Pathophysiology and prevention of photoaging

the role of melanin, reactive oxygen species and infiltrating neutrophils

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Pathofysiologie en preventie van veroudering van de huid onder invloed van zonlicht

de rol van melanine, reactieve zuurstofdeeltjes en
infiltrerende neutrofiele granulocyten
(met een samenvatting in het Nederlands)

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Carpe diem

Contents

Chapter 1

General Introduction

I. Clinical and microscopic characteristics of photoaged skin

'Photoaging' or 'extrinsic aging' is the process by which sunlight or artificial ultraviolet radiation (UV) gradually induces clinical and histological changes in the skin. Photoaging, in fact, superimposes intrinsic physiological skin-aging otherwise known as 'chronoaging'.[1] The clinical appearance of photoaged skin can vary considerably between individuals. Typical photoaged skin, however, is characterized by dryness, a rough texture, irregular pigmentation, telangiectasia, yellowish color, plaque-like thickening, loss of skin tone, deep creases and fine wrinkles.[2] One of the primary affected sites is the back of the neck which is easily and often unintentionally exposed to the sun.[3]

The skin changes seen in UV-protected chronoaged skin differ considerably from typical photoaged skin. UV-protected chronoaged skin is relatively smooth, pale and atrophic.[1;4]

Histological changes in the skin due to chronic UV exposure involve the epidermis and the dermis. The epidermis may be relatively normal or show alterations such as epidermal hyperplasia or atrophy, disappearance of dermal papillae, thickening of the basement membrane, focally increased numbers and irregular distribution of melanocytes and melanosomes, atypical keratinocytes, parakeratosis, and thickening of the stratum corneum. These epidermal changes are responsible for the irregular pigmentation and roughening of the skin surface.[2] Ultimately UV-induced damage to the epidermal cells can lead to malignant transformation.[5;6]

The most conspicuous dermal histological defect, and the hallmark of photoaged skin, is an accumulation of elastotic material in the mid and upper dermis (so-called solar elastosis). By electron microscopy, fully developed photoaged skin shows alternating areas of fibrous, granular and homogenous elastotic material.[3;7;8] The fibrous areas are believed to consist of increased numbers of thickened and tangled elastic fibers. The granular and homogenous areas are thought to be the result of fragmentation of these thickened and tangled fibers. Other observations in the mid and upper dermis include the presence of deformed collagen fibers, a decrease in the total amount of collagen, increased amounts of ground substance, and dilated blood vessels.[1;4]

In normal skin, elastic and collagen fibers form a complex network providing a biomechanical scaffold for cell attachment and anchorage of macromolecules. Elastic and collagen fibers thus provide tensile strength and resistance to the skin and help maintain its shape and form.[9;10]

Collagen molecules, the building blocks of collagen fibers, are synthesized by fibroblasts. A collagen molecule comprises three polypeptide chains (α-chains) which form a unique triple helix structure.[9;11] The formation of collagen fibrils and fibers is basically a self-assembly process which is mostly determined by the intrinsic properties of the individual collagen molecules.[9] There are more than twenty genetically distinct collagen

molecules (collagen types) in human tissues. Numerous collagen types can be found in the skin including types I, III, IV and VII.[11;12] Collagen molecules constitute ±70% of dry weight of the skin.

Elastic fibers are also synthesized by fibroblasts. In the upper dermis the elastic fiber system is organized in a characteristic fashion: so-called elaunin fibers form bundles running parallel to the epidermis and bundles originating in the middle dermis running radially towards the epidermis. Terminal elastic fibers, known as oxytalan fibers, run perpendicular to the epidermis and end in the basement membrane zone.[7;10] Elastic fibers consist of two major components: elastin and microfibrils.[10;13;14] Elastin makes up 90% of a mature, normal elastic fiber.[7] Microfibrils are biochemically made up of fibrillin-1, fibrillin-2, fibrillin-3 and other microfibril-associated glycoproteins.[13] During the synthesis of elastic fibers microfibrils are layed down as scaffold on which elastin is deposited.[7;10;15] On electron-microscopic examination, the cross section of a young elastic fiber shows an oval or flattened, speckled structure. The electron-dense speckles are formed by concentrations of microfibrils which are surrounded by amorphous, relatively electron-lucent elastin. The periphery of the elastic fiber is also rich in electron-dense microfibrils. As the elastic fiber matures, the electron-dense speckles can become obscured by further deposition of elastin.[7;10] The ratio of elastin to microfibrils ('level of elastinization') decreases as elastic fibers approach the epidermis. Elaunin fibers are only partially 'elastinized' while oxytalan fibers consist purely of microfibrils.[7]

