erythema, sunburn cells, DNA damage, UV-induced epidermal hyperproliferation, and mast cell infiltration in human skin. Furthermore, the histological data show a trend toward Langerhans cell preservation and reduced vasodilatation. PL is an extract from a fern plant grown in Central America, that has been used for centuries by native Americans for the treatment of malignant tumors.[15] Elaborate studies have shown that PL has anti-oxidative properties,[16,17] immunomodulatory properties,[26-28] and anti-tumoral activity.[15,29] Based on anecdotal observations, PL had been used safely for over 30 years for the treatment of inflammatory skin diseases.[30-33]

In this study PL decreased skin sensitivity to UVR after intake of only two doses. This decrease was mild but significant, and can be compared to the effect obtained after repeated administration of other anti-oxidants.[6-8] The clinically visible erythema reduction is histologically supported by the decrease in vasodilatation, which plays a role in erythema formation. PL does not have a specific absorption peak or band in the UVB or UVA region,[17] and we believe the photoprotective effect observed in this study to result from PL's anti-oxidative properties. The reduced epidermal hyperproliferation in PL-treated skin ($p<0.001$), demonstrated by a lower percentage of Ki67 positive epidermal cells (Fig. 3c), also shows PL's photoprotection, as an exposure to UVR is known to induce epidermal hyperproliferation[34]. It is believed that the resulting epidermal hyperproliferation is a protective response by skin against additional damage.[34] Figure 2 illustrates that the clinical photoprotective effect decreased as time between exposure and PL intake increases. It is known that skin anti-oxidants can be depleted by exposure to UVR,[35] but this depletion occurs locally at the irradiated skin site.[36] We performed each photo-test separately while the rest of the skin was protected from UVR, therefore our results suggest a rapid absorption and early bio-availability of oral PL, subsequently leading to an early photo-protection that lasts up to 2 hrs after administration.

Although erythema is generally considered a marker for UVR damage, anti-oxidants decreasing erythema do not automatically provide photoprotection against long-term UVR effects such as skin (pre-)cancer formation.[37-39] Currently, retinoids are the main oral chemopreventive agents successfully used in skin cancer prevention,[40,41] but this occurs at doses that are associated with relevant side-effects such as increases in cholesterol levels and mucocutaneous toxicity.[42-43] Our histological results show that the photoprotective effect of PL extends beyond just decreasing the erythema reaction. CPDs are mutagenic and lead to carcinogenesis[44,45] and subsequently their prevention is highly desirable. It is interesting that PL decreases CPDs as these lesions are not thought to result from oxidative damage.[2] However, DNA repair enzymes are susceptible to damage through oxidative stress,[46] and it is possible that the anti-oxidative properties of PL reduced this damage, allowing a better DNA repair and subsequently leading to lower numbers of CPDs. Others have found reduced amounts of CPDs in mice immediately after UVR exposure with topical antioxidant use, with a similar rate of CPD

reduction afterward in treated and control group and they concluded that this could not be due to enhanced repair.[47] However, DNA repair is known to be a cellular process that takes place continuously[48] and therefore we believe that enhanced repair might be one of the factors involved. Nonetheless, additional research work is warranted to further investigate this issue. Reduction of CPDs has been shown to reduce skin cancer development.[49] The significant decrease of CPDs ($p<0.001$) by PL is therefore promising because this is the first report of an oral anti-oxidant to decrease DNA damage, and it suggests that PL might be able to prevent long-term skin damage such as skin cancer. Additionally, PL significantly decreased sunburn cells ($p<0.05$). Sunburn cells emerge when epidermal cells have suffered irreparable damage such as DNA damage,[50] and their numerical reduction shows the photoprotective ability of PL to decrease UVR induced cell damage, most likely including DNA damage. We also found a trend towards Langerhans cell preservation in PL-treated skin. A study with a 10 week B-carotene supplementation lead to Langerhans cell preservation after physiological doses of UVR.[51] However, our study is the first to report of an oral anti-oxidant showing a tendency towards Langerhans cell preservation after exposure to 2 times the MED with intake of only 2 doses ($p>0.05$). In PL-treated skin exposed to PUVA the Langerhans cell preservation was significant.[19] Langerhans cell depletion is thought to play a role in the development of skin cancer, as they have been shown to be vital for the induction of tumor specific immunity against UVR induced tumors[52] and their presence has been shown to be indispensable for tumor rejection.[53] Finally, cell proliferation has been proposed as a predictive biomarker for carcinogenesis.[54] PL reduced epidermal proliferation as seen by the lower percentage of Ki67 positive keratinocytes. The combined histological data is promising as it suggests that oral PL might help in the prevention of long-term UVR skin damage, such as skin cancer. *In vivo* animal studies have shown that PL may be effective in decreasing the prevalence of UVB-induced skin cancer,[29] and carefully conducted studies in humans will have to be performed in order to determine a long term cancer preventive effect in humans. On the other hand, ultraviolet radiation also affects resident dermal cells. Among other dermal cells, mast cells are located in the papillary dermis, adjacent to blood and lymphatic vessels and in close proximity to peripheral nerves.[55] These cells may be implicated in UVB-induced skin immune suppression

and photoaging.[56,57] The ability of PL to decrease dermal mast cell infiltration might lead to a reduction of these two phenomena.

In conclusion, oral PL is an effective systemic chemophotoprotective agent as it leads to a significant decrease of UV-induced erythema, sunburn cell formation, DNA damage, epidermal hyperproliferation, and dermal mast cell infiltration. A trend towards Langerhans cell preservation and reduced vasodilatation was also seen. These short-term results are promising as they suggest that PL might be able to protect against long-term UV-induced skin damage. Further studies are needed to investigate this issue.

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Chapter 6

Orally administered *Polypodium leucotomos* extract decreases psoralen-UVA-induced phototoxicity, pigmentation and damage of human skin

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Abstract

The use of PUVA in patients of skin phototype I-II is limited by side-effects of acute phototoxicity and possible long-term carcinogenesis. We sought to assess oral *Polypodium leucotomos extract* (PL) in decreasing PUVA-induced phototoxicity of human skin on a clinical and histologic level. Ten healthy patients with skin phototypes II-III were exposed to PUVA alone (using 0.6 mg/kg oral 8-methoxypsoralen) and to PUVA with 7.5 mg/kg oral PL. Clinically, phototoxicity was always lower in PL-treated skin after 48-72 hours ($p<0.005$), and pigmentation was also reduced four months later. Histologically, PL-treated skin showed a significant numerical reduction of sunburn cells ($p=0.05$), preservation of Langerhans cells ($p\leq0.01$), decrease of tryptase positive mast cell infiltration ($p<0.05$), and decrease of vasodilation ($p\leq0.01$). No differences were found in Ki67 positive proliferating cells. In conclusion, PL is an effective chemophotoprotector against PUVA-induced skin phototoxicity and leads to substantial benefits of skin protection against damaging effects of PUVA as evidenced by histology.

Introduction

Psoralen-UVA (PUVA) therapy (UVA, 320-400 nm) was first reported for the treatment of psoriasis,[1] and its efficacy was soon confirmed by controlled clinical trials in a large patient series, both in the USA and Europe.[2, 3] These original findings contributed to the development of photomedicine and established the clinical benefit of PUVA for over 30 skin disorders,[4] especially for severe psoriasis,[5] and early stage mycosis fungoides.[6] However, besides cross-linking with DNA leading to decreased epidermal proliferation and immunomodulation,[7-9] UVA activated psoralens also produce reactive oxygen species (ROS) and free radicals by reacting with oxygen.[10] This leads to acute skin phototoxicity,[11] manifested as erythema, edema, pain, and patient discomfort. The hyperpigmentation that develops after PUVA therapy contributes to therapeutic tolerance of UVA, and thus induces the need to increase the UVA dose repeatedly in order to maintain the therapeutic effect, leading to higher total UVA irradiation doses.[12] PUVA also leads to Langerhans cell depletion, inducing local immunodeficiency of skin.[13] This may be one of the reasons why high dose PUVA exposure has been correlated with an increased risk of epithelial skin cancer.[14–18] These factors are limitations in the use of PUVA therapy for skin disorders.

The tropical fern, *Polypodium leucotomos* (PL), has long been believed by native Americans to have anti-tumoral and anti-inflammatory effects.[19] Previous studies have shown that PL acts as an anti-oxidant by quenching free radicals, membrane-lipid peroxidation and ROS such as hydroxyl radical, singlet oxygen, and superoxide anion.[20, 21] Preliminary data showing that orally administered PL decreased skin phototoxicity and resulted in Langerhans cell preservation of human skin when exposed to 8-methoxypsoralen (8-MOP) and sunlight was very promising as it suggested that the addition of PL to PUVA therapy might improve this therapeutic modality by decreasing phototoxicity and epidermal damage.[22]

The objective of this study was to investigate under carefully controlled laboratory conditions whether PUVA therapy could be improved by the addition of PL. We clinically assessed the effectiveness of oral PL in preventing the acute PUVA-induced phototoxic reaction. We also studied the protective effect of this combination on various histological parameters, such as sunburn cells, Langerhans cells, mast cells, proliferating cells and vasodilatation.

Patients and methods

Patient selection

Ten healthy volunteers with skin phototype II or III were included in this open-label study after they read and signed a written informed consent form and protocol approved by an Institutional Review Board of Massachusetts General Hospital. The study was conducted over a period of one year in our laboratories. The group consisted of 6 men and 4 women with ages ranging from 24 to 47 years old. We excluded patients with a personal or family history of skin cancer, a history of photosensitivity, or taking any drug that might alter the response of skin to ultraviolet radiation (UVR). We used the skin of the back for these studies, which had to be free of any blemishes and not exposed to sunlight or artificial UV radiation (tanning booth) for at least 8 weeks prior to the study.

Radiation source and exposure conditions

A 1000 watt high-pressure Xenon arc lamp (Oriel Corp., Stratford, CT) emitting a collimated beam and equipped with a 2 mm WG-335 filter (Schott, Germany) and a first surface mirror (Edmund Scientific, Barrington, NJ) was used as a UVA radiation source (320-400 nm). A high velocity fan was used to eliminate any impact of heat from the infrared radiation of the lamp on the skin. The intensity of the UVA radiation was measured with a calibrated radiometer (International Light, Newburyport, MA) before each experiment when the lamp had warmed up for 30 minutes, and after each experimental protocol to ensure stability of the UVA output, which was usually around 3 mW/cm^2. Photo-tests were performed with adhesive UVR-reflecting aluminum stickers containing 6 exposure windows each 3.3 cm^2 in size (DV Die Cutting Inc., Danvers, MA). One photo-test consisted of exposure of each skin site to UVA doses increasing with 12-50% to obtain an exposure range with regular incremental UVA doses. A dose equivalent to the 2x minimal phototoxic dose (MPD) was always included in each photo-test of the volunteer. From the 6 skin sites, either the 1st, 2nd or 3rd site was exposed to the MPD dose, and either the 4th , 5th or 6th site was exposed to the 2x MPD. During exposures the rest of the skin was covered by an opaque UV-protective cloth. The distance between the exposed skin and the lamp was kept constant by stabilizing the back skin against an aluminum template located at a fixed distance from the exit port of the lamp.

Study design

Prior to the start of the study each patient's MPD was assessed. All patients were instructed to have no more than a low fat light meal before the exposure. Patients received a dose of 0.6 mg/kg 8-MOP, and were exposed to UVA radiation 1 hour (hr) and 30 minutes later. The MPD was defined as the minimal dose of UVA inducing confluent erythema with 4 sharp borders of the exposed skin site. This MPD was further used throughout the following study protocol.

In the first part of the study protocol patients were exposed to PUVA without receiving oral PL. After intake of oral 8-MOP (0.6mg/kg), the skin of the back was exposed 4 times to the same photo-test at: i)1 hour, ii) 1 hr and 30 minutes, iii) 2 hrs, and iv) 2 hrs and 30 minutes to assure optimal phototoxicity. The phototoxic reaction of PUVA exposed skin was evaluated 48 hrs after UVA exposure. Biopsies were taken from 4 patients after 48 hrs and from 3 patients 72 hrs after exposure.

After completion of this first part of the protocol, patients received the first dose of oral PL (7.5 mg/kg body weight) the evening before the second part of the protocol. The next day each patient received the dose of oral 8-MOP and the second dose of oral PL, after which the same 4 photo-tests were repeated as performed in the first part of the protocol, i.e. after 1 hr, 1 hr and 30 minutes, 2 hrs, and 2 hrs and 30 minutes of ingestion of 8-MOP and oral PL. Skin phototoxicity was again evaluated after 48 hrs, and skin biopsies were taken after 48 hrs from 4 patients, and after 72 hrs from 3 patients. Specifics about the biopsies are given further on in the "Histology" section. In addition, follow-up pictures up to 4 months after the experimental protocol were obtained from 6 patients for clinical evaluation of the photoprotective effect of PL on PUVA-induced pigmentation. Oral 8-MOP (Methoxsalen or Oxsoralen Ultra®, ICN, Costa Mesa, CA) was administered to each patient in a dose of 0.6 mg/kg, as used in conventional PUVA therapy. The capsules containing PL (180 mg each) were supplied by I.F.Cantabria, S.A. (Madrid, Spain) and were administered orally in a dose of 7.5 mg/kg body weight.

Clinical evaluation

Clinical evaluation of the phototoxic response of every exposed skin site was performed by at least 2 experienced investigators of PUVA therapy using a scoring system for the intensity of erythema and edema ranging from grades

0 to 4 (0= no erythema; 1= trace erythema; 2= visible, not confluent erythema, no sharp borders; 3= confluent erythema with 4 sharp borders and no edema: MPD; 4= intense erythema with edema). The mean of the grades of each skin site was calculated for all patients at each time-point of exposure. Differences in pigmentation of exposed sites were assessed qualitatively during the next 4 months.

Histology

A 3-mm punch biopsy was obtained during the first part of the protocol from the skin site exposed to the 2x MPD, from the photo-test at the time-point of maximal phototoxicity. The biopsy in the second part of the protocol was taken from the "mirrored" skin site, i.e. exposed to the same fluence, from the same photo-test time-point. All skin specimens were fixed in 10% buffered formalin and embedded in paraffin for sectioning and microscopic evaluation. Three μm sections were routinely stained with hematoxylin and eosin (H&E) for histologic gross evaluation and quantitative assessment of sunburn cells.

Immunohistochemistry

Five μm sections were used for immunohistochemical analysis. They were deparaffinized with xylene and rehydrated with graded ethanols. Endogenous peroxidase was blocked with hydrogen peroxide, and the samples were then rinsed in phosphate-buffered saline (PBS). To yield adequate intensity signals with the respective antibodies, heat-induced antigen retrieval was carried out by microwave pretreatment in citric acid buffer (10 mM; pH 6.0) for 20 minutes. For reduction of background labeling, the sections were blocked for 30 minutes in normal horse serum. The sections were later incubated overnight at 4°C with the commercially available antibodies: i) prediluted anti-CD1a for Langerhans cells (Immunotech, clone O10, cat. No. 1590, Marseille, France) [23]; ii) 1:150 diluted anti-Ki67 for proliferating keratinocytes (DAKO, clone MIB-1, cat. No. M72470, Glostrup, Denmark) [24]; iii) 1:100 diluted anti-tryptase for mast cells (DAKO, clone AA1, cat. No. M7057)[25]; and iv) 1:10 diluted anti-CD31 for endothelial cells (DAKO, clone JC/70A, cat. No. M0823).[26] After washing with PBS, sections were incubated with biotinylated secondary antibody, avidin-biotin-peroxidase complex (ABC Elite; Vector Laboratories, Burlingame, CA), and then 3,3'-diaminobenzidine. Each section was counterstained with

hematoxylin, dehydrated, and covered with a coverslip. Positive and negative controls were also included in each staining run.

Quantitative histological analysis

All parameters listed below were quantified in a blinded fashion.

Sunburn cells: Sunburn cells were defined as cells with a hypereosinophilic cytoplasm and a dense, small, dark, and irregularly formed nucleus in comparison to neighbouring cells, and located in the epidermis away from areas of blistering or crush artefacts.[27] Sunburn cells were quantified in 4 entire sections per specimen (10-11 fields per section) with a light microscope at a magnification of 40x. Using an eyepiece micrometer, the average number of sunburn cells per mm epidermal length was calculated.

Proliferating cells: Ki67 immunoreactivity (proliferating cells) was quantified with a Visilog image analysis system. The equipment used included a Nikon Elipse E400 microscope (Nikon, Japan) with a 20x objective lens, a digital color camera (Polaroid), and a Visilog image processing and analysis system version 5.2 (Noesis, France). This program is able to differentiate and count hematoxylin-stained objects and Lag-red/brown (DAB-stained) objects. The percentage of proliferating epidermal cells was calculated by dividing the number of immunoreactive nuclei by the total number of epidermal nuclei.

Langerhans cells and mast cells: CD1a positive cells (Langerhans cells) and tryptase positive cells (mast cells) were quantified by light microscopy using a 40x objective lens and an eyepiece micrometer. A cell with a nucleus and clear immunoreactivity was considered a positive cell. In each biopsy the number of positive cells was counted in 8-10 consecutive fields, and then calculated per mm^2 of epidermal or upper dermal surface, respectively. To determine the number of mast cells, the micrometer and grid were aligned on one edge along the epidermal dermal junction.

Microvessels: Using a 40x objective lens, 8-10 consecutive fields were counted. CD31 positive cell (endothelial cell) clusters consisting of more than two cells as well as large microvessels were included in the microvessel count.[28] The number of microvessels per mm^2 dermis was counted, and vasodilatation assessed as a percentage of the total surface area occupied by vessels divided by the total dermal surface area.

Statistical analysis

Clinically scored phototoxicity grades were analyzed using the ANOVA test. Mann-Whitney tests were performed to compare the number of sunburn cells, Langerhans cells, mast cells, proliferating cells, and microvessels as well as vasodilatation in PL-treated and untreated skin specimens. Analyses were performed on combined data obtained from biopsies taken at 48 and 72 hrs. A *p*-value of 0.05 or smaller was considered statistically significant.