On electron-microscopic examination of photoaged skin the lower dermis shows relatively normal elastic fibers with a speckled amorphous core surrounded by electron-dense microfibrils. Depending on the degree of UV damage the following changes of elastic fibers can be observed in the mid and upper dermis: (1) enlarged electron-dense zones within the elastic fiber, (2) reduced peripheral electron-dense microfibrils, (3) loss of fibrous structure, (4) alternating areas with granular and amorphous elastotic material.[7]

Immunohistochemical light- and electron-microscopic studies of photoaged skin consistently show elastin positivity of the elastotic material in photoaged skin indicating that it originates from elastic fibers. However, there are contrasting reports on microfibril staining in photoaged skin. Although some studies have described fibrillin-1 protein staining, Suwabe *et al.*[16] did not find significant fibrillin-1 staining in degenerated elastic fibers of severely photoaged facial skin, and Matsuta *et al.*[3] could not detect anti-microfibril HB-8 (HB-8, anti-human thoracic duct lymphocyte monoclonal antibody which is cross reactive to elastic fiber micorfibrils) staining in photodamaged skin in the neck.

The origin of the elastotic material in photoaged skin has been (and still is) subject of much debate. Hypotheses include that the elastotic material could be (1) a degradation product of elastic fibers,[17] (2) a degradation product of collagen fibers,[18] (3) a degradation product of both collagen and elastic fibers,[19] and (4) an abnormal product of UV-stimulated fibroblasts.[20] Braverman *et al.*[7] found decreased amounts of collagen in areas of

elastosis, but found no morphological evidence that collagen degeneration participated in the production of the elastotic material. Based on the immunohistochemical staining patterns of multiple markers of the extracellular matrix (ECM), several groups concluded that the elastotic material in photoaged skin must mainly be derived from degenerated elastic fibers.[3;16;17] Debelle and Tamburro[14] stated that: 'mature elastin is extremely stable, and its turnover is so slow it can be considered that elastin lasts for the entire lifespan of the host'.

In summary, photoaging involves many changes in the epidermis and the dermis. The ECM damage in the dermis, so-called solar elastosis, is the most conspicuous feature of photoaged skin.

II. Acute and chronic UV-induced events and the pathophysiology of photoaging

Acute and chronic effects of UV

Most of the harmful effects of sunlight are attributed to the ultraviolet part of the solar spectrum (wavelengths 100-400 nm).[21;22] Solar UV is classically divided into UVA (320-400 nm), UVB (280-320 nm) and UVC (100-280 nm). UVA is further subdivided into UVA1 (340-400 nm) and UVA2 (320-340 nm).[23] The stratospheric ozone layer, formed 10-40 km above the earth's surface, prevents all UVC and 70-90% of UVB from reaching the earth's surface. Individual exposure to the remaining solar UV depends on geographical location, altitude, time of the year, time of the day and cloud cover.[24]

UV can damage cellular and extracellular components in the skin.[25;26] A single exposure to UV can lead to a clinical sunburn or 'sunburn reaction'. This is characterized by the four cardinal signs of an inflamed tissue: rubor, tumor, calor and dolor. Histologically, an inflammatory infiltrate consisting of various cell types is observed.[27] The presumable function of a sunburn reaction is to remove and repair UV-damaged tissue. Chronic exposure to UV can lead to the clinical and histological changes discussed above (i.e., photoaged skin) and (pre)malignant skin lesions.

Although the pathophysiology of photoaging has been studied extensively and attractive hypothetical models have been proposed, the exact mechanisms (and their relative contribution) are not yet fully understood. Furthermore, the precise action spectrum for photoaging of human skin has not yet been determined. UVA could be more important than UVB.[28] This is based on the following data: daily and year-round exposure to UVA is 10-100 times higher than exposure to UVB, UVA penetrates the skin more deeply,[29] and UVA is a powerful inducer of reactive oxygen species (ROS) in the skin.[30] On the other hand, UVB photons are 600-1000 times more energetic than UVA photons[31] and, despite being strongly filtered by the epidermis, 10-15% of UVB still penetrates into the dermis.[21] Furthermore, UVB is also capable of inducing oxidative damage in the skin.[32] Finally, UVB is believed to be mainly responsible for the sunburn reaction, sun tanning and photocarcinogenesis.[5;28;31]

Many studies investigating the acute and chronic effects of UV have used artificial UVB and/or UVA sources to conduct their experiments. Moreover, many studies are limited to *in vitro* work or were performed using animals. These studies have provided valuable data, yet one cannot unreservedly extrapolate these experimental results to sunlight-induced acute and/or chronic changes in human skin. There is a growing trend towards using solar simulator UV-sources which emit both UVB and UVA and whose emission spectra approach that of sunlight.[21] Using solar simulators and performing experiments *in vivo* with human subjects are probably superior tools to investigate sunlight-induced skin changes, and are most likely to generate results relevant to photoaging of

human skin. Therefore, next to natural sunlight, solar-simulating radiation (SSR) should be the radiation of choice when investigating photoaging *in vivo.*

UV-induced photoproducts, ROS, signaling pathways, and inflammatory response

The damaging effects of UV on the skin are both direct and indirect.[25;26] UV directly induces DNA-photoproducts, transforms trans- to cis-urocanic acid, and is a powerful generator of ROS.[21] These direct actions trigger a cascade of events which ultimately give rise to the acute and chronic signs (clinical and histological) of UV-damaged skin. The precise sequence of all the events that take place and their relative contribution to the photoaging process remains a topic of study.

The heterocyclic bases of DNA are major UV-absorbing chromophores in the skin.[33;34] Absorption of UV-photons leads to the formation of DNA photoproducts. The main directly induced DNA photoproducts are cyclobutane pyrimidine dimers and 6-4 photoproducts.[33;35] The latter, although quantitatively less important, are more mutagenic. Thymine dimers form the major portion UV-induced cyclobutane pyrimidine dimers.[33] UV, particularly UVA, also induces DNA photoproducts through generation of ROS which react with nucleic acids.[33] Keratinocytes and other affected cells in the skin are generally well-equipped to repair DNA-photoproducts. However, excessive and/or recurrent DNA damage can overwhelm the repair-systems resulting in permanent mutations in coding or regulatory DNA sequences.[5;6] These permanent mutations can eventually give rise to malignant tumors, particularly when they occur in tumor suppressor genes or oncogenes.[5;6] In addition to gene mutations, through induction of proinflammatory and immunoregulatory cytokines and chemokines, DNA photoproducts appear to trigger other events surrounding UV-induced skin changes, including immune suppression.[21]

Trans-urocanic acid is another major UV-absorbing chromophore in the skin.[36] It is formed by deamination of histidine and accumulates in the epidermis due to a lack of the catabolic enzyme urocanase. Urocanic acid is a normal constituent of all skin phototypes (SPT, see further). Trans-urocanic acid absorbs UV and is transformed to cis-urocanic acid. Cis-urocanic acid can induce mast-cell degranulation, inhibit respiratory burst activity of neutrophils, and is believed to play an important role in UV-induced immune suppression.[21;36;37]

There is considerable evidence that ROS initiate many of the events following irradiation of the skin (summarized in Table 1). *In vivo*, under normal circumstances, low levels of ROS are generated continuously. These ROS are involved in signaling pathways, cell-activation, cell-proliferation and cell-differentiation.[38-40] The body defends itself against excessive endogenous or exogenous oxidative stress with antioxidant enzymes and non-enzymic antioxidants.[38;41] During UV exposure, similar to DNA damage and repair, this protective system can be overwhelmed resulting in direct and indirect damage to cellular and extracellular components, including the ECM proteins.[42-45]