Results

Clinical results

PL significantly decreased the acute PUVA-induced phototoxic reaction and also diminished the subsequent cutaneous pigmentary response (Figure 1). PL-treated skin sites always showed a statistically significant lower grade of erythema and edema than skin sites exposed to PUVA alone at all time-points of exposure ($p<0.005$) (Figure 2), indicating a decreased phototoxic reaction of PL-treated skin. The PUVA-induced hyperpigmentation was clearly decreased in 4 out of the 6 patients who received a follow-up examination after 4 months (Figure 1). This decrease in pigmentation coincided with a decrease in the acute phototoxic reaction.

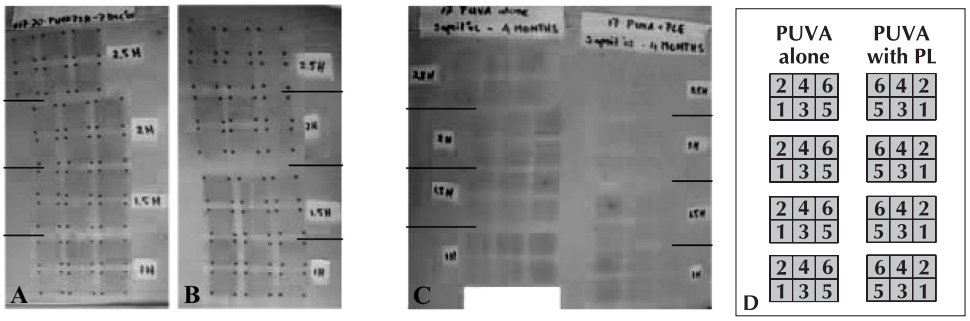

Figure 1. (A) Phototoxic reaction 48 hrs after exposure to PUVA alone. (B) Phototoxic reaction after 48 hrs of PUVA with PL. (C) Pigmentation reaction 4 months after exposure, showing skin sites exposed to PUVA alone on the left side and skin sites exposed to PUVA with PL on the right side. Pictures show decreased erythema and pigmentation in PL-treated skin, indicating that PL decreases the phototoxic and pigmentary response. (D) Schematic representation of skin sites in the pictures. Numbers in squares indicate relative UVA intensities of each site (1 = lowest UVA intensity, 6 = highest UVA intensity). Sites exposed to PUVA without and with PL are mirrored.
(☞ page)

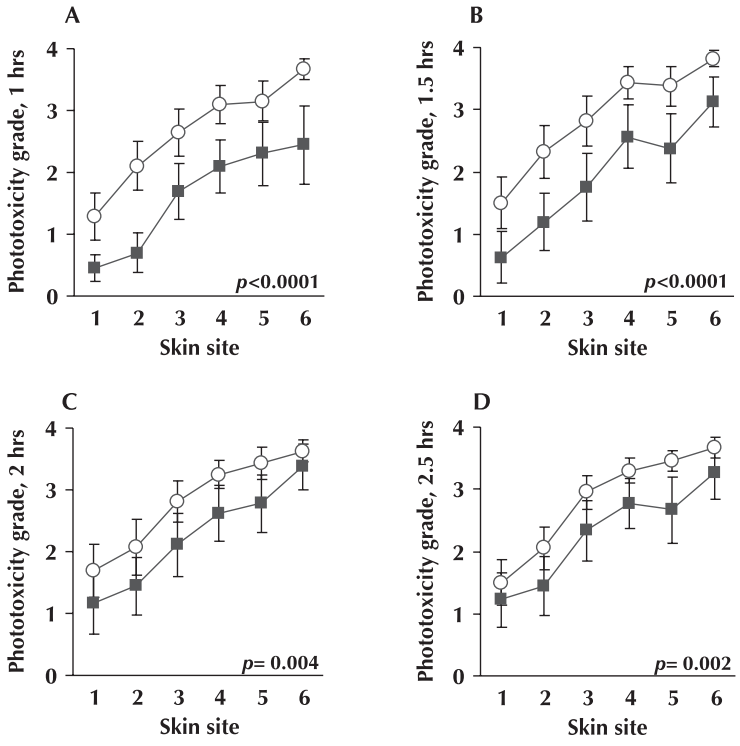

Figure 2. Mean of the phototoxicity grade at 48 hrs (*y*-axis) of each exposed skin site (*x*-axis) from the 10 patients. Shown are the mean of grades of skin sites exposed to 8-MOP without PL (open circles) and with PL (closed squares) after: (A) 1 hr; (B) 1.5 hrs; (C) 2 hrs; and (D) 2.5 hrs of UVA. Error bars represent standard error of the mean (SEM). PL-treated skin always shows statistically significant lower phototoxicity grades at all 4 time-points of exposure.

Histological results

When comparing H&E stained sections from skin exposed to PUVA alone and PL-treated skin, some gross morphological changes became apparent. PUVA exposed skin without PL showed noticeably more maturation disarray, microvesiculation, and vacuolization of keratinocytes, while these findings of epidermal damage were not as prominently found in PL-treated skin (Figure 3a). The number of sunburn cells/mm epidermis was significantly lower in PL-treated skin when compared to PL-untreated skin (p=0.05) (Figure 3a). PL-treated skin showed significantly less depletion of Langerhans cells/mm^2 epidermis in response to PUVA when compared to PL-untreated skin ($p \leq 0.01$) (Figure 3b). In addition, Langerhans cells in PL-untreated skin were increased in size and showed a loss of dendritic morphology, while in PL-treated skin these cells preserved their size and dendritic appearance. PL pretreatment

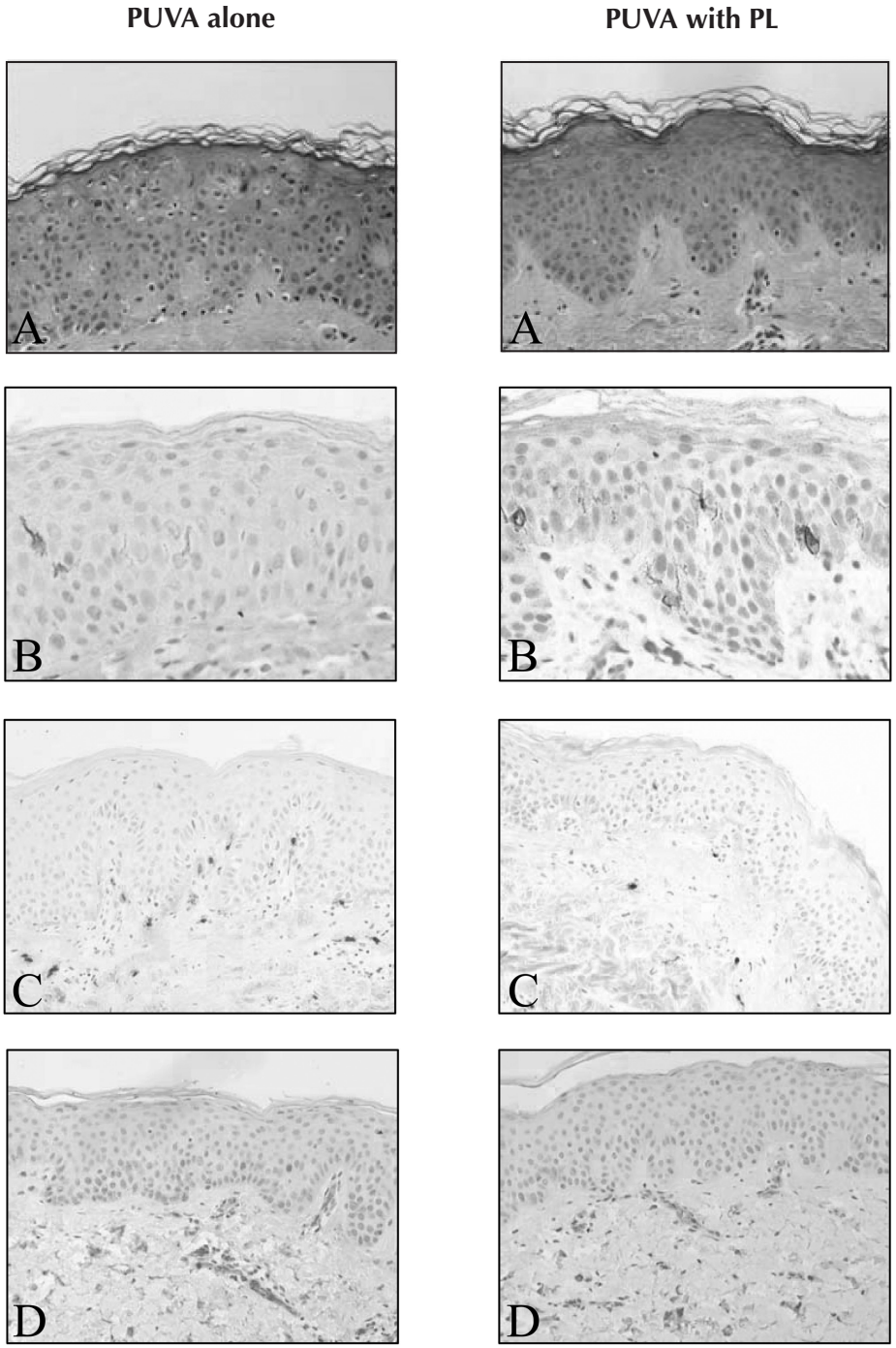

Figure 3. Histology from paired biopsies of skin treated with PUVA alone (left columns) and PUVA with PL (right columns). PL-treated skin shows: (A) less sunburn cells, maturation disarray, microvesiculation, and vacuolization. (B) Langerhans cell numerical and morfological preservation. (C) Less dermal mast cells infiltration. (D) Less blood vessel dilatation. (☞ page)

Table I. Quantitative overview of histologic parameters and *p*-values between skin treatments

Histologic parameter	PUVA alone Mean (SEM)	PUVA with PL Mean (SEM)
Sunburn cells/mm epidermis	16.6 (1.5)	11 (2.5) [a]
Langerhans cells/mm^2 epidermis	20.64 (3.68)	34.24 (3.52) [b]
Mast cells/mm^2 upper dermis	132.16 (8.96)	108.8 (9.6) [a]
Number of microvessels/mm^2 dermis	109.41 (9.55)	100.32 (6.93)
Vasodilatation (% vessel surface area)	1.76 (1.24)	1.24 (0.08) [b]
% Ki 67 positive epidermal cells	16.8 (1.21)	15.9 (1.47)

a= $p \leq 0.05$, b= $p \leq 0.01$. For each slide the number of Langerhans cells and mast cells were counted in at least 10 view fields (ocular grid, 0.0625 mm^2 ; x400)

also resulted in a statistically significant reduction of tryptase positive mast cells in the upper dermis compared to PL-untreated skin ($p \leq 0.05$) (Figure 3c). The total number of CD31 positive microvessels/mm^2 dermis did not show a statistically significant difference in both groups. However, there was a statistically significant decrease in the surface area occupied by microvessels in PL-treated *vs.* untreated skin ($p \leq 0.01$) (Figure 3d), indicating that PL was able to decrease vasodilatation induced by PUVA. There was no statistically significant difference in epidermal proliferation seen by Ki67 immunoreactivity in PL-treated *vs.* untreated skin. Table I gives an overview of all histologic parameter counts and statistical results.

Discussion

The high popularity of PUVA therapy has somewhat declined since its discovery in the seventies.[29] New developments in the area of phototherapy have made the phototoxicity and patient discomfort associated with PUVA less acceptable and attractive. When considering safety aspects it should be recognized that the long-term risks of PUVA are known because of careful prospective follow-up studies, while data on long-term effects of newer types of phototherapy are based mainly on animal studies and mathematical models, and actual long-term effects need yet to be assessed.[30, 31] In spite of these issues, PUVA remains a significant therapeutic option, as its effectiveness continues to be recognized,[32–34] and it still is the most effective UV-based therapy for patients with extensive psoriasis[5] and severe plaque form psoriasis.[35] Therefore, improvement of this therapy is clinically relevant.[36]

In this study we combined PUVA with the oral administration of an extract of the fern *Polypodium leucotomos*. PL had been used in the management of inflammatory skin diseases [37–39] for over 30 years without significant side-effects.[40] In animal models, toxicology tests have shown that PL is non-carcinogenic, non-toxic and non-teratogenic (unpublished data).

This study shows that oral administration of PL lead to a significant reduction of phototoxicity, i.e. erythema and edema in PUVA-treated individuals ($p<0.005$) (Figure 1). This observation was made at all time-points of PUVA exposure (Figure 2). This decrease in cutaneous phototoxicity should make PUVA considerably more tolerable and comfortable for patients. It is believed to result mainly from the anti-oxidative properties of PL,[20, 21] which can also be concluded from the histologic data presented in this communication (Table I, Figure 3). Oral PL caused a marked reduction in sunburn cells in PUVA exposed skin ($p=0.05$), and significant numerical preservation of Langerhans cells ($p\leq0.01$) with conservation of morphology. PUVA exposure induces sunburn cell formation by either oxidative damage or direct DNA damage.[41] PUVA is also known to lead to Langerhans cell depletion in psoriatic[13] as well as in normal skin[42] due to generated oxidative stress.[43] We believe that the decrease in sunburn cells and the preservation of Langerhans cells result from the potent quenching of oxidative stress by oral PL,[20, 21] similar to the presumed working mechanism of other topically administered anti-oxidants.[20, 22, 44, 45] ROS and free radicals can also lead to lipid peroxidation of endothelial cell membranes, resulting in vasodilation, increased vasopermeability and increased synthesis of eicosanoids.[8, 46] The anti-oxidative ability of PL to inhibit lipid peroxidation[20] lead to a significant decrease in vasodilatation ($p\leq0.01$), clinically expressed in reduced phototoxicity, and less pain and burning sensations in PL-treated skin as spontaneously reported by our study patients. PL also reduced the dermal mast cell infiltration significantly ($p\leq0.05$). As previous studies showed that mast cells do not play a major role in the development of the PUVA phototoxic reaction,[47, 48] we believe that the mast cell reduction most likely did not contribute to the observed reduction in phototoxicity. The number of proliferating cells indicated by Ki67 did not differ in the two treatment regimens. This may either be because the biopsies were taken too early (at 48 to 72 hrs) to detect any changes in Ki67 expression, or because PUVA does not have a clear effect on Ki67 expression, as previously shown by repeated PUVA exposures of normal skin of psoriatic patients.[49]

Our observations made in the acute phase after PUVA give rise to interesting hypotheses about the long-term benefits PUVA plus PL may have. There is an increased risk of developing squamous cell carcinoma in PUVA exposed skin that seems to be directly related to the total cumulative UVA dose received.[14–17] In high dose PUVA-treated patients even an increased risk of malignant melanoma has been reported, [50] although this has not as yet been confirmed by other studies,[16, 18, 51] and the American follow-up study[50] did not have a control population and did not make a distinction between patients with a foregoing increased risk of melanomas.

Any long-term effect of the combination of PL and PUVA will have to be carefully studied. Nevertheless, based on these study results we would like to hypothesize that the combination of PL and PUVA may contribute to improve the safety of PUVA. First of all, PUVA-induced DNA damage, resulting in sunburn cells, occurs either directly through cross-linking of psoralens in DNA, or through damage induced by generated ROS and free radicals. [9, 41] The significant reduction of sunburn cells by PL may very likely be partly due to a reduction of the latter type of DNA damage. As ROS and free radicals damaging DNA have been implicated in the promotion of carcinogenesis,[52, 53] a decrease of this damage may increase the safety of PUVA. A second argument is the significant preservation of Langerhans cells. Langerhans cell depletion is thought to play a role in the development of skin cancer, as they have been shown to be vital for the induction of tumor specific immunity against UVR induced tumors,[54] and their presence has been shown to be indispensable for tumor rejection.[55] Commonly accepted chemical skin cancer promotors also work by depleting Langerhans cells, giving transformed cells the chance to grow uninhibited by any immunological surveillance.[56] Carcinogenesis resulting from PUVA follows the pattern induced by carcinogenesis promotors,[57] and it can be assumed that this is, at least in part, the result of Langerhans cell depletion. As PL leads to a significant preservation of Langerhans cells and thus an improved immunological status of PUVA-exposed skin, it is possible that this will lead to a decrease of the chance of developing skin cancer when compared to PUVA alone. Preliminary studies have shown that PL reduces UVR induced carcinogenesis in animal models.[58] Noteworthy is that PL decreased the hyperpigmentation typically induced by PUVA. Although clinical evaluation of the pigmentation response was not systematically performed in all study patients, we found a decrease of hyperpigmentation

in 4 out of the 6 cases evaluated, always corresponding with a decrease in acute phototoxicity. Cellular membranes and DNA are two major UVR targets involved in melanogenesis in response to injury. DNA damage by itself increases melanogenesis,[59] and the observed decrease in pigmentation may reflect reduced DNA damage by quenching of ROS by PL. Exposure to PUVA also increases diacylglycerol,[46] which stimulates melanogenesis through activation of protein kinase c.[59] As oxidative stress generates diacylglycerol,[60] the quenching of ROS by PL can be another mechanism leading to reduced pigmentation. Reduction of this PUVA-induced hyperpigmentation is important, because when pigmentation is not the primary therapeutic goal, it can become a limiting factor by increasing the tolerance of skin to UVR.[12] This makes it necessary to increase UVA doses during the therapy in order to maintain the therapeutic response. By reducing this hyperpigmentation, the need for increasing UVA doses will also be reduced, thus leading to a lower total UVA dose for clearance of psoriasis. As the height of the total UVA dose is considered the main risk factor for developing squamous cell carcinoma,[14–18] it is highly likely that the addition of PL will decrease the risk of developing skin cancer, thus markedly improving the safety profile of PUVA. Supporting this hypothesis are the results of a preliminary study in psoriatic patients, in which the addition of PL resulted in a significant reduction of PUVA sessions and a significant reduction in the total cumulative UVA dose required for clearance of psoriasis when compared to PUVA alone.[61] From this study we can also conclude that PL does not interfere with the therapeutic effectiveness of PUVA for psoriasis.[61]

In conclusion, PL is the first oral agent to decreases the acute phototoxicity and the subsequent development of hyperpigmentation induced by PUVA, and leads to a significant Langerhans cell preservation, decrease of sunburn cell formation, vasodilation and mast cell infiltration. These results show that PL is an effective chemophotoprotector against PUVA-induced skin phototoxicity and leads to substantial benefits of skin protection against damaging effects of PUVA as seen by histology. The observations made in the acute phase warrant long-term follow-up studies as they suggest that the combination of PUVA and PL may result in a therapeutic modality with less long-term adverse side-effects.