Table 1. Direct and indirect effects and/or involvement of UV-induced ROS*

Event ■ Affecting * Via → Leads to •					Ref
■ activation of NF-κβ	• induction of proinflammatory cytokines including IL-1, TNF-α, IL-6, IL-8 • expression immunoregulatory proteins • expression of cell survival proteins				21;26;28;48-50
■ lipid membrane oxidation	→ arachidonic acid release	• prostaglandins, prostacyclin and leukotrienes synthesis			
	→ ceramide release	→ increased nuclear transcription complex AP-1 activity	→ decreased TGF-β receptors and/or TGF-β signaling and other mechanisms	• reduced collagen I/III synthesis • keratinocyte proliferation • upregulation of MMPs	28;43;44
■ activation of cell surface receptors, including receptors for EGF, IL-1, insulin, keratinocyte growth factor, and TNF-α, partly through inhibition of protein-tyrosine phosphatase-κ	→ stimulation of stress-associated mitogen-activated protein kinases p38 and JNK				
■ inhibition of PTEN ■ activation of Akt	→ pi3 kinase pathway				
■ SMAD7 induction	→ decreased TGF-β induced SMAD2-3 signaling				
■ protein oxidation	• loss of structural protein function • loss or gain of protein/enzyme activity • increased or decreased susceptibility to degradation				28;42;51
■ mitochondrial damage (mtDNA 'common deletion')	• decreased mitochondrial function • cell apoptosis				28;30;52;52
■ nucleic acid oxidation	* genomic DNA * telomeres (when telomeres become critically short or in the event of loop disruption)	• mutations in coding or regulatory DNA sequences • tumor suppressor protein p53 activation • cell apoptosis or senescence			5;6;21;21;28;48;53
■ nitric oxide synthesis	• vasodilation • stimulation of melanogenesis				54;55
■ oxidation and/or polymerization of existing melanin or melanogenic precursors	• immediate pigment darkening • persistent pigment darkening				56

*non-exhaustive list of events

Generation of ROS by UV requires the absorption of photons by endogenous and/or exogenous photosensitizer molecules. Examples of endogenous photosensitizers are trans-urocanic acid, riboflavin, NADPH, porphyrins, quinones and bilirubin.[34;43] Absorption of photons leads to an excited state of the photosensitizer which subsequently reacts with another substrate (type I reaction) or with oxygen (type II reaction).[23] Type I reac-

tions produce radical ions, type II reactions produce ROS. Examples of ROS are singlet oxygen, superoxide anion, hydrogen peroxide and hydroxyl radical.[46] The hydroxyl radical, which is formed via superoxide anion and hydrogen peroxide, is highly active and has been detected in UV-exposed murine skin.[47]

Following UV exposure, one of the first observable events is a vasodilatory response resulting in an increased blood flow. Vasodilation and resultant erythema follow a biphasic pattern. Erythema is observed immediately following or during UV exposure and is seen again 1 hour after exposure, reaching a peak intensity after 24-48 hours.[21;26] Although keratinocytes are primarily affected and stimulated by UV, all nucleated cells in the skin are capable of producing cytokines when stimulated or activated by UV. Key mediators of UV-induced erythema are thought to be prostaglandins (PGE2, PGF2α and other arachidonic acid metabolites) and nitric oxide (NO).[21;48;54] Other inflammatory substances, however, probably also contribute to UV-induced erythema. In the initial phase of erythema development UV-stimulated release of vasoactive mediators by mast cells could play an important role. Resident mast cells can release proinflammatory and immunoregulatory cytokines (e.g., histamine, TNF-α) in response to UV exposure.[57] The precise mechanisms behind UV-induced mast cell degranulation are unknown. Photosensitizers may be involved or, as Kulka *et al.*[58] showed, particular neuropeptides (substance-P, VIP) may initiate mast cell degranulation and synthesis of chemokines.

NO, which is produced by keratinocytes following UV exposure and, as mentioned above, appears to play an important role in UV-induced erythema, may also be a key mediator in UV-induced melanogensis.[55]

As early as 30 minutes after UV exposure, depending on the dose and wavelengths of UV, dyskeratotic or apoptotic keratinocytes (sunburn cells) can be observed in the lower half of the epidermis. UV-induced apoptosis or 'programmed cell death' protects the body against UV-induced carcinogenesis.[6] 'Immediate apoptosis', which is observed within 30-60 minutes after UV exposure, is caused by direct targeting of mitochondria by ROS. 'Intermediate apoptosis', starting within 4 hours of UV damage, involves receptor-triggered mechanisms (e.g., FasR). UV-induced genomic DNA damage is believed to be the trigger of 'delayed apoptosis' which commences later than 4 hours after UV exposure and entails new protein synthesis.[30]