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Chapter 7

Treatment of vitiligo vulgaris with narrow-band UVB and oral *Polypodium leucotomos* extract: a randomized double-blind placebo controlled study

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Abstract

The first choice treatment for vitiligo vulgaris is narrow-band UVB (NB-UVB), but no satisfactory treatment exists. We investigated if *Polypodium leucotomos* (PL), an anti-oxidative and immunomodulatory plant extract, improves NB-UVB induced repigmentation. Fifty patients with vitiligo vulgaris randomly received 250 mg oral PL or placebo 3 times daily, combined with NB-UVB twice weekly for 25 to 26 weeks. Repigmentation was higher in the PL group *vs*. placebo in the head-and-neck area (44% *vs*. 27%, p=0.06). Small repigmentation increases (p=n.s.) were observed for the trunk (6% increased repigmentation), extremities (4%), and hands-and-feet (5%) in the PL group *vs.* placebo. Patients attending more than 80% of required NB-UVB sessions showed increased repigmentation in the head-and-neck area in the PL group *vs.* placebo (50% *vs.* 19%, p<0.002); no significant differences were seen in the other body areas. Patients with skin types 2 and 3 showed more repigmentation in the head-and-neck area in the PL group *vs.* placebo (47% *vs.* 21%, p=0.01), and no significant differences were seen in the other body areas. No conclusions could be drawn on skin types 4 and 5 due to low patient numbers. In conclusion, there is a clear trend towards an increase in repigmentation of vitiligo vulgaris affecting the head-and-neck area when NB-UVB phototherapy is combined with oral PL. This effect may be more pronounced in light skin types.

Introduction

Vitiligo is a common pigmentary skin disorder of unknown etiology characterized by milk-white depigmented macules resulting from loss of epidermal melanocytes. Due to the uncertain pathogenic mechanism, therapeutic approaches are mainly based on theoretical grounds. The hypothesis that loss of melanocytes in vitiligo occurs through an auto-immune process is currently the most well-supported.[1] Analyses of serum of vitiligo patients revealed high frequencies of high avidity melanocyte-specific CD8+ cytotoxic T cells [2, 3], correlating with the activity of the disease.[4] Cytotoxic T cells have also been detected in peri-lesional skin of active vitiligo and melanoma-associated vitiligo[5, 6, 7, 8, 9], suggesting a cell mediated auto-immune process. Another theory with substantial evidence is that melanocyte loss in vitiligo results through cytotoxic metabolites of melanogenesis possibly related to increased epidermal oxidative stress. Several studies have shown disturbances in the anti-oxidative system in vitiligo in blood and skin[10, 11, 12, 13] and increased sensitivity of melanocytes to oxidative stress.[14, 15]

According to the last guidelines for the treatment of vitiligo vulgaris, narrow-band UVB (NB-UVB) is the first treatment of choice in generalized vitiligo in adults[16]. Our institute was the first to report that NB-UVB leads to similar to better repigmentation with less adverse events compared to PUVA therapy in adults[17] and is also effective in children.[18] Since then, its efficacy for vitiligo has also been widely confirmed.[19, 20, 21]

We and others have previously reported that an extract of the tropical fern plant *Polypodium leucotomos* (PL) has anti-oxidative and immunomodulating properties. Topical and oral PL has been shown to quench free radicals, lipid peroxidation and reactive oxygen species.[22, 23, 24] PL also induces a shift from a type 1 T-cell cytokine profile to a type 2 T-cell cytokine profile, decreasing production of IL-2, IFN-γ and TNF-α, and enhancing production of IL-10, suggesting immunomodulation through inhibition of cell-mediated immunity mechanisms.[25, 26]

Based on these data, the aim of this study was to investigate if orally administered PL improves repigmentation induced by NB-UVB phototherapy in patients with vitiligo vulgaris. This repigmentation was assessed separately in different anatomic sites, namely the head-and-neck area, the trunk (including buttocks), extremities (without hands and feet), and hands-and-feet together,

as vitiligo is known for its different tendencies for repigmentation according to different body localizations.

Patients and methods

Patient selection

Fifty patients aged 18 years or older with a clinical diagnosis of vitiligo vulgaris were recruited from our out-patient clinic and/or the Dutch Vitiligo Patient Association to participate in this prospective double-blind randomised placebo controlled clinical trial performed in the Netherlands Institute for Pigment Disorders from 2003 to April 2004 after signing a written informed consent form. Exclusion criteria were: a history of skin cancer, abnormal photosensitivity, pregnancy or lactation, segmental vitiligo, phototherapy or sun exposure 3 months prior to enrolment, use of topical treatments during the study and starting vitamin intake during the study.

Study design

Patients were consecutively included and randomized to take either PL capsules (Industrial Farmaceutica Cantabria, SA, Madrid, Spain) of 250 mg 3 times daily (morning, noon, evening) or placebo capsules containing the same ingredients except for PL. To ensure the reliability of blinding, medication was packaged in identical (numbered) containers holding identical capsules. Medication was dispensed by MMH, while randomization was performed by a third party that possessed the code during the entire study. The randomization remained blinded for the study investigators and patients. Randomization occurred via an automated computerized method using Clipper, Version 5.2c and the library Nantucket tools II.

NB-UVB phototherapy was performed twice weekly, on 2 non-consecutive days using Phillips TL-01 100W/01 light tubes (Phillips, The Netherlands) emitting UVB with a spectrum of 310 to 315 nm and a maximal wavelength of 311 nm. The irradiance was measured at the beginning of the study and the fluence was adjusted accordingly. The starting dose was between 210 and 360 mJ/cm^2 according to skin type, and doses were gradually increased until a mild, pink erythema developed 5-12 hours after the exposure in vitiligo lesions. Lesions needing more exposure were exposed longer after protecting

the other areas that had already received the appropriate dose. The entire study duration was 25 to 26 weeks.
Clinical investigations were conducted according to the Declaration of Helsinki principles and the protocol was approved by the Medical Ethical Committee of the Academic Medical Center Amsterdam.

Data collection

Patients were monitored at the beginning (W0), after 6 weeks (W6), 12 weeks (W12) and 26 weeks (W26) of the study. At W6 and W12 the protocol was reviewed with the patient. At W0 and W26 the following data were collected:
Detailed digital photography of all vitiligo lesions was performed. Photography was performed under standardized conditions of location, background and lighting. We photographed the entire body of the patient in a systematic fashion by subdividing the body in specific areas like the head and neck area, trunk (front and back including buttocks), upper arm, lower arm, hands, upper leg, lower leg, and feet by using anatomical features as reference points for these subdivisions. With this system we could obtain identical pictures at the start (W0) and end (W26) of the study that could allow comparison of the pigmentary changes occurring at all anatomic sites.
In a physician global assessment we graded the severity of the vitiligo on a 4-point grading scale, i.e. very severe, severe, more or less severe, not so severe, by taking all factors into account (extent and location of macules, gender, visibility etc.). The relevance of the obtained repigmentation at W26 in the head-and-neck area was also determined.
In a patient global assessment patients were asked to score the severity of their disease by giving a grade between 0 and 10, and to express any inconveniences they experienced from taking capsules.
Patients completed the Skindex-29 to assess the effect of treatment on quality of life. The Skindex-29 yields 3 scale scores representing 3 specific aspects of quality of life, i.e. the effect of skin disease on the patient's functioning, on his/her emotions, and effect on the quality of life due to physical symptoms experienced from the skin disorder, as well as a sum score representing the average of these 3 scale scores.[27] Scores are transformed to a 0 to 100 scale, in which a high score indicates a better quality of life.

Serum was obtained from blood samples centrifuged at 1000 g for 20 minutes and stored at -20°C. Il-6, Il-10, IFN-γ and TNF-α were determined using ELISA kits (Sanquin Reagents, Amsterdam, The Netherlands).
The cumulative dose of NB-UVB received during the study was determined at W26.

Repigmentation assessment

A total of approximately 1700 pictures collected at W0 and W26 were scored by 2 dermatologists (WW and external consultant) experienced in evaluating vitiligo, both blinded for the treatment received by the patient. In a preparatory introduction session the scoring method was explained and discussed to obtain consensus between dermatologists. To decrease the interobserver variability, the scoring dermatologist had to visually divide the vitiligo area in 4 quarters and visually estimate the increase or decrease in pigmentation at W26 in comparison to W0. This lead to a repigmentation percentage reflecting the gain or loss in pigmentation at W26 compared to W0. When at W0 the area affected by vitiligo was of too little clinical significance according to the scoring physicians, this area was not evaluated and the picture was disregarded. The percentage of repigmentation is reported per 4 different parts of the body, namely i) the head-and-neck area, ii) the trunk (including buttocks), iii) extremities (without hands and feet), and iv) hands-and-feet together.

Outcome measures

The primary outcome measure was the percentage of repigmentation at W26 in the oral PL group compared to placebo (intention-to-treat analysis). Secondary outcome measures were: i) Repigmentation in patients attending more than 80% of required NB-UVB sessions. ii) Influence of constitutive pigmentation on repigmentation by comparing the effect of PL in light *vs.* dark skin types. Skin types 2 and 3 were considered "light skin types" and skin types 4 and 5 "dark skin types", consistent with the classification of these skin types as melanocompromised and melanocompetent, respectively.[28] iii) Quality-of-life changes measured with the Skindex-29 by calculating the difference in scores obtained at W0 and W26, and global patient evaluation by the patient global assessment. iv) Evaluation of the physician global assessment. v) The effect of treatment on cytokines in serum.

Statistics

Prior to the study, we performed a power analysis to calculate the sample size. We assumed an average repigmentation rate of 56% with NB-UVB alone, with a calculated standard deviation of 15 after 26 weeks.[17] An absolute increase of 15% regimentation with NB-UVB plus PL compared to NB-UVB alone was considered clinically relevant. A sample size of 30 patients per group (total of 60 patients) would be needed for a power of 80% with an alpha of 0.05 to detect this difference, assuming a common standard deviation of 20 using the student t-test. All statistical analyses were performed using the Mann-Whitney test.

Results

Patient characteristics

Forty-nine patients completed the total study period of 25 to 26 weeks. One patient was lost to follow-up by not attending the last visit for unknown reasons, despite repeated contacting efforts. A flow diagram of patient treatment and analysis is shown in Figure 1, and patient characteristics are given in Table 1.

Repigmentation of NB-UVB with oral PL *vs.* placebo

The means and SD of the percentage of repigmentation obtained at W26 with NB-UVB and PL *vs.* placebo are shown in Figure 2. The PL group shows a trend towards more repigmentation compared with the placebo group in all 4 body area categories. This trend is most prominent in the head-and-neck area where differences are nearly statistically significant (p=0.06; 95% CI 31-58 *vs.* 15-40). A small increase in repigmentation is observed in the other 3 body areas with PL compared to placebo (p>0.05: Trunk 95% CI 26-46 *vs.* 19-40; extremities 95% CI 20-40 *vs.* 14-38; hands-and-feet 95% CI 14-33 *vs.* 7-30). The mean cumulative dose was similar for the PL and placebo group, that is 49 and 52 J/cm^2 respectively.

When abandoning the intention-to-treat analysis by analyzing data from patients following more than 80% of the NB-UVB sessions required for the study, we found statistically significant more repigmentation in the head-and-neck area in the PL group compared to the placebo group (p<0.002;

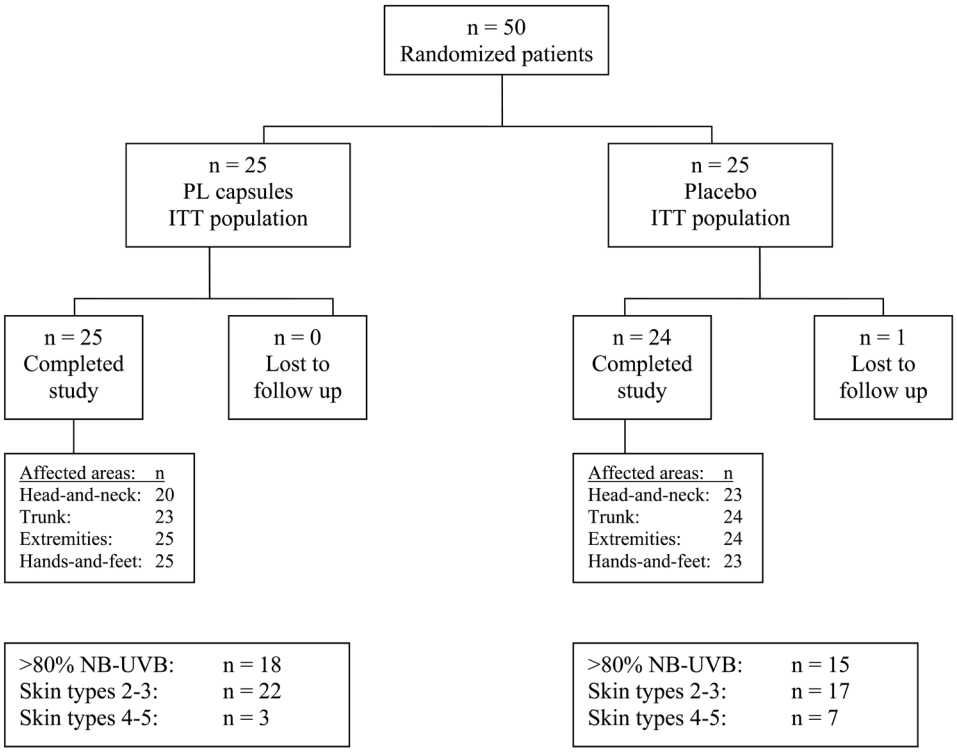

Figure 1. Flow diagram. ITT, Intention to treat.

95% CI 36-64 *vs.* 7-31) (Figure 3). The repigmentation with PL was also more pronounced in the other 3 body areas, but no statistical significant differences were seen ($p>0.05$: Trunk 95% CI 27-52 *vs.* 15-43; extremities 95% CI 23-45 *vs.* 12-40; hands-and-feet 95% CI 15-37 *vs.* 4-30).

Analysis of repigmentation in light skin types (skin type 2 and 3) and dark skin types (skin type 4 and 5) separately, showed that repigmentation was significantly higher in the head-and-neck area for patients with light skin types taking PL compared to placebo ($p=0.01$; 95% CI 32-62 *vs.* 7-35) (Figure 4). In the other 3 body areas no significant differences were found ($p>0.05$: Trunk 95% CI 25-48 *vs.* 18-43; extremities 95% CI 19-42 *vs.* 12-39; hands-and-feet 95% CI 13-35 *vs.* 6-31). No conclusions could be drawn on dark skin types due to low patient numbers (Figure 4).

Table 1. Patient characteristics

Characteristic	PL	Placebo
Number of patients (n=49)	25	24
Number of females (n=33)	15 (60%)	18 (75%)
Mean age at inclusion in years (range)	38.6 (22-58)	46.3 (28-65)
Mean duration of disease in years (range)	21.7 (2-51)	20.5 (1-48)
Mean age of first signs vitiligo in years (range)	16.8 (0-49)	25.8 (0-64)
Mean VIDA score ± SD	2.6 ± 1.6	2.6 ± 1.7
Affected body areas (n)		
Head-and-neck	20	23
Trunk	23	24
Extremities	25	24
Hands and feet	25	23
Affected body area % by vitiligo (n)		
0-25%	17 (68%)	18 (75%)
26-50%	5 (20%)	5 (21%)
51-75%	0 (0%)	1 (4%)
76-100%	3 (12%)	0 (0%)
Distribution of skin types (n)		
II	3 (12.0%)	2 (8.3%)
III	19 (76.0%)	15 (62.5%)
IV	3 (12.0%)	4 (16.7%)
V	0 (0%)	3 (12.5%)

VIDA score[18] (vitiligo disease activity score) indicates activity of the disease as assessed by the patient. Score of 2: active disease in the past 6 months; score of 3: active disease in the past 3 months. SD= standard deviation

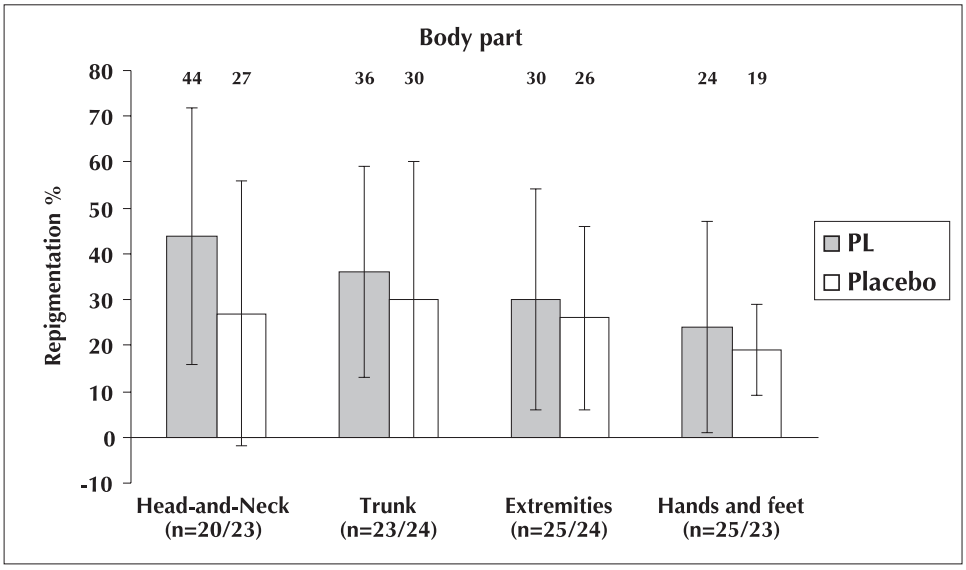

Figure 2. Repigmentation with NB-UVB at W26 in patients receiving PL *vs.* placebo. Number above column is the mean percentage of repigmentation; bars represent SD. The number (n) under the body parts on the X-axis are number of patients per group having involvement of that body part (n=PL/placebo).