Langerhans cells quickly migrate from the epidermis upon UV exposure. 72 hours after UV exposure (depending on the dose and wavelengths of UV) only 10% may remain in the epidermis.[26]

As mentioned earlier, the UV-induced inflammatory infiltrate contains different cell types. These cells, in order of entrance, are neutrophilic granulocytes, macrophages and T-lymphocytes.[27;59-61] Leucocytes enter the skin a few hours after irradiation and the response generally resolves within 48-72 hours. Inflammatory cell-type, cell-numbers and the duration of inflammation are, however, dose-dependent and dependent on the UV

source.[62] Mobilization of inflammatory cells largely depends upon specific chemoattractant stimuli (e.g., IL-8, LTB4, PAF, C5a) and appropriate adhesion molecule expression (e.g., E-selectin, P-selectin, ICAM-1, L-selectin, β2 integrins).[63;64] UV-activated keratinocytes are capable of producing numerous cytokines including IL-1, TNF-α, IL-6, IL-8, and Gro-α. These cytokines, together with other soluble factors, are responsible for the recruitment of the inflammatory cells after UV irradiation.[21;65;66] Dermal endothelial cells have been shown to express adhesion molecules after UV exposure.[66;67]

In summary, UV can: (1) damage cellular and extracellular components including nucleic acids, proteins, lipids and bilipid membranes, (2) activate signaling pathways that are related to proliferation, differentiation, senescence and tissue degradation, (3) cause depletion of cellular antioxidants and antioxidant enzymes, (4) activate the neuroendocrine system and stimulate release of neuroendocrine mediators, (5) cause increased synthesis and release of proinflammatory mediators from a variety of skin cells, and (6) induce an inflammatory infiltrate. With respect to photoaging, UV-induced mediators may either directly damage the ECM or indirectly contribute to the skin changes observed: i.e., induced skin-infiltrating cells and stimulated resident cells may release their own mediators which can further damage the ECM.

Matrix metalloproteinases and photoaging

Matrix metalloproteinases (MMPs) are a group of structurally related zinc-dependent endopeptidases.[68] Endopeptidases are enzymes that break peptide bonds within a protein molecule, in contrast to exopeptidases which cleave only the end parts of a polypeptide chain. The family of MMPs comprises over 20 members. MMPs are classified as collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs and others[69] (Table 2).

MMPs are multifunctional proteins. Collectively they can degrade essentially all extracellular matrix components including collagen fibers, elastic fibers, glycoproteins and proteoglycans.[70;71] Furthermore, they are able to process many bioactive molecules: MMPs can cleave cell surface receptors, release apoptotic ligands, and activate or inactivate cytokines, chemokines and growth factors.[71] MMPs are believed to play an important role in cell-proliferation, cell-differentiation, cell-migration, cell-adhesion, apoptosis and host defense.[72] Keratinocytes, fibroblasts, endothelial cells, macrophages, mast cells, neutrophils and eosinophils are all capable of producing MMPs.[73] MMPs can be induced in the skin by UVA, UVB, SSR, infrared radiation and natural sunlight.[74-78]

Each MMP has specific action loci whereby particular amino acid sequences are cleaved.[70] However, the substrate specificity of MMPs can be broad and, consequently, cross reactivity between different MMPs is common (Table 2). The relative proteolytic capacity of different MMPs with respect to a particular substrate has not yet been fully elucidated. Not all the MMPs listed in Table 2 have been studied in the context of photo-

Table 2. Human matrix metalloproteinases and their extracellular matrix substrates[69-71]