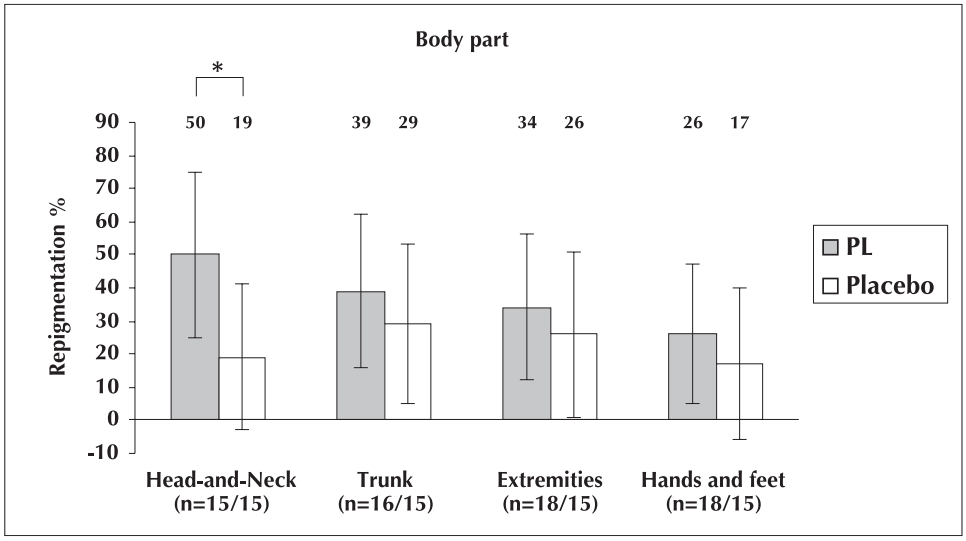

Figure 3. Repigmentation with NB-UVB at W26 in patients following more than 80% of NB-UVB sessions in the PL *vs.* placebo group. Number above column is the mean percentage of repigmentation; bars represent SD. The number (n) under the body parts on the X-axis are number of patients per group having involvement of that body part (n=PL/placebo). *p <0.002.

Physician Global Assessment

The degree of severity of vitiligo was considered more or less equal in both groups at W0, but shifted to being less severe in the PL group compared to the placebo group at W26 (Figure 5).

When assessing the relevance of the obtained repigmentation in the head-and-neck area from the physicians point of view, the repigmentation was considered clinically relevant in 72% of cases in the PL group while this was the case for 43% of cases in the placebo group.

Patient global assessment and quality-of-life with Skindex-29

The mean score for severity of the disease was 7.5 for both the PL group and placebo group at W0. At W26, the PL group graded the severity with a mean of 6.2, while the placebo group scored a 6.0 (p>0.05). Taking of capsules was not considered bothersome for 81% of the total study group, while 4% did not like having to take capsules 3 times a day. The remaining patients did not know the answer. Some patients experienced mild and transient itching (PL group: n=10, placebo group: n=5) and dryness of skin (PL group: n=5, placebo group: n=3) due to NB-UVB exposure. Four patients in the PL group and 5 patients in the placebo group reported mild gastrointestinal complaints due to capsule intake.

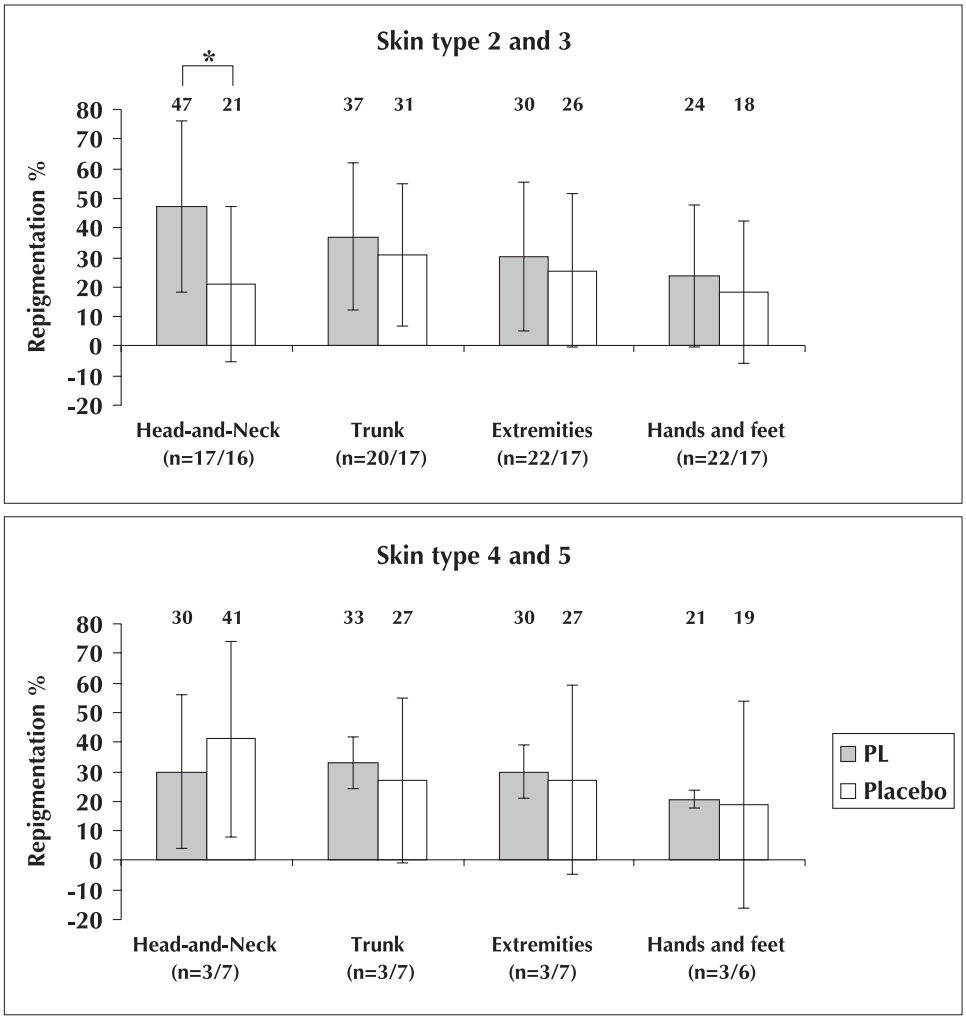

Figure 4. Repigmentation with NB-UVB at W26 in patients with light skin types (skin type 2 and 3) and dark skin types (skin type 4 and 5) in the PL *vs.* placebo group. Number above column is the mean percentage of repigmentation; bars represent SD. The number (n) under the body parts on the X-axis are number of patients per group having involvement of that body part (n=PL/placebo). *p =0.01.

One patient was excluded from the Skindex-29 analysis due to insufficient knowledge of Dutch language to understand the questions (patients analyzed: PL group: n= 24, placebo group: n=24). Improvement or worsening of quality-of-life was determined by calculating the difference in scores obtained at W0 and W26. A positive number indicates improvement, while a negative number indicates worsening of quality-of-life. Between the PL group and placebo group no significant differences were found in change

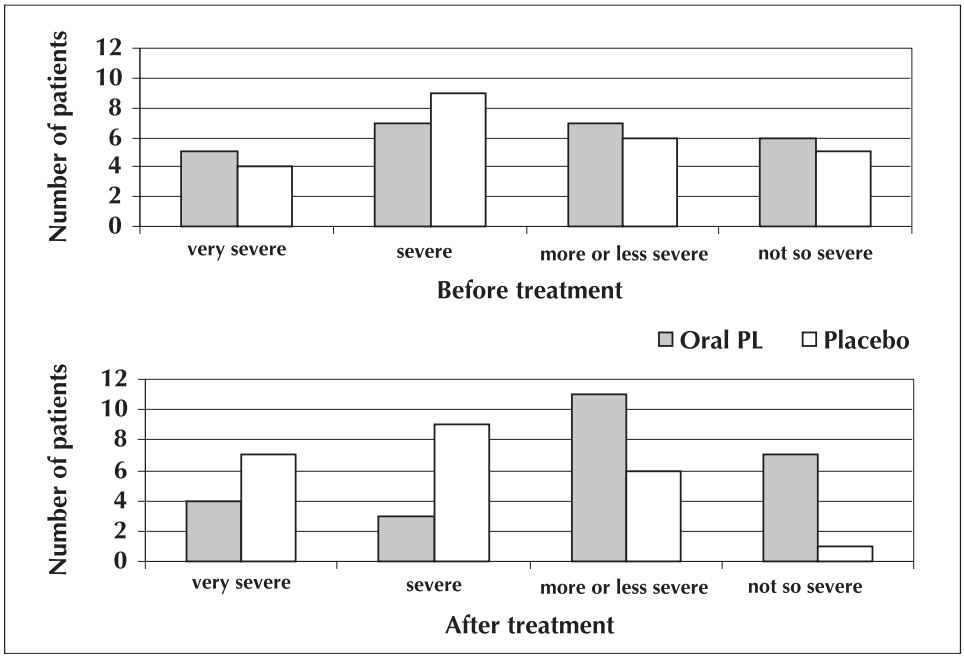

Figure 5. Physician global assessment**.** PL group: n= 25, placebo group: n=24.

of quality-of-life in the subscales emotions, symptoms or functioning, nor in the sum score representing the average of the 3 scale scores after the 6 months treatment (p>0.05). The calculated differences found were 6 *vs.* 7 for the subscale emotions, -2 *vs.* -6 for the subscale symptoms, 5 *vs.* 3 for the subscale functioning, and 4 *vs.* 2. for the sum score in the PL *vs.* placebo groups, respectively.

Blood analysis

IL-10, IFN-γ and TNF-α could only be detected in 7, 2 and 1 patient(s), respectively. No cytokines were detectable in serum of the other patients, which would be similar in normal subjects. No clear trends were observed in the detected cytokines and numbers were too small to draw any conclusion. IL-6 could not be detected in any serum sample of the first or last visit.

Discussion

The rationale behind this study is based on our current knowledge and understanding of pathophysiological mechanisms behind vitiligo vulgaris and

the properties of *Polypodium leucotomos* (PL). PL is a tropical fern plant that has long been known by Native Americans who believe it has anti-tumoral and anti-inflammatory effects.[29] An extract of PL has been used for many years as (adjuvant) treatment for inflammatory skin disorders.[30, 31, 32] This plant extract has no reported side-effects[33], and toxicology tests performed in mice, rats and rabbits show that PL is non-carcinogenic, non-toxic and non-teratogenic (data not published). Previous studies show that PL has immunomodulatory properties. *In vivo* and *in vitro* experiments with PL showed shifting from a T cell type 1 cytokine profile to a T cell type 2 cytokine profile, as the production of IL-2, IFN-γ and TNF-α is decreased, while the production of IL-10 is enhanced. Interestingly, the production of IL-6 can be completely abolished by PL.[25, 26] Furthermore, PL can inhibit T cell proliferation, delay rejection of skin grafts and inhibit the delayed-type hypersensitivity reaction.[26, 34] These observations suggest that PL modulates mechanisms involved in cell-mediated immunity.[26] Besides its immunomodulating properties, PL has anti-oxidant properties. Topically or orally administered PL leads to quenching of free radicals, lipid peroxidation and reactive oxygen species such as the hydroxyl radical, singlet oxygen, superoxide anion, and hydrogen peroxide[22, 23, 24]. These anti-oxidative properties are believed to be the main cause of the observed protection from oral PL against PUVA and UV-induced damage in human skin, perceived as a decrease of erythema and psoralen-induced phototoxicity, as well as preservation of Langerhans cells and decreased sunburn cells after topical or oral administration.[35, 36, 37] These data prompted us to investigate whether PL can be used as an adjuvant therapy in the treatment of vitiligo, as it would interfere with 2 of the major etiological theories of vitiligo. In fact, PL has been anecdotically used as a monotherapy in the management of vitiligo.[38]

We found that addition of PL to NB-UVB lead to increased repigmentation in the head-and-neck area, that was nearly statistically significant (p=0.06) (Figure 2). This effect was enhanced and statistically significant in patients receiving more that 80% of required NB-UVB session (Figure 3). The total number of patients with lesions in the head-and-neck area analyzed in the intention-to-treat analysis was 43. If a higher number of patients would have been included, a statistically significant result may have been obtained. The effect in the other anatomic sites was smaller (Figure 2&3). Repigmentation was assessed per separate anatomic site as it is known that vitiligo has different tendencies for repigmentation in different localizations. One total

score for total body repigmentation would therefore not give an accurate overview of repigmentation. In our study, the hands-and-feet showed the lowest repigmentation percentages, which concurs with the known tendency of these areas to show the worst repigmentation effect with any treatment modality.

Besides the shift in the physician global assessment severity scoring from a more or less homogenous situation at W0 towards a tendency of a less severe rating of patients in the PL group and a more severe rating of patients in the placebo group at W26 (Figure 5), the repigmentation obtained in the head-and-neck area also was more often considered clinically relevant in the PL group (72%) than in the placebo group (43%). However, when patients had to grade the overall severity of their vitiligo on a 0 to 10 scale, no clear differences were observed in their scores at W26 between PL and placebo (6.2 *vs.* 6.0, respectively). Also in the Skindex-29 analysis, no significant differences were observed in the quality-of-life between patients taking PL *vs.* placebo. This shows that although from our medical point of view repigmentation was considered relevant, patients did not perceive the difference between the 2 treatments as relevant. A possible explanation could be the disappointing effect of repigmentation on the hands-and-feet area. Since hands, together with the face, are one's most visible areas of skin, the lack of effectiveness on the hands could possibly have counteracted the positive effect of gained pigmentation in the head-and-neck area in the patient's perception. This however was not investigated separately. The decrease in quality-of-life on the subscale symptoms in both groups can be explained by the reported side effects such as itching and dry skin induced by NB-UVB exposure.

There are several factors that can explain the observed increased repigmentation in vitiligo resulting from PL. TNF-α and IL-6, both known inhibitors of human melanocyte proliferation and melanogenesis, have been shown to be increased in vitiligo skin when compared to normal skin.[39, 40] PL decreases the production of both these cytokines[25, 26], therefore abolishing this inhibitory factor, allowing increased repigmentation through stimulation by NB-UVB. TNF-α is also known to increase the expression of ICAM-1 on melanocytes, facilitating binding of lymphocytes to melanocytes.[13] Increased levels of ICAM-1 have indeed been found on melanocytes of vitiligo patients. In addition to this binding being an undesirable effect on itself based on the auto-immune theory, infiltrating lymfocytes increase the local concentration

of superoxide anion, thus contributing to increased oxidative stress in skin. Besides decreasing the production of TNF-α, PL also is a strong quencher of superoxide anion[23], thus abolishing both these above mentioned events. Several studies have shown an impaired anti-oxidative system with a decrease in several cutaneous anti-oxidants[10, 11, 12, 13] and signs of increased lipid peroxidation and oxidative stress in patients with vitiligo.[10, 12] Melanocytes of vitiligo patients have also shown increased sensitivity to external oxidative stress.[14, 15] The ability of PL to quench free radicals and lipid peroxidation[22, 23, 24] could help to decrease the anti-oxidative stress in vitiligo, thus restoring the impaired anti-oxidative balance and explaining the observed facilitated repigmentation of NB-UVB by PL. Other more circumstantial evidence is the development of vitiligo as a side-effect in successful eradication of human malignant melanomas with high doses of IL-2[41, 42, 43] and anti-cancer therapy with IFN-γ[44] implying involvement of these cytokines in the induction of vitiligo. PL decreases the production of both IL-2 and IFN-γ. Also, in a mouse animal model in which a vitiligo-like coat color developed after CD4+ T cell depletion with subsequent melanoma destruction, CD8+ T cells were found around hair bulbs in skin, and a T helper type 1-dominant cytokine profile was detectable in tumor-draining lymph nodes.[45] PL induces a shift from a type 1 T cell cytokine profile to a type 2 T cell cytokine profile.[25]

We found a statistically significant increase in repigmentation in the head-and-neck area in patients with light skin types taking PL (Figure 4). This increased effect in light skin types can also be explained in view of the theory of anti-oxidative imbalance in vitiligo, as light skin types are thought to have less endogenous anti-oxidants than dark skin.[46] Therefore, light skinned individuals would be more susceptible to anti-oxidative stress and would have more benefit of an exogenous anti-oxidant such as PL. However, a separate study specifically designed to address this question should be performed to confirm this observation. The seemingly absent effect of PL in dark skin types has to be interpreted in view of the low number of patients with dark skin type (n=3 in PL group), combined with the high variability of the mean (SD). This makes it impossible to draw conclusions on these data, and a separate study with larger patient groups should be performed to study the effect of PL on dark skin types. However, hypothetically darker skin types would benefit less from an exogenous anti-oxidant like PL as they assumingly posses more endogenous anti-oxidants.

One aspect that should be considered is that we did not assess whether administration of PL lead to an arrest of vitiligo activity. This may have shown a clearer effect of PL on vitiligo. Another aspect that should be considered when interpreting the results of this study is the NB-UVB phototherapy regimen. Due to variations in frequency and length of phototherapy regimens applied for vitiligo, our results may not apply to other treatment regimens.

In conclusion, addition of oral PL to the treatment of vitiligo vulgaris with NB-UVB shows a trend towards an increased repigmentation in the head-and-neck area, that nearly reaches statistical significance. This effect is more pronounced and significant in patients with light skin types. The observed effect might be attributed to PL's immunomodulating and anti-oxidative properties, in agreement with the 2 major etiological hypothesis of vitiligo resulting from an auto-immune process or increased epidermal oxidative stress. Therefore, oral PL could be a useful addition to NB-UVB therapy in vitiligo patients with involvement of the facial and neck skin. This agent could also be especially useful in patients with light skin types. Larger trials are warranted to confirm these observations.

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Chapter 8

Summary and conclusions
Nederlandse samenvatting

Summary

Pigmentation is one of the most important visible and recognizable characteristics of the human being. Skin color has been and is still associated with healthiness, social status and beauty ideals. For that reason disorders in this area can often lead to great psychological distress and social impact in those affected. On the other hand, attempts to influence one's skin color via sun exposure or bleaching agents can have damaging effects on skin and ultimately lead to skin disorders such as skin cancer or permanent discoloration. The management of pigmentary disorders constitutes a substantial part of the dermatological practice and often remains a therapeutical challenge to the dermatologist.

The aim of the studies presented in this thesis was to enlarge our knowledge on pigmentation, in particular on how to optimize the detection of pigmentation and to study methods to safely modulate and influence pigmentation, especially when it concerns ultraviolet radiation (UVR)-induced pigmentation. The pigmented guinea pig animal model is a well-established and frequently employed pigmentation model to study the effects of UVR on skin and modulation of UVR-induced pigmentation, because their skin tans comparably to human skin after exposure to UVR. Traditionally, the effect of pigment modulating agents or procedures is evaluated by visual and/or histological inspection. The disadvantage of these methods is that visual inspection usually offers only a rough estimate of the occurring changes. Histological evaluation overcomes these imperfections by giving a detailed view of the changes on cellular level, but implies destruction of the skin sample of interest by biopsy for histological examination, preventing serial examination of the same area.

We therefore sought to optimize this animal model by investigating whether we could use the *in vivo* reflectance confocal microscope (RCM) to non-invasively follow the processes occurring in UVR-induced pigmentation of the skin of the pigmented guinea pig (**chapter 2 - Detection of UV-induced pigmentary and epidermal changes over time using *in vivo* reflectance confocal microscopy**). The *in vivo* RCM is a microscope that can be placed directly on living skin of humans or animals (see Introduction – figure 4), allowing immediate, real time, *in vivo* visualization of skin on a cellular level. Imaging is based on detection of back scattered light that is imaged by a detector.

A near-infrared laser is shone perpendicularly on the skin and part of the illuminating light will penetrate the skin. Part of these photons will be absorbed, but part of the light will be reflected and will exit the skin again (see Introduction – figure 1). It is on this reflectance that the confocal microscope is based, hence the name reflectance confocal microscopy. The reflected light is imaged by a detector. However, light will be reflected from different depths within the object. By placing a pinhole in front of the detector one can reject all reflecting light coming from planes that are not of interest, the out-of-focus planes, thereby allowing visualisation of one point of interest within a specimen. In order to visualise more than just one spot, but a certain plane within the specimen (similar to a histological section), a scanner rapidly scans the light beam in horizontal and vertical direction, thereby visualising a field of adjacent illuminated spots, creating a horizontal field-of-view. Images in RCM therefore are *en face* (horizontal) images, in contrast to the vertical images in conventional histological sections. By moving the laser beam deeper into skin, one can create a continuum of horizontal images from stratum corneum up to the upper level of the reticular dermis. Contrast in images is based on inherent differences in refractive indices of cellular structures in skin, resulting in black and white imaging with cellular detail. Since melanin is highly refractile, it is the best endogenous contrast agent in skin, causing melanocytes and other melanin-containing structures to be visible as bright and white structures on RCM images. To induce UVR-induced pigmentation, 4 pigmented guinea pigs were exposed to UVR from a solar simulator for 7 days. RCM was performed during the irradiation period and during the follow-up period. We found that RCM could clearly distinguish 3 major changes in UVR-exposed skin, namely i) an increase in melanocytes size, dendricity, and apparent number; ii) an increase of melanin content within keratinocytes; and iii) an increase in epidermal thickness, which was also quantified and found to be statistical significantly increased in irradiated skin *vs.* control skin. The pigmentary changes observed with RCM were confirmed by histology at the end of the study. We also found that RCM was sensitive enough to detect pigmentary changes in skin before these were clinically visible to the naked eye as a tanning response. We therefore concluded that the RCM is a non-invasive imaging technique that is sensitive enough to repeatedly study UVR-induced epidermal pigmentary changes and UVR-induced epidermal

thickness in this guinea pig animal model, making it a useful addition to this pigmentary animal model.

In the second study (**chapter 3 - Topical application of a protein kinase C inhibitor reduces skin and hair pigmentation**) we used the same animal model to study pigment modulation using a topical pigment modulating agent called bisindolylmaleimide (BIS), a selective protein kinase C (PKC) inhibitor with a higher selectivity for PKC-β. PKC-β plays a crucial role in the melanogenesis cascade as its activation by diacylglycerol and calcium leads to the phosphorylation of two serine residues on the cytoplasmic tail of the melanosome-bound tyrosinase, causing activation of tyrosinase and hence increasing pigmentation. BIS should therefore inhibit tyrosinase activation and negatively influence melanogenesis. We investigated the effect of topically applied BIS on constitutive and UVR-induced pigmentation in the guinea pig animal model. We also looked at the effect of BIS on the tyrosinase activity of cultured human melanocytes *in vitro*, and on hair color of black mice. Compared to control, we found that topical application of BIS to guinea pig skin decreased the UVR-induced tanning response. This was evaluated by clinical evaluation, histological evaluation, as well as with the *in vivo* RCM. Images of the RCM of irradiated skin showed the expected increased melanization of epidermal keratinocytes and increased brightness and dendricity of melanocytes, as was observed in the previous study, while these changes were clearly less noticeable in BIS-treated skin. BIS also had an effect on non-irradiated constitutive pigmentation as its application lead to reduced basal epidermal melanization as seen in Fontana-Masson stained histological sections. Addition of BIS to cultured melanocytes showed a blockage of induction of tyrosinase activity that should result from its activation by PKC-β. Finally, topical BIS lightened the color of regrowing hair in depilated non-irradiated skin of black mice. We concluded that topical application of the PKC inhibitor BIS is effective to reduce UVR-induced pigmentation and constitutive pigmentation in epidermis en hair. These studies give further insight in that PKC-β activity is necessary for tyrosinase activity, and it may offer interesting possibilities in the treatment of unwanted hyperpigmentation of human skin and/or in lightening hair color.

In the next study (**chapter 4 - Confocal histopathology of irritant contact dermatitis *in vivo* and the impact of skin color (black vs. white)**), we used the RCM to study the role skin color may have on the susceptibility to develop

irritant contact dermatitis (ICD), as it is believed that black skin may be more resistant to develop ICD than white skin. Although this difference in susceptibility may not be strictly related to differences in pigmentation, it was nonetheless interesting to investigate the concept that differences in skin color or ethnicity may have an impact on the propensity to develop certain dermatological conditions. The hypothesis is that black skin provides a more resistant barrier because of a denser, more compact stratum corneum. The development of ICD is a dynamic process, which makes evaluation with the RCM ideal as one can repeatedly image the same skin site in order to follow the changes occurring during ICD development. Healthy volunteers with skin phototypes II and III (white skin) and skin phototypes V and VI (black skin) were exposed to 1% and 4% sodium lauryl sulphate (SLS) on several sites on the ventral forearm for 6 hours and 24 hours. RCM imaging was performed: i) immediately after removal of the 4% patch that had been on 6 hours, ii) immediately after removal of the 1% and 4% patch that had been on 24 hours, and iii) after 48 hours (24 hours after removal of 1% and 4% patch). Normal skin and a control site exposed to saline solution for 24 hours served as negative and positive controls. As transepidermal water loss may be used as a measure for stratum corneum barrier function this was also determined. Participants with white skin showed more severe clinical reactions than those with black skin. RCM could detect microscopic changes such as parakeratosis, spongiosis, perivascular inflammatory infiltrate, microvesicle formation, and a marked increase in melanization of basal keratinocytes. These changes could also be seen in patch tests showing no clinical evidence of an irritation response. The observed changes were confirmed by histological analysis of biopsies taken at the end of the study. Also, white skin had greater mean increases in transepidermal water loss after exposure to 4% SLS than black skin. We concluded that this study may support the theory that black skin is more resistant to irritants than white skin, and it illustrates that RCM is a valid detection method with good correlation to conventional histology in detecting histopathological changes occurring in ICD.

Since oxidative stress can play a role in melanogenesis, in the second part of this thesis we investigated the effect of an extract of *Polypodium leucotomos* (PL) in modulating UVR-induced effects such as pigmentation in humans. PL is an extract from a fern plant grown in Central America endowed with anti-oxidant and immunomodulatory properties. *In vitro* studies have shown that PL is an

effective antioxidant by quenching reactive oxygen species, mainly superoxide anion, and by partly inhibiting lipid peroxidation and hydrogen peroxide. Human studies showed that topically and orally administrated PL decreased the acute sunburn response and resulted in Langerhans cell preservation in human skin when exposed to natural sunlight and PUVA photochemotherapy. PL also has immunomodulating properties. *In vivo* and *in vitro* studies with PL showed a shift from a T cell type 1 cytokine profile to a type 2 cytokine profile, as the production of IL-2, IFN-γ and TNF-α was decreased and the production of IL-10 was enhanced. Interestingly, the production of IL-6 could be completely abolished. Furthermore, PL could inhibit T cell proliferation, delay rejection of skin grafts and inhibit the delayed-type hypersensitivity reaction, suggesting modulation of mechanisms involved in cell-mediated immunity.

In the first study of this series (**chapter 5 - Oral *Polypodium leucotomos* extract decreases ultraviolet-induced damage of human skin**) we started by studying the effects of orally administered PL on human skin exposed to UVR from a solar simulator under carefully controlled laboratory conditions. Nine healthy volunteers with skin phototype II and III were recruited for this study. After initial minimal erythema dose (MED) testing, small sites of the volunteer's back were exposed to incremental UVR doses, including a site receiving 2 to 3 times the MED. Clinical evaluation of erythema and edema reaction was performed 24 hours after exposure, and in most volunteers a biopsy for histological and immunohistochemical examination was obtained from the site exposed to 2 to 3 times the MED after 24 hours; in other volunteers biopsies were taken after 72 hours. Volunteers then received 7.5 mg/kg oral PL the night before, and on the day of the experiment itself. In order to determine a time course of effectiveness of PL, UVR irradiation took place after 30 minutes, 1 hour, 1.30 hours, 2 hours and 3 hours of PL administration. Clinical evaluation was again performed after 24 hours and biopsies were obtained from the site receiving the same UVR dose in the same volunteers at the same time points after exposure as in the experiment without PL, from test time point showing maximal erythema. We found that oral PL lead to a statistically significant decrease in the UVR-induced erythema reaction evaluated at 24 hours after UVR exposure, which we ascribe to PL's anti-oxidant properties. Although this decrease was mild, it can be compared with the effect obtained after prolonged administration of other anti-oxidants such as vitamin C and

E. The photoprotective effect on erythema decreased as time between PL intake and exposure increased, and it was significant up to 2 hours after PL administration, suggesting a rapid absorption and early bioavailability of PL. Histologically, we found that biopsies taken from PL treated skin taken 24 hours after UVR exposure showed less epidermal damage with a statistically significant reduction in sunburn cells. More importantly, we found a statistically significant decrease in cyclobutane pyrimidine dimers, indicating that oral PL protects against UVR-induced DNA damage. At 72 hours, we also found a statistically significant decrease in Ki67 positive epidermal cells indicating decreased epidermal proliferation and hyperplasia, which is an epidermal protective response to UVR-induced damage. The reduction in this response reflects the reduced damage inflicted to skin. Combined data from biopsies taken at 24 and 72 hours showed statistically significant decrease in dermal mast cells. A trend was seen regarding preservation of Langerhans cell numbers and morphology in UVR exposed skin treated with PL, but these results were not statistically significant. The dermal surface area occupied by microvessels also showed a trend towards a decrease in PL-treated *vs.* untreated skin, corresponding with the observed decrease in clinical erythema reaction. These results showed that the administration of only 2 doses of oral PL leads to an effective systemic chemophotoprotection against erythema and several histological parameters including DNA damage resulting from UVR from a solar simulator.

Next, we investigated the effect of oral PL on PUVA induced phototoxicity and pigmentation in healthy human skin (**chapter 6 - Orally administered *Polypodium leucotomos* extract decreases psoralen-UVA-induced phototoxicity, pigmentation, and damage of human skin**). PUVA photochemotherapy was first reported for the treatment of psoriasis, but later its efficacy was shown for several skin disorders. Because of its strong hyperpigmentation response, it is also used to treat the pigmentary disorder vitiligo. Psoralens are believed to cross-link with DNA, leading to decreased proliferation and immunomodulation, but UVA-activated psoralens also produce reactive oxygen species and free radicals by reacting with oxygen. We therefore investigated whether the anti-oxidant properties of PL could lead to a decrease in the PUVA-induced oxidative damage and pigmentation in human skin. Ten healthy volunteers with skin types II to III were recruited for this study. The minimal phototoxic dose (MPD) was assessed before the study. For the first part of the study,

volunteers received a dose of 0.6 mg/kg 8-methoxypsoralen, and 6 small sites of the back were exposed to incremental UVA doses, including a site receiving 2 times the MPD. This phototest occurred 4 times, namely 1 hour, 1.30 hours, 2 hours and 2.30 hours after 8-methoxypsoralen intake. The phototoxic reaction of PUVA-exposed skin was evaluated 48 hours after UVA exposure and from most volunteers a biopsy was obtained from the site exposed to 2 times the MPD, from the phototest showing maximal phototoxicity; in other volunteers biopsies were taken after 72 hours. Subsequently, volunteers took 7.5 mg/kg oral PL the night before, and again on the day of the experiment itself, together with 8-methoxypsoralen. Then the same 4 phototests were repeated as performed in the first part of the study. Phototoxicity was again evaluated after 48 hours, and biopsies were taken from the mirrored skin sites at the same time points after exposure as in the experiment without PL. Follow-up was performed in 6 patients up to 4 months after the experiments for clinical evaluation of the photoprotective effect of PL on PUVA-induced pigmentation. This study showed that oral PL lead to a statistically significant decrease of PUVA-induced skin phototoxicity at all timepoints of exposure. Oral PL also decreased PUVA-induced pigmentation in 4 of the 6 observed volunteers. Analysis of combined data from biopsies taken at 48 and 72 hours showed that PL lead to better preservation of epidermal architecture, and statistically significant less sunburn cells, preservation of epidermal Langerhans cells, less dermal mast cells, and less vasodilation. A trend towards a decrease in Ki67 positive epidermal cells was seen. These results showed that oral PL is a powerful antioxidant that leads to effective protection against PUVA-induced skin phototoxicity and pigmentation, and leads to substantial benefits of protection against damaging effects of PUVA as evidenced by histology.

In the last chapter (**chapter 7 - Treatment of vitiligo vulgaris with narrow-band UVB and oral *Polypodium leucotomos* extract: a randomized double-blind placebo controlled study**) the use of PL in the treatment of the pigmentary skin disorder vitiligo vulgaris was evaluated. The etiology of vitiligo is not known, but it is believed to be a cell-mediated auto-immune disease, and/or to result from an increase in oxidative stress in skin. Mainly because of this last hypothesis, we wanted to investigate whether the anti-oxidative properties of PL could be employed in the repigmentation process induced by narrow-band UVB (NB-UVB) phototherapy. The previous studies had already shown that PL can exert its antioxidant effects in skin when administered orally. Although at first it

may seem contradictory to use PL to *increase* NB-UVB induced pigmentation in vitiligo, while PL had shown to *decrease* PUVA-induced pigmentation, the explanation of this hypothesis can be found in the different mechanisms of pigment induction and the role oxidative stress plays in vitiligo and PUVA-induced pigmentation. In vitiligo, oxidative stress is thought to be the cause of the depigmentation, as increased oxidative stress would impair the conversion of phenylalanine to tyrosine, thereby further increasing oxidative stress and creating metabolites toxic to melanocytes, leading to their destruction. Neutralization of this increased oxidative stress by an antioxidant such as PL would restore the balance and allow NB-UVB phototherapy to lead to *increased* repigmentation. On the other hand, PUVA-induced pigmentation is thought to result, as least in part, from the oxidative stress that arises from UVA activated psoralens, so neutralization of this oxidative stress would lead to *decreased* pigmentation. Besides the anti-oxidative effects, PL has immunomodulatory effects that would counteract the cell-mediated autoimmunity thought to play a role in the etiology of vitiligo.

To test this hypothesis, 50 patients with vitiligo vulgaris with skin types II to V were randomly allocated to receive 250 mg oral PL or placebo 3 times daily, combined with NB-UVB twice weekly for 25 to 26 weeks. We found that the percentage of repigmentation was higher (44% *vs.* 27%) in the head-and-neck area in the PL group compared to the placebo group, that was almost statistically significant (p=0.06). Analysis of only patients attending at least 80% of required NB-UVB sessions showed statistically significant differences in repigmentation percentages in the head-and-neck area (50% *vs.* 19%, p<0.002). Analysis of patients with skin types II and III also showed statistically significant differences in repigmentation percentages in this area (47% *vs.* 21%, p<0.01). Due to small patient numbers we could not draw conclusions on patients with skin types IV and V. Small repigmentation increases were observed for the rest of the body in all analyzed groups. We concluded that oral PL could be a useful addition to NB-UVB phototherapy in patients with vitiligo vulgaris involvement of the facial and neck skin. Its effect may be more pronounced in light skin types.

Concluding remarks and future perspectives

This thesis describes two interesting novel concepts.

The first one is the concept of non-invasive microscopic evaluation of skin using the *in vivo* reflectance confocal microscope (RCM). Over the last decades, significant advances have been made to improve non-invasive imaging techniques to study human diseases, with clear examples being ultrasonography, magnetic resonance imaging (MRI) and computed tomography (CT). These novel techniques needed time to be accepted and mastered before they were implemented for regular usage in daily medical settings, but the way medicine is currently practiced would be unimaginable without them. RCM is a promising new technique to non-invasively visualize human skin at quasi-histopathological level. Because skin is visualized in horizontal sections in stead of vertical sections as in routine histology, this technique requires a new approach in the way we interpret skin histology, which with proper training is not difficult to master. This combined with the fact that the RCM machinery is easy to use makes that RCM holds the potential elements for wide spread usage in medical practice.

In the field of skin cancer, a considerable amount of research has been performed with RCM to characterize tumors such as malignant melanoma and basal cell carcinoma. It is quite well imaginable that with additional research and improvement of the imaging properties of the RCM machinery, at some point this technique will be used in clinical settings as an aid to diagnose and determine the potential malignancy of a skin tumor, much like the dermatoscope is currently used today. In Mohs micrographic surgery, RCM could also fulfill a valuable role. Mohs surgery has been shown to reduce recurrence rates of diverse skin tumors, but its use is limited because it is very time-consuming due to preparation of excised tissue for histopathological analysis. As fresh *ex vivo* skin sections can be directly visualized with RCM, one can imagine that serial excised sections can quickly be scanned for the presence of tumor tissue using RCM, and that only sections that are tumor-free as diagnosed with RCM are processed as frozen sections for final confirmation by light microscopic analysis. This application of the RCM could greatly accelerate the process of Mohs surgery and one can hope that at some point RCM will be implemented as a regular tool in this technique. For research purposes, RCM can be a particularly valuable tool because of

its capacity to repeatedly study the same skin spot (this thesis). This capacity, combined with the fact that melanin is the best endogenous contrast agent for RCM showing up bright and white on RCM images, makes RCM a particularly valuable research tool to study aspects pertaining pigmentation (this thesis).
RCM imaging is still in its infancy. With all the significant work that has been -and is still being- performed to further elucidate the potential of this technique and to improve the technical aspects of the machinery, RCM may possess the potential to revolutionize the way dermatological conditions are evaluated today.