MMPs	ECM substrates
Soluble-types	
Collagenases	
MMP-1, MMP-8, MMP-13	Gelatin, Collagens (I, II, III, VII, X), entactin, tenascin, aggrecan
Gelatinases	
MMP-2, MMP-9	Gelatin, collagens (I, IV, V, VII, X, XI), elastin, fibrillin, fibronectin, vitronectin, laminin, aggrecan
Stromelysins	
MMP-3, MMP-10	Gelatin, collagens (III, IV, V, IX, X, XI), fibronectin, laminin, entactin, tenascin, aggrecan, decorin, fibrin/fibrinogen
MMP-11	Fibronectin, laminin, aggrecan
Matrilysins	
MMP-7	Gelatin, collagens (III, IV, V, IX, X, XI), elastin, fibrillin, fibronectin, laminin, entactin, tenascin, aggrecan, decorin
MMP-26	Gelatin, collagen type IV, fibronectin, fibrinogen
Others	
MMP-12	Gelatin, collagen IV, elastin, fibrillin, fibronectin, vitronectin, laminin, entactin, fibrin/fibrinogen, aggrecan
MMP-19	Gelatin, Collagen IV, fibronectin, laminin, entactin, tenascin, aggrecan
MMP-20	Amelogenin, cartilage oligomeric matrix protein, aggrecan
MMP-21	Gelatin
MMP-23	Gelatin
MMP-27	-
MMP-28	-
Membrane-types	
Transmembrane	
MMP-14, MMP-15, MMP-16, MMP-24	Gelatin, collagen, fibronectin, vitronectin, aggrecan
GPI-anchored	
MMP-17	Gelatin, fibrin
MMP25	Gelatin, collagen IV, fibronectin, laminin, fibrin/fibrinogen

aging but in theory, if induced in the skin, virtually all could contribute to the photoaging process.

The above mentioned collagenases are capable of hydrolyzing fibrillar collagen and, once cleaved in the triple helical domain, collagen can further be broken down by

gelatinases and stromelysins. The combined actions of interstitial collagenase (MMP-1), gelatinase A (MMP-2), gelatinase B (MMP-9) and stromelysin 1 (MMP-3) can fully degrade collagen type I (Dermal collagen consists of ±80% collagen type I and 10-20% collagen type III[11]) and components of the elastic network.[70] These MMPs, together with neutrophil collagenase (MMP-8), macrophage elastase (MMP-12) and matrilysin (MMP-7), have particularly been studied with respect to the pathophysiology of photoaging. MMP-8, which is a major product of neutrophils, has similar substrates compared to MMP-1: these substrates include collagens type I, II, III, VII, X, gelatin, various glycoproteins and proteoglycans.[71] MMP-8, however, cleaves collagen type I faster than type III, while MMP-1 shows greater selectivity for collagen type III compared to type I.[73] MMP-12 is mainly produced by macrophages.[73;79] Its substrates includes fibronectin, fibrin/fibrinogen, laminin, and proteoglycans. Furthermore, MMP-12 together with MMP-2, MMP-7 and MMP-9, are capable of damaging components of the elastic-fiber network.[69;80-82]

The integrity of healthy human skin is largely dependent on a tightly regulated homeostasis of the collagen and elastic fiber networks.[83] Under normal conditions, the activity of MMPs is controlled by transcriptional regulation, pro-enzyme activation, and inhibitors of MMPs. MMPs are inhibited by α2-macroglobulin and tissue inhibitors of metalloproteinases (TIMPs).[69;70] The latter multifunctional proteins can inactivate specific MMPs. An imbalance between MMP and TIMP synthesis, resulting in an excess of (activated) MMPs, can lead to extracellular matrix degradation. Investigators have shown that following UV exposure certain MMPs are upregulated and particular TIMPs are downregulated.[75;84;85] Thus, the cells and mediators involved in UV-induced synthesis, activation and release of MMPs and TIMPs could play an important role in photoaging.

Neutrophils and photoaging

Neutrophils are the most abundant type of white blood cells and are a hallmark of tissue inflammation.[86] Neutrophils react within an hour of tissue injury and rapidly migrate toward the site of tissue damage. Neutrophils are capable of releasing a wide variety of products including proteolytic enzymes, antimicrobial products, ROS, cytokines and chemokines. These products are stored in azurophilic-, specific-, tertiary-, and secretory vesicles, or are newly formed.[87-89] Carl Nathan[90] collected considerable scientific evidence to support the following views with respect to the functions of neutrophils: (1) neutrophils often have an important role in launching immune responses, (2) neutrophils help to heal tissues as well as destroying them, (3) neutrophils give instructions with as much specificity as a lymphocyte or neuron, albeit with a specificity of a different kind, (4) neutrophils integrate information with a circuitry of awe-inspiring design, to tailor its responses to its spatial and temporal context, (5) neutrophils offer potential opportunities for selective pharmacological intervention, to both promote and restrain inflammation.