The second novel concept in this thesis is the concept of oral photoprotection and pigment modulation of human skin by the anti-oxidant *Polypodium leucotomos* (PL). The concept of oral photoprotection in itself is not new, as several investigators have studied other oral agents, mainly vitamins, for their photoprotective capabilities. However, PL is the first oral agent that with ingestion of only two doses leads to significant systemic photoprotection, clinically shown by a decrease in sunburn response, and histologically shown by a decrease in DNA damage, as seen by reduced thymine dimer formation, and decreased sunburn cell formation (this thesis). Furthermore, the same dosage of PL protects skin against the acute phototoxic effects of PUVA, and the later emerging PUVA-induced pigmentation. Histologically, PL showed to protect skin against PUVA-induced Langerhans cell depletion and sunburn cell formation (this thesis). These histological observations also give rise to interesting hypotheses on possible long term benefits that PL may have in protecting skin against UVR-induced skin cancer. However, only long term follow up studies could answer this question.
Oral PL may serve as a supplement to current topical photoprotecting sunscreens, offering improvement of photoprotection by providing a uniform and easy to achieve whole body protection. The effects of prolonged intake of PL should be investigated, as the photoprotective capabilities of other antioxidants such as vitamine C and E are shown to increase after prolonged ingestion, resulting in a photoprotective effect that on certain parameters is comparable to that of only two doses of PL. The search for an oral photoprotective agent will continue as the advantages of oral over topical photoprotection are obvious and explain the interest of many investigators in this concept. Further investigations towards the active compound in

PL may offer possibilities to chemically reproduce the compound and enhance its protective nature, thereby taking a step towards a true systemic photoprotective agent.

Another aspect of PL is its use as a supplement to phototherapy in order to either decrease phototherapy side effects or to modulate phototherapy-induced pigmentation. An open controlled study investigating the combination of oral PL with PUVA in the treatment of psoriasis vulgaris showed that the cumulative UVA dose needed for clearance of skin lesions was significantly lower in patients taking PUVA with PL compared to patients receiving only PUVA. As the carcinogenic risk of PUVA is clearly associated with the total cumulative UVA dose, combined with the fact that addition of PL decreases PUVA-induced damage on a histological level (this thesis), this shows that the addition of PL improves the safety of PUVA without impairment of its effectiveness. Furthermore, we have shown that pigmentation of vitiligo vulgaris of the facial and neck skin can be increased when narrow-band UVB phototherapy is combined with oral PL showing improvement of the effectiveness of narrow-band UVB phototherapy (this thesis). Further studies are needed to investigate the potential of PL in improving safety and therapeutic outcome of other phototherapeutical modalities.

Nederlandse samenvatting

De pigmentatie van de huid is één van de belangrijkste zichtbare kenmerken van een individu. De huidskleur werd en wordt nog steeds geassocieerd met sociale status, gezondheid en een bepaald schoonheidsideaal. Daarom leiden pigmentstoornissen frequent tot grote psychosociale problemen bij de aangedane persoon. Vaak beïnvloeden mensen ook zelf de gezonde huidskleur door bijvoorbeeld zonblootstelling en gebruik van bleekmiddelen, wat kan leiden tot huidafwijkingen zoals huidkanker en permanente depigmentaties. De behandeling van pigmentstoornissen vormt een substantieel onderdeel van het werk van een dermatoloog en is vaak een uitdaging door de complexiteit van de huidaandoeningen en het gebrek aan effectieve therapeutische middelen.

De primaire doelstelling van dit proefschrift is om de kennis op het gebied van de pigmentatie van de huid te vergroten. De nadruk ligt hierbij op onderzoek naar technieken waarmee de detectie van pigmentatie kan worden geoptimaliseerd en op onderzoek naar methoden om pigmentatie op een veilige manier te moduleren, met name pigmentatie ten gevolge van ultraviolette (UV) straling. Het gepigmenteerde cavia proefdiermodel is een frequent gebruikt model in studies naar het effect van UV straling op de huid en modulatie van UV-geïnduceerde pigmentatie. De huid van de gepigmenteerde cavia vertoont namelijk hetzelfde pigmentatie reactiepatroon na UV bloostelling als de menselijke huid. Van oudsher wordt het effect van pigmentmodulatie geëvalueerd door klinische inspectie en/of histologische analyse van de huid. Het nadeel hiervan is dat klinische inspectie slechts een grove weergave geeft van de opgetreden veranderingen. Dit wordt verholpen door histologische analyse wat een gedetailleerde weergave biedt van de veranderingen op cellulair niveau. Het gevolg hiervan is echter dat de onderzochte huid voor verder onderzoek verloren gaat door de biopsie, waardoor het herhaaldelijk onderzoeken van dat specifieke huiddeel onmogelijk wordt gemaakt. In het eerste hoofdstuk van dit proefschrift werd gezocht naar optimalisatie van dit pigmentatie proefdiermodel door te onderzoeken of de *in vivo* reflectance confocale microscoop (RCM) op non-invasieve wijze de UV-geïnduceerde pigmentatie van de huid kan detecteren en vervolgen (**hoofdstuk 2 - Detection of UV-induced pigmentary and epidermal changes over time using *in vivo* reflectance confocal microscopy**). De *in vivo* RCM is een microscoop

die direct op de levende huid van mens of dier kan worden geplaatst (zie Introduction – figure 4), waarbij deze huid direct op cellulair niveau kan worden afgebeeld. Deze techniek is gebaseerd op reflectie van fotonen door verschillende huidstructuren. De straling van een laser wordt loodrecht op de huid geschenen, waarna de fotonen de huid deels zullen penetreren en deels in de huid zullen worden geabsorbeerd. Een deel zal echter ook worden gereflecteerd door verschillende huidstructuren (zie Introduction – figure 1). Het is deze reflectie van licht waarop de confocale microscoop is gebaseerd, vandaar de naam "reflectance" confocale microscoop. Deze gereflecteerde fotonen worden gedetecteerd door een detector, waarna er vervolgens een digitale afbeelding van wordt gemaakt. Er zullen echter fotonen vanuit verschillende dieptes uit de huid worden gereflecteerd, maar door een kleine opening vóór de detector te plaatsen kan men de fotonen die afkomstig zijn uit lagen in de huid die niet bestudeerd moeten worden afstoten. Hierdoor kan je één specifiek punt in de huid bekijken zonder daadwerkelijk in de huid te snijden. Om niet slechts één punt maar een heel vlak in de huid te kunnen bekijken (vergelijkbaar met een histologische coupe) zal de laser straal door een scanner in hoog tempo in horizontale en verticale richting worden bewogen, waarbij een reeks aaneengesloten punten worden verkregen, wat resulteert in de afbeelding van één horizontaal vlak. RCM afbeeldingen zijn daardoor horizontale weergaven van de huid, in tegenstelling tot de verticale coupes die gebruikelijk zijn in de histologie. Door het focuspunt van de straal op en neer in de huid te bewegen kan men een continuüm aan horizontale plaatjes van de huid creëren, beginnend bij het stratum corneum tot aan de bovenste lagen van de reticulaire dermis. Het contrast in de afbeeldingen wordt bepaald door verschillen in de brekingsindex van de verschillende cellulaire structuren en organellen, met als gevolg zwart-wit afbeeldingen met cellulair detail. Melanine heeft een hoge brekingsindex, waardoor dit het beste endogene contrastmiddel van de huid is, omdat melanocyten en melanine bevattende cellen wit oplichten in confocale afbeeldingen. Voor deze studie werden vier gepigmenteerde cavia's zeven dagen blootgesteld aan UV straling van een artificiële zonlicht-bron (solar simulator) om UV-geïnduceerde pigmentatie te bewerkstellingen. RCM werd tijdens en na de bestralingsperiode verricht. Uit de resultaten blijkt dat RCM 3 belangrijke UV-geïnduceerde veranderingen kon detecteren, namelijk i) een toename in grootte, dendrietvertakkingen en globale aantallen van melanocyten, ii) een

toename van melanine in keratinocyten, en iii) een toename van de epidermale dikte, die bij kwantificatie statistisch significant dikker werd bevonden in de bestraalde *vs*. de controle huid. De met RCM gemaakte observaties werden bevestigd door histologische analyse van de huid aan het eind van de studie. De RCM kon ook pigmentatie veranderingen in de huid detecteren voordat deze klinisch zichtbaar waren met het blote oog. We concluderen dat de RCM een non-invasieve beeldvormende techniek is met voldoende gevoeligheid om op herhaaldelijke wijze UV-geïnduceerde pigmentveranderingen en epidermale hyperplasie te detecteren in dit cavia proefdiermodel. Dit maakt dat de RCM een nuttige aanvulling is op dit bekende pigmentatie model.

In de tweede studie (**hoofdstuk 3 - Topical application of a protein kinase C inhibitor reduces skin and hair pigmentation**) werd in hetzelfde proefdiermodel gekeken naar pigmentmodulatie door topicale applicatie van bisindolylmaleimide (BIS). BIS is een selectieve protein kinase C (PKC) remmer met een hoge selectiviteit voor PKC-β. PKC-β speelt een belangrijke rol in de melanogenese, omdat activatie van PKC-β door diacylglycerol en calcium leidt tot phosphorylatie van twee serine residuen in het cytoplasma domein van tyrosinase. Dit leidt tot activatie van tyrosinase en daardoor tot verhoogde melanogenese. BIS zou derhalve remmend werken op activatie van tyrosinase en dus de melanogenese negatief beïnvloeden. Het effect van topicale applicatie van BIS op de basale en de UV-geïnduceerde pigmentatie werd onderzocht in het cavia proefdiermodel. Tevens werd het effect van BIS op tyrosinase activiteit van gekweekte melanocyten *in vitro* en op de haarkleur van zwarte proefdiermuizen *in vivo* onderzocht. De resultaten lieten zien dat topicale applicatie van BIS remmend werkt op de UV-geïnduceerde pigmentatie van de caviahuid in vergelijking met de controle huid. Dit werd middels klinische en histologische analyse vastgesteld, alsmede met de RCM. RCM afbeeldingen van de bestraalde caviahuid toonden een duidelijke toename van melanine en dendrietvertakkingen van melanocyten (zoals gezien was in de vorige studie), terwijl deze veranderingen duidelijk minder aanwezig waren in de met BIS behandelde huid. BIS applicatie verminderde ook de pigmentatie van de basale (niet-bestraalde) huidskleur, wat zichtbaar was als verminderde pigmentatie van de basale laag van de epidermis in Fontana-Masson gekleurde histologische coupes. Bij toevoeging van BIS aan gekweekte melanocyten trad een blokkade van inductie van tyrosinase activiteit op, wat had moeten optreden door activatie van tyrosinase door

PKC-β. Applicatie van BIS leidde in teruggroeiend haar van onthaarde zwarte muizen tot een verminderde pigmentatie en dus lichtere haarkleur. Wij concluderen dat topicale applicatie van de PKC remmer BIS effectief is in het verminderen van de UV-geïnduceerde en basale pigmentatie in epidermis en haren. Deze studies tonen dat PKC-β noodzakelijk is voor de activatie van tyrosinase, wat interessante nieuwe mogelijkheden biedt tot modulatie van ongewenste pigmentatie van huid en haar.

In de volgende studie (**hoofdstuk 4 - Confocal histopathology of irritant contact dermatitis *in vivo* and the impact of skin color (black vs. white)**) werd met RCM bestudeerd of huidskleur een rol speelt in de gevoeligheid om orthoërgisch contact eczeem (OCE) te ontwikkelen. Hoewel dit verschil mogelijk niet een direct gevolg is van verschillen in huidpigmentatie, is het toch interessant om het concept dat verschillen in huidskleur en etniciteit van invloed kunnen zijn op de gevoeligheid om bepaalde huidziekten te ontwikkelen nader te bestuderen. De hypothese stelt dat de donkere huid resistenter zou zijn tegen het ontwikkelen van OCD wegens een dichter en compacter stratum corneum. Omdat het ontwikkelen van OCD een dynamisch proces is, is de RCM ideaal om dit te bestuderen, omdat je herhaaldelijk hetzelfde stukje huid kan onderzoeken en daardoor de veranderingen optredend gedurende het ontstaan van OCD kan detecteren. Gezonde vrijwilligers met huidtype II en III (blanke huid) en huidtype V en VI (donkere huid) werden blootgesteld aan 1% en 4% sodium lauryl sulphate (SLS) op de ventrale zijde van de onderarm gedurende 6 en 24 uur. RCM beeldvorming vond plaats: i) direct na de verwijdering van de 4% patch na applicatie van 6 uur, ii) direct na verwijdering van de 1% en 4% patch na applicatie van 24 uur, en iii) na 48 uur (24 uur na verwijdering van 1% en 4% patch). Controle huid 24 uur blootgesteld aan NaCl oplossing en normale huid dienden als negatieve en positieve controles. Het transepidermale waterverlies werd ook bepaald omdat dit gebruikt kan worden als maat voor de barrièrefunctie van het stratum corneum. De resultaten toonden dat de blanke huid ernstigere OCD reacties ontwikkelde dan de donkere huid. De RCM detecteerde microscopische veranderingen zoals parakeratose, spongiose, perivasculaire infiltraten, micro-vesikels en een toename in pigmentatie van de basale keratinocyten. Deze veranderingen werden ook gedetecteerd in patchtesten die klinisch geen OCD respons vertoonden. De observaties gemaakt met RCM werden bevestigd door histologische analyse verricht aan het eind van de studie. De

blanke huid toonde ook meer transepidermaal waterverlies bij blootstelling aan 4% SLS dan de donkere huid. Op basis van deze studie concluderen wij dat er aanwijzingen lijken te zijn die de hypothese ondersteunen dat de donkere huid resistenter zou zijn tegen ontwikkeling van OCD. Tevens blijkt de RCM een valide methode te zijn om de histopathologische veranderingen van OCD te detecteren en heeft het een goede correlatie met histologie.
In het tweede deel van deze thesis werd gekeken naar het effect van *Polypodium leucotomos* extract (PL) op UV-geïnduceerde huidveranderingen, waaronder pigmentatie. PL is een varensoort dat in Midden-Amerika wordt gekweekt en dat beschikt over antioxidatieve en immunomodulerende eigenschappen. *In vitro* studies met PL toonden dat het een effectieve antioxidatieve werking heeft middels neutralisatie van reactieve zuurstofradicalen. Onderzoek met menselijke vrijwilligers toonde dat topicale en orale PL de zonnebrandreactie van de huid na UV blootstelling verminderde en dat er behoud was van Langerhans cellen in huid blootgesteld aan zonlicht en PUVA photochemotherapie. PL heeft ook een immunomodulerende werking. *In vivo* en *in vitro* studies met PL toonden een verschuiving van het type I naar type II T cel cytokine profiel, met een verminderde productie van IL-2, IFN-γ en TNF-α en meer productie van IL-10. Opvallend genoeg kon de productie van IL-6 volledig geremd worden. PL leidde ook tot remming van T cel proliferatie, vertraagde afstoting van huidtransplantaten en remming van het vertraagde type overgevoeligheidsreactie. Deze observaties suggereren dat PL ingrijpt op cellulair gemedieerde immuunmechanismes. In de eerste studie van deze serie (**hoofdstuk 5 - Oral *Polypodium leucotomos* extract decreases ultraviolet-induced damage of human skin**) werd het effect van orale PL op menselijke huid blootgesteld aan UV onderzocht, onder gecontroleerde laboratorium omstandigheden. Negen gezonde vrijwilligers met huidtype II en III werden geïncludeerd. Na bepaling van de minimale erytheem dosis (MED) werden kleine testvlakken van de rug blootgesteld aan oplopende UV doses van een solar simulator. Hierbij werd één testvlak blootgesteld aan 2 tot 3 maal MED. Na 24 uur vond klinische evaluatie van de reacties plaats. Van de meeste vrijwilligers werd toen ook een biopt voor histologie en immunohistochemie afgenomen van het testvlak blootgesteld aan 2-3 MED; bij andere vrijwilligers werd het biopt pas na 72 uur afgenomen. Voor het tweede deel van de studie kregen de vrijwilligers 7.5 mg/kg orale PL op de avond voor en op de dag van experiment zelf. Dezelfde phototest werd verricht, maar om een tijdsbeloop

van de effectiviteit van PL te bepalen werd dit gedaan na 30 minuten, 1 uur, 1.30 uur, 2 uur en 3 uur van PL toediening. De klinische evaluatie vond weer plaats na 24 uur en biopten werden genomen van dezelfde vrijwilligers op dezelfde dag na UV blootstelling als in het experiment zonder PL, van het testvlak blootgesteld aan dezelfde UV dosis als in het eerste experiment, behorend tot de phototest met de sterkste erytheem reactie. De resultaten toonden dat orale PL leidde tot een statistisch significante vermindering van UV-geïnduceerd erytheem optredend na 24 uur, wat wij toeschreven aan de antioxidatieve werking van PL. Hoewel het effect klein was, is het vergelijkbaar met het effect van andere antioxidanten zoals vitamine C en E na langdurige toediening. Het photoprotectieve effect van PL nam af naarmate de tijd tussen PL inname en UV blootstelling toenam, maar het bleef statistisch significant tot en met twee uur na inname. Deze observatie suggereert een snelle absorptie en biologische beschikbaarheid van PL. De histologische analyse toonde vermindering van epidermale schade in de PL behandelde huid, met een statistisch significante reductie in het aantal sunburn cellen. Belangrijker nog is dat er statistisch significant minder cyclobutane pyrimidine dimeren waren in de PL behandelde huid, wat aangeeft dat orale PL bescherming bood tegen UV-geïnduceerde DNA schade. In de biopten genomen 72 uur na blootstelling vonden we een statistisch significante afname van Ki-67 positieve epidermale cellen, duidend op verminderde epidermale proliferatie en hyperplasie. Omdat epidermale hyperplasie door UV blootstelling gezien wordt als een beschermingsmechanisme van de huid tegen verdere UV straling duidt vermindering van deze respons op minder doorgemaakte epidermale schade. De gecombineerde data van biopten genomen na 24 en 72 uur lieten een statistisch significante vermindering van dermale mestcellen zien. Een trend tot het behoud van aantallen en morfologie van Langerhans cellen in UV blootgestelde huid behandeld met PL werd gezien, maar de verschillen waren niet significant. Ook werd een trend gezien tot een vermindering van de oppervlakte ingenomen door de microvasculatuur, wat overeenkomt met de klinisch geobserveerde verminderde erytheem respons. Wij concluderen dat toediening van slechts 2 doses orale PL kan leiden tot een effectieve systemische photoprotectie tegen de zonnebrandreactie en diverse histologische parameters waaronder DNA schade optredend door UV blootstelling.