It has long been recognized that neutrophils can damage tissues.[91;92] Furthermore, neutrophils and their products have been identified as a major cause of tissue destruction in a number of conditions, including acute lung injury and emphysema,[93;94] rheumatoid arthritis,[95] periodontitis,[96;97] and wound infection[98]. Despite their potency, however, neutrophils have only been reported as likely important contributors to photoaging in a murine model.[99]

An important immunohistochemical marker and major product of neutrophils, is neutrophil elastase.[100;101] Together with cathepsin G and proteinase-3, neutrophil elastase belongs to the group of serine proteinases produced by neutrophils.[94] These serine proteinases are stored in azurophilic granules and are normally rapidly and irreversibly inhibited by powerful plasma antiproteinases (α1-antitrypsin, α2-macroglobulin and secretory leucoproteinase inhibitor) present in blood and the interstitium.[92] Neutrophil elastase is a potent proteolytic enzyme capable of cleaving elastin, fibrillin, collagen (types I-IV), fibronectin, laminin, vitronectin and proteoglycans.[94;102;103] The biological functions of neutrophil elastase include: (1) involvement in migration of neutrophils by means of focal proteolysis, (2) killing of microorganisms, and (3) degradation or activation of various proteins including ECM proteins, receptors, cytokines and chemokines.[94;101;104]

Neutrophils also express MMPs, namely; MMP-8 and MMP-9. MMP-8 and MMP-9 are stored in specific and tertiary vesicles in an inactive form.[105] MMP-12 expression by neutrophils has also been reported.[106]

The probable mechanisms by which neutrophil-derived proteolytic enzymes are activated and prevented from being inactivated by antiproteinases, and cause ECM damage are reviewed by Weiss[92] and Chua.[104] It involves a complex interaction with nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-derived oxygen metabolites and it occurs at or just outside the plasma membranes of neutrophils. The following section summarizes the events that take place: activated neutrophils generate ROS by means of the membrane-associated NADPH oxidase enzyme complex. Following a series of reactions catalyzed by superoxide dismutase and myeloperoxidase and involving superoxide anion and hydrogen peroxide, hypochlorous acid is formed. Hypochlorous acid is a highly active product and instantaneously reacts with amines (present in the immediate vicinity of the neutrophil or released by the cell itself) forming a group of oxidants known as chloramines. These chloramines are responsible for breaking down the antiproteinase shield surrounding tissue-infiltrating neutrophils (chloramines oxidize and inactivate plasma antiproteinases such as α1-antitrypsin) and are responsible for activating particular proteolytic enzymes released from neutrophil secretory vesicles (chloramines oxidize and activate MMP-8 and MMP-9). In addition, MMP-8 and MMP-9 can themselves hydrolyse and inactivate α1-antitrypsin and neutrophil elastase can inactivate TIMPs. MMP-8, MMP-9 and neutrophil elastase released from neutrophils are thus able to damage the ECM. In conditions where there is a deficiency of antiproteinases (e.g., α1-

antitrypsin deficiency) or in case of massive neutrophil infiltration (e.g., infected wounds with abscess formation) proteolytic enzymes are active beyond the immediate vicinity of the neutrophil and cause accelerated and/or severe tissue destruction.

In summary, neutrophils are potent cells capable of generating and releasing multiple mediators which can directly or indirectly damage the ECM. Since neutrophils infiltrate the skin following UV exposure they should be studied more closely with respect to the pathophysiology of photoaging.