In de volgende studie hebben we onderzocht of orale PL van invloed was op PUVA-geïnduceerde phototoxiciteit en pigmentatie van de gezonde huid (**hoofstuk 6 - Orally administered *Polypodium leucotomos* extract decreases psoralen-UVA-induced phototoxicity, pigmentation, and damage of human skin**). In het begin werd PUVA photochemotherapie voornamelijk gebruikt voor de behandeling van psoriasis, maar later bleek deze methode effectief te zijn voor diverse huidziektes. Omdat PUVA leidt tot een sterke hyperpigmentatie respons van de huid, heeft het ook een plaats verworven in de behandeling van de pigmentstoornis vitiligo. Na toediening vindt een verbinding plaats tussen het psoralen en het DNA in de huid, resulterend in een verminderde proliferatie en immunomodulatie. Echter, de UVA-geactiveerde psoralenen leiden door hun reactie met zuurstof ook tot reactieve zuurstofradicalen en vrije radicalen. Wij onderzochten of de antioxidatieve werking van PL kon leiden tot vermindering van oxidatieve schade en pigmentatie van de PUVA behandelde huid. Tien gezonde vrijwilligers met huidtype II en III werden geïncludeerd. De minimale phototoxische dosis (MPD) werd voor het starten van de studie bepaald. Voor het eerste deel van de studie kregen de vrijwilligers 0.6 mg/kg 8-methoxypsoralen toegediend. Vervolgens werden 6 kleine testvlakken op de rug bestraald met oplopende UVA doses, met inbegrip van blootstelling van één testvlak aan 2 MPD. Deze phototest werd vier keer herhaald, namelijk 1 uur, 1.30 uur, 2 uur en 2.30 uur na inname van 8-methoxypsoralen. Na 48 uur werd de phototoxische reactie klinisch beoordeeld en werd van de meeste vrijwilligers een biopt genomen van het 2 MPD testvlak van de phototest op het tijdstip met de sterkste phototoxische reactie; van andere vrijwilligers werd dit biopt na 72 uur genomen. Voor het tweede deel van de studie kregen de vrijwilligers 7.5 mg/kg orale PL de avond voor de studie, en weer op de dag van de studie maar dan samen met 8-methoxypsoralen. Dezelfde 4 phototests van het eerste deel van de studie werden herhaald. De phototoxische reactie werd weer na 48 uur geëvalueerd, en biopten werden verkregen van de gespiegelde testvlakken zoals in het eerste deel van de studie. Zes vrijwilligers werden tot vier maanden na de blootstelling geobserveerd voor klinische evaluatie van het effect van PL op PUVA-geïnduceerde pigmentatie. De resultaten van deze studie lieten zien dat orale PL leidde tot een statistisch significante vermindering van phototoxiciteit door PUVA op alle tijdstippen van PUVA blootstelling. Orale PL leidde ook tot een verminderde pigmentatie door PUVA in 4 van de 6

bestudeerde vrijwilligers. De analyse van de gecombineerde data van de biopten genomen na 48 uur en 72 uur liet zien dat PL leidde tot behoud van de epidermale architectuur. Tevens werd er statistisch significant minder sunburn cellen, behoud van epidermale Langerhans cellen, minder dermale mestcellen en minder vasodilatatie gezien. Een trend tot een verminderd aantal Ki-67 positieve epidermale cellen werd ook gezien. Wij concluderen dat orale PL een krachtig antioxidant is, dat een effectieve bescherming biedt tegen PUVA-geïnduceerde phototoxiciteit en pigmentatie van de huid, en dat het een aanzienlijke bescherming biedt tegen de schadelijke effecten van PUVA op cellulair niveau in de huid.

In het laatste hoofdstuk (**hoofdstuk 7 - Treatment of vitiligo vulgaris with narrow-band UVB and oral *Polypodium leucotomos* extract: a randomized double-blind placebo controlled study**) is het gebruik van PL in de behandeling van de pigmentstoornis vitiligo vulgaris nader onderzocht. De etiologie van vitiligo is onbekend, maar de hypothese is dat het een cellulair gemedieerde auto-immuun ziekte is en/of het gevolg is van een toegenomen oxidatieve stress toestand in de huid. Vooral in het kader van deze laatste hypothese wilden wij onderzoeken of de antioxidatieve werking van PL tot een toename in repigmentatie van vitiligo behandeld met smalspectrum UVB (NB-UVB) kon leiden. De vorige studies hadden immers aangetoond dat PL als antioxidant werkzaam is in de huid na orale toediening. Hoewel het op het eerste gezicht tegenstrijdig lijkt om PL te gebruiken om met NB-UVB *meer* pigmentatie in vitiligo te bewerkstelligen terwijl er juist *minder* pigmentatie optreedt met PL en PUVA, is dit toch te verklaren door de verschillende mechanismen van pigment inductie en door de rol van oxidatieve stress in vitiligo en PUVA-geïnduceerde pigmentatie. In vitiligo zou oxidatieve stress de oorzaak van de depigmentatie zijn, daar de verhoogde oxidatieve stress zou leiden tot een verstoring in de omzetting van phenylalanine tot tyrosine, met hierdoor verdere toename van oxidatieve stress en ontstaan van metabolieten die toxisch zijn voor de melanocyt, resulterend in hun destructie. Door deze oxidatieve stress te neutraliseren met een antioxidant zoals PL zou de balans in de huid weer hersteld kunnen worden waardoor NB-UVB phototherapie de kans krijgt om *meer* repigmentatie te induceren. Bij PUVA speelt de oxidatieve stress die ontstaat bij UVA geactiveerde psoralenen waarschijnlijk mede een rol in het ontstaan van pigmentatie na PUVA. Derhalve zou het neutraliseren van deze oxidatieve stress leiden tot *minder* pigmentatie. Naast

de antioxidatieve werking heeft PL ook immunomodulerende eigenschappen die zouden kunnen ingrijpen op de cellulair gemedieerde autoimmuniteit die mogelijk een rol speelt in het ontstaan van vitiligo.
Om onze hypothese te testen werden 50 vitiligo patiënten met huidtypes II tot V geïncludeerd. Randomisatie vond plaats waarbij de ene helft van de groep drie maal daags 250 mg orale PL kreeg en de andere helft placebo, gecombineerd met wekelijks twee NB-UVB sessies gedurende 25 tot 26 weken. De resultaten toonden dat er hogere repigmentatie percentages waren voor het hoofd-hals gebied in de PL groep in vergelijking met de placebo groep (44% *vs.* 27%), waarbij dit verschil grensde aan statistische significantie (p=0.06). De analyse van alleen die patiënten die ten minste 80% van de benodigde NB-UVB sessies gevolgd hadden toonde wel een statistisch significant verschil in repigmentatie percentages in het hoofd-hals gebied (50% *vs.* 19%, p<0.002). De analyse van alleen patiënten met een lichte huidskleur (huidtype II en III) toonde ook statistisch significant meer repigmentatie in het hoofd-hals gebied (47% *vs.* 21%, p<0.01). Door het kleine aantal patiënten met huidtype IV and V konden geen conclusies over deze groep getrokken worden. Voor vitiligo lesies gelocaliseerd op de rest van het lichaam werden slechts kleine repigmentatie verschillen gezien in alle geanalyseerde groepen. Wij concluderen dat orale PL een nuttige toevoeging kan zijn aan de NB-UVB behandeling van vitiligo vulgaris van het hoofd-hals gebied. Dit positieve effect lijkt versterkt aanwezig te zijn in patiënten met een lichte huidskleur.

Conclusies en toekomstperspectieven

Deze thesis beschrijft twee interessante nieuwe concepten.
Het eerste is het concept van non-invasieve microscopische analyse van de huid met de *in vivo* reflectance confocale microscoop. De laatste jaren is aanzienlijke vooruitgang geboekt met de optimalisatie van non-invasieve beeldvormende technieken om menselijke ziektes te bestuderen. Duidelijke voorbeelden hiervan zijn de echografie, magnetic resonance imaging (MRI) en computed tomography (CT). Het zou tegenwoordig ondenkbaar zijn om het medische vak uit te oefenen zonder deze technieken. Echter, er was in eerste instantie wel tijd nodig om deze technieken te leren accepteren en

beheersen voordat ze als regulier onderdeel van het diagnostisch arsenaal werden beschouwd. De RCM is een veelbelovende nieuwe techniek die het mogelijk maakt om de huid op non-invasieve wijze te onderzoeken op quasi-histopathologisch niveau. Ook deze techniek vereist een nieuwe benadering van het interpreteren van de histologie van de huid omdat de huid in horizontale vlakken (coupes) wordt afgebeeld in plaats van de verticale coupes die we gewend zijn bij de traditionele histologie. Echter, met goede training is deze manier van kijken niet moeilijk te leren. Daarbij komt dat het instrumentarium van de RCM eenvoudig te bedienen is, zodat de RCM zeer geschikt is om gebruikt te kunnen worden in de medische praktijk.

Op het gebied van huidkanker is reeds een aanzienlijke hoeveelheid onderzoek verricht naar het karakteriseren van tumoren zoals maligne melanomen en basaalcelcarcinomen met de RCM. Het is indenkbaar dat met verder onderzoek en verbetering van de beeldvormende kwaliteit de RCM op een bepaald moment gebruikt kan worden als een klinisch hulpmiddel om de potentiële maligniteit van huidafwijkingen te helpen bepalen, vergelijkbaar met hoe de dermatoscoop tegenwoordig wordt gebruikt. De RCM kan ook een rol spelen bij Mohs chirurgie. Recidiefpercentages van diverse huidtumoren zijn lager na behandeling met Mohs chirurgie, maar het gebruik van deze techniek wordt beperkt doordat het erg tijdrovend is door de bewerking van het weefsel voor directe histopathologische analyse. Daar ook verse *ex vivo* excisie preparaten onder de RCM gelegd kunnen worden voor directe visualisatie, zou men met de RCM meerdere geëxcideerde coupes snel kunnen beoordelen op aan- of afwezigheid van tumorweefsel en zouden alleen die coupes die met de RCM tumor-vrij bevonden zijn verder verwerkt kunnen worden tot vriescoupes voor lichtmicroscopische analyse. Op deze wijze kan RCM het proces van Mohs chirurgie aanzienlijk versnellen en hopelijk kan de RCM op een bepaald moment geïmplementeerd worden als onderdeel van deze techniek. In onderzoeksverband is de RCM waardevol omdat hetzelfde huidgebied herhaaldelijk bestudeerd kan worden (deze thesis). Deze eigenschap gecombineerd met het feit dat melanine het beste contrast levert door wit op te lichten in RCM afbeeldingen maakt RCM waardevol om pigmentatie gerelateerde aspecten van de huid te onderzoeken (deze thesis).

De techniek van de RCM staat nog in de kinderschoenen. Gezien de aanzienlijke hoeveelheid onderzoek die reeds is - en nog steeds wordt - gedaan om de

mogelijkheden van deze techniek verder te exploreren en de technische aspecten ervan te verbeteren, beschikt deze techniek over de potentie om de wijze waarop dermatologische aandoeningen heden ten dage worden gediagnosticeerd revolutionair te veranderen.

Het tweede nieuwe concept in deze thesis is het concept van orale photoprotectie en pigment modulatie van menselijke huid door middel van het antioxidant *Polypodium leucotomos* (PL). Het concept van orale photoprotectie is op zichzelf niet nieuw daar meerdere onderzoekers studies hebben verricht naar de photoprotectieve werking van andere orale middelen, met name vitamine preparaten. PL is echter wel het eerste oraal toedienbare middel dat na inname van slechts 2 doses leidt tot een significante systemische photoprotectie, wat klinisch zichtbaar is als een vermindering van de zonnebrandreactie, en histologisch als een vermindering van DNA schade, te zien aan een verminderd aantal thymine dimeren en sunburn cellen (deze thesis). Ook leidt toediening van dezelfde PL dosis tot bescherming tegen de acute phototoxiciteit van PUVA, en tegen de hyperpigmentatie respons die zo kenmerkend voor PUVA therapie is. Histologisch zien we bescherming tegen het PUVA-geïnduceerde Langerhans cel verlies en de inductie van sunburn cellen (deze thesis). Deze histologische observaties prikkelen tot interessante hypothesen over de photoprotectieve werking die PL op de lange termijn kan hebben tegen UV-geïnduceerde huidkanker, maar dit kan alleen onderzocht worden in lange termijn follow-up studies.
Orale PL zou als supplement kunnen dienen bij de huidige topicale sunscreens leidend tot een verbeterde photoprotectie doordat de orale toedieningsvorm op simpele wijze een egale bescherming voor het gehele lichaam biedt. Het effect van langdurige inname van PL dient onderzocht te worden omdat de photoprotectieve eigenschappen van andere antioxidanten zoals vitamine C en E toenemen bij langdurige inname, resulterend in een photoprotectief effect dat op bepaalde punten vergelijkbaar is met het effect van PL na inname van slechts 2 doses.
De zoektocht naar een oraal photoprotectief middel zal voortduren omdat orale photoprotectie duidelijke voordelen heeft boven topicale photoprotectie. Dit verklaart ook de interesse van verschillende onderzoeksgroepen in dit concept. Verder onderzoek naar de actieve stoffen in PL zal wellicht mogelijkheden bieden om deze stoffen chemisch te reproduceren en te

verbeteren, waarbij een duidelijke stap in de richting van een echt systemisch photoprotectief middel zou worden gezet.
Een ander aspect is het gebruik van PL als supplement bij phototherapie, zowel om de bijwerkingen te verminderen als om phototherapie-geïnduceerde pigmentatie te moduleren. Een open gecontroleerde studie naar de combinatie van orale PL met PUVA in de behandeling van psoriasis vulgaris liet zien dat de cumulatieve UVA dosis noodzakelijk om complete remissie van de huidafwijkingen te bewerkstelligen statistisch significant lager was in de patiëntengroep die PUVA met PL kreeg dan bij patiënten die alleen PUVA kregen. Omdat het risico op huidkanker door PUVA duidelijk geassocieerd is met de cumulatieve UVA dosis en toevoeging van PL leidt tot vermindering van de PUVA-geïnduceerde schade op histologisch niveau (deze thesis), wijst dit erop dat de veiligheid van PUVA verbeterd kan worden door PL zonder afname van effectiviteit. Wij hebben aangetoond dat de pigmentatie van vitiligo vulgaris van het hoofd-hals gebied toeneemt als smalspectrum UVB phototherapie wordt gecombineerd met orale PL, waardoor de effectiviteit van deze phototherapie wordt verbeterd (deze thesis). De mogelijkheden om met PL de veiligheid en effectiviteit van andere phototherapeutische therapieën te verbeteren dienen verder te worden onderzocht.

Chapter 9

Curriculum vitae
Bibliografie
Dankwoord / Words of thanks

Curriculum vitae

Maritza (Pina) Middelkamp Hup werd geboren op 29 juli 1976 te Amsterdam als tweede kind in een gezin van 4 dochters. In 1979 verhuisde zij naar Curaçao, Nederlandse Antillen, waar zij haar VWO opleiding afrondde aan het Radulphus College. In 1994 begon zij met de opleiding geneeskunde aan de Erasmus Universiteit te Rotterdam. Haar afstudeeronderzoek en eerste publicatie voltooide zij aan de afdeling kinderdermatologie onder begeleiding van prof. dr. Arnold Oranje. Na behalen van het artsexamen in maart 2001 verhuisde zij naar Boston, U.S.A., om als research fellow onderzoek te doen aan de Wellman Laboratories of Photomedicine van de Harvard Universiteit, onder begeleiding van dr. Salvador González, prof. dr. Madhu Pathak en prof. dr. Thomas Fitzpatrick. Deze onderzoeken vormden de basis van haar proefschrift "Detection, modulation and impact of skin pigmentation". In juni 2002 begon ze als arts-assistent dermatologie in het Nederlands Instituut voor Pigmentstoornissen van het AMC, waar zij het voortzetten van haar onderzoeken combineerde met klinisch werk, onder supervisie van dr. W. Westerhof. In oktober 2004 begon ze met haar opleiding dermatologie op de afdeling dermatologie van het AMC onder begeleiding van prof. dr. J.D. Bos, welke ze verwacht af te ronden in april 2008.

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Dankwoord / Words of thanks

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Colour figures

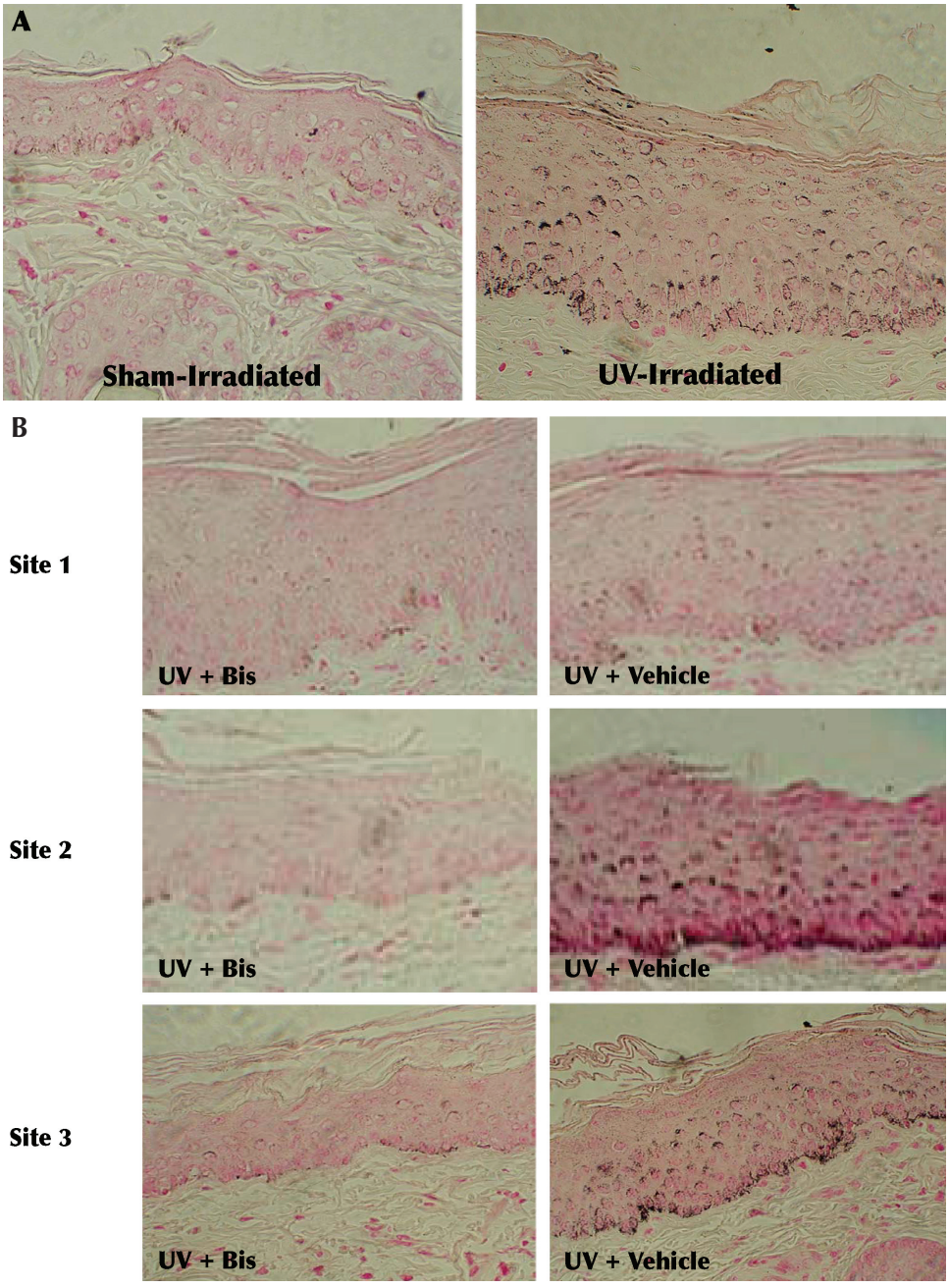

A
Sham-Irradiated
UV-Irradiated
B
Site 1
UV + Bis
UV + Vehicle
Site 2
UV + Bis
UV + Vehicle
Site 3
UV + Bis
UV + Vehicle

Figure 4

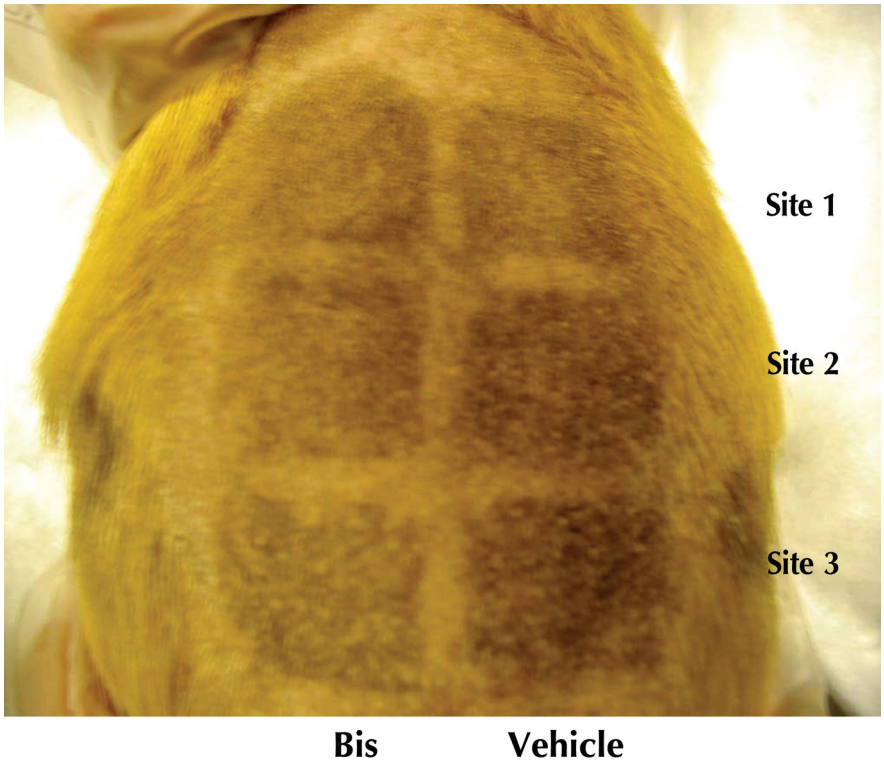


Figure 5

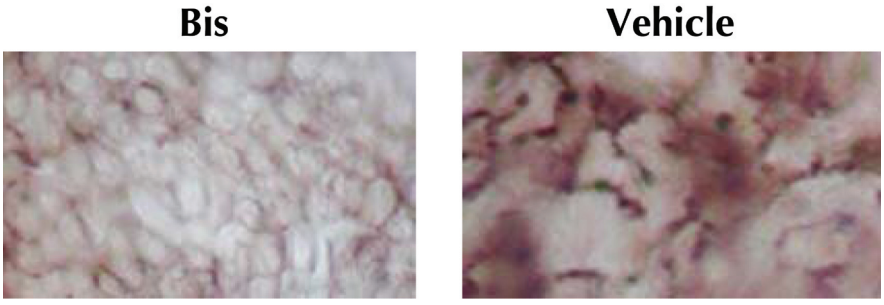


Figure 6

Chapter 3

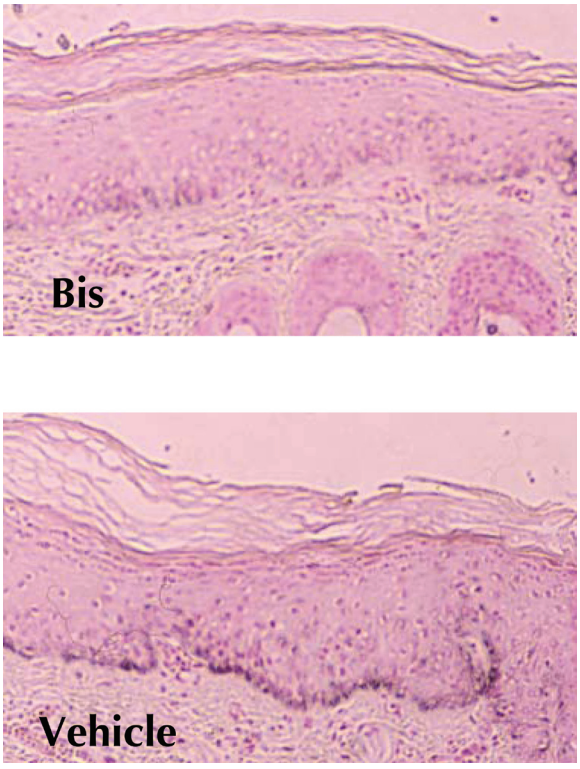


Figure 8

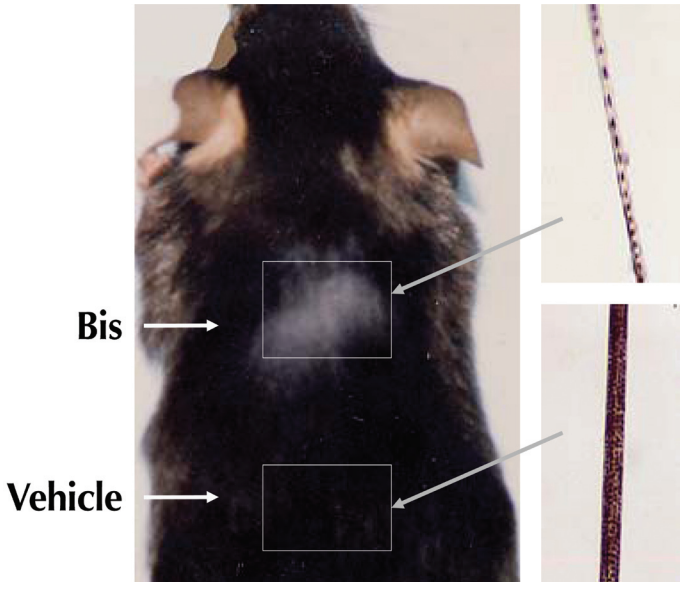


Figure 9

Chapter 4

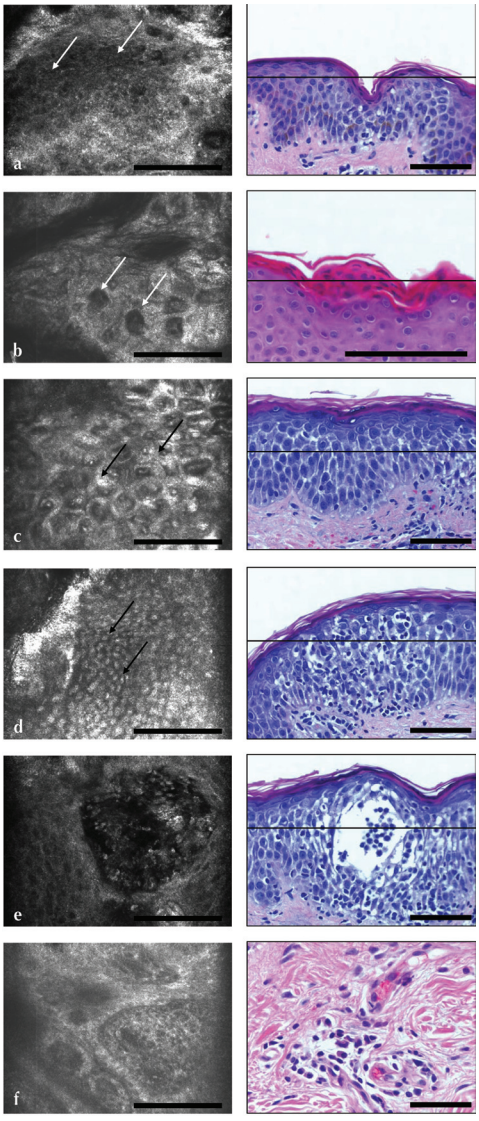


Figure 1

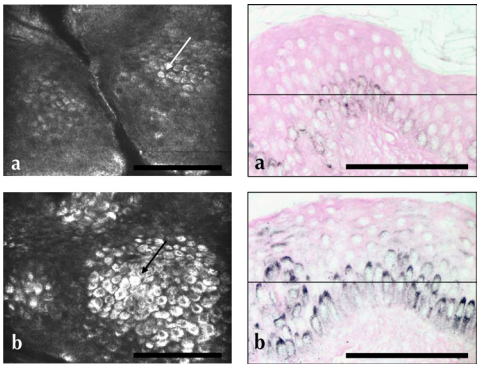
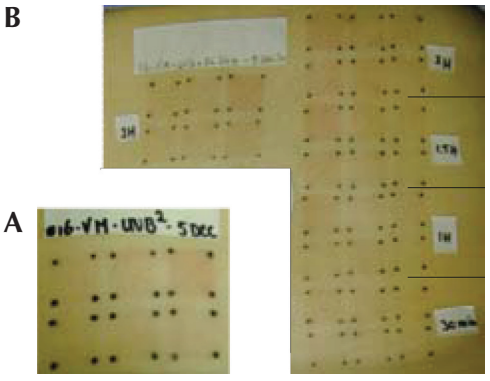


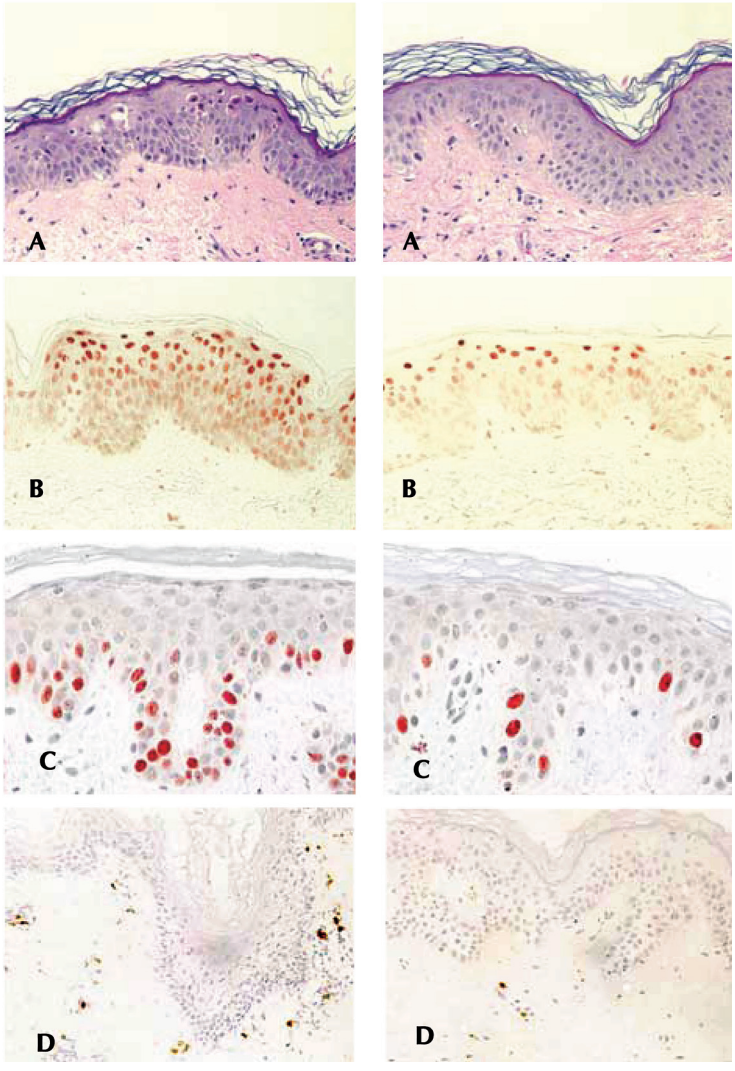
Figure 2

Chapter 5



SS alone

SS with PL



Chapter 6

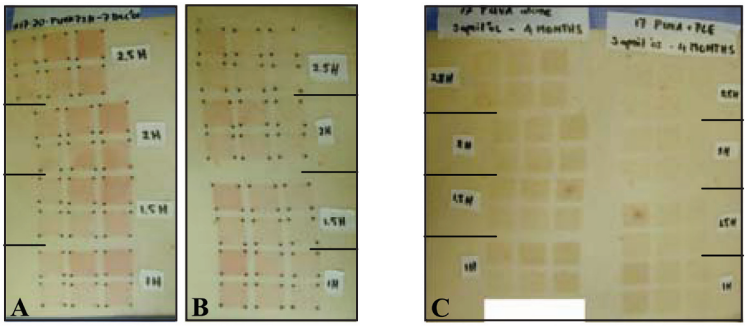


Figure 1

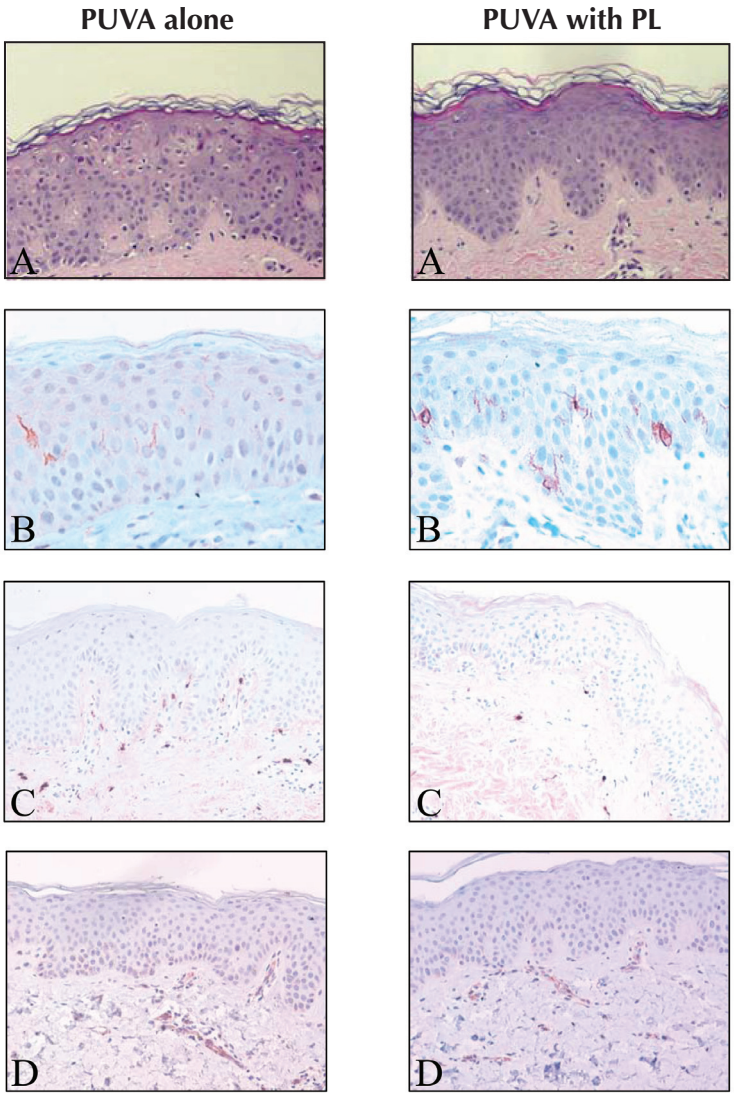


Figure 3