Current hypothetical model of photoaging

A hypothetical model for the pathophysiology of photoaging has been presented by Fisher *et al.*[76;85] They suggested that skin damage after a single exposure to UV is only partially repaired and that microscopic damage accumulates after each exposure, eventually leading to the clinical and histopathological signs of photoaged skin. Fisher *et al.*[76;85] postulated that this microscopic damage is caused by specific MMPs. They showed that MMP-1, MMP-3 and MMP-9 are induced following exposure of human skin to suberythemogenic doses of UVB. As mentioned above, these MMPs are capable of breaking down skin collagens. On the basis of (1) the localization of immunohistochemically stained MMPs, (2) *in situ* hybridisation of mRNA, (3) *in situ* zymography, and (4) literature data, they concluded that the MMPs were fibroblast and keratinocyte derived. The expression of MMP protein by fibroblasts following UV exposure was based on the morphology of MMP-positive cells in *in vivo* experiments and on *in vitro* data. In their *in vivo* studies, MMP mRNA was mostly located in the epidermis and MMP protein was also mainly induced in this compartment.[85] They concluded that keratinocytes are the major source of MMPs. Because the ECM damage observed in photoaged skin is located mostly in the mid and upper dermis, they proposed that keratinocyte-derived MMPs diffuse from the epidermis into the dermis (where most of the MMP enzyme activity had been detected). In a separate study, Fisher *et al.*[77] studied neutrophil-derived MMP-8. MMP-8 was induced in skin exposed to 2 MED of UVB and 2 MED of SSR. On the basis of the finding that trans-retinoic acid failed to inhibit MMP-8 expression following UV exposure, but did inhibit degradation of collagen type I (indirect detection method), they concluded that MMP-8 was not enzymatically active and therefore did not contribute to photoaging.

Thus, the current hypothetical model proclaims that fibroblast- and particularly keratinocyte-derived MMPs are key mediators in the pathophysiology of photoaging (Figure 1).

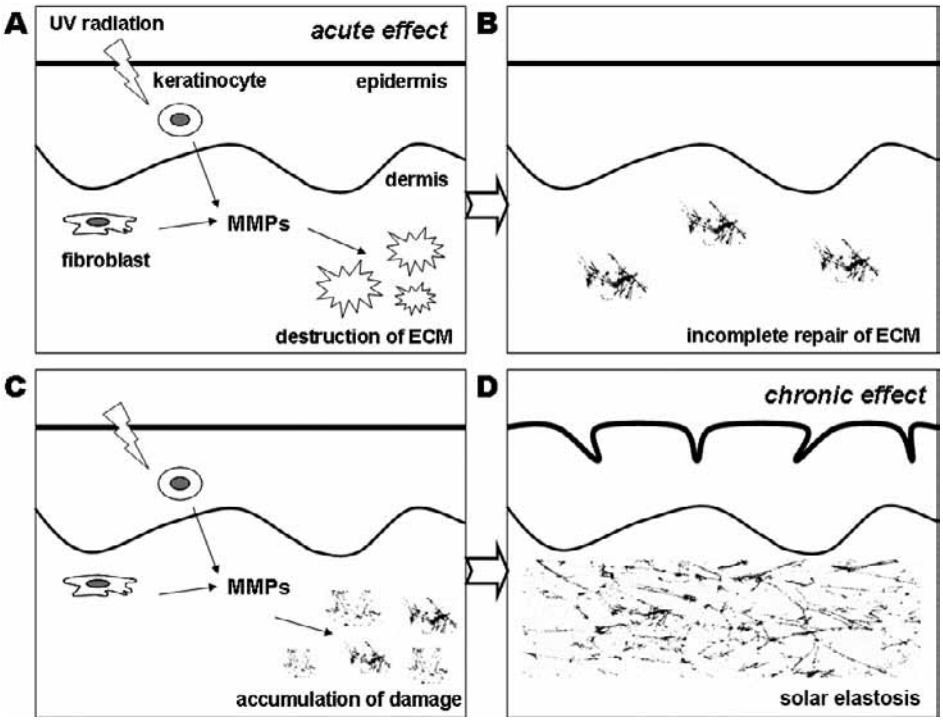

Figure 1. Current hypothetical model for the pathophysiology of photoaging. UV-stimulated keratinocytes and fibroblasts express and release MMP-1, -3 and -9. Keratinocytes are the major source of MMPs. MMPs degrade collagen fibers in the mid and upper dermis. Panels A and B represent short-term effects of UV-induced damage to the skin. Panels C and D represent the long-term effects of UV-induced damage to the skin.

III. Skin phototypes (SPT) and natural defenses against sunlight/UV

Natural defenses of human skin against sunlight/UV[107]

The basic mechanisms by which the skin protects itself against the damaging effect of sunlight/UV are summarized below:

(1) The stratum corneum absorbs, reflects and scatters UV. UV-absorbing products in the stratum corneum include urocanic acid and particular amino acids of keratins such as histidine, tyrosine and tryptophan.
(2) Genetically determined constitutive and facultative (see further) skin pigmentation. Melanin absorbs and scatters UV, and is a free radical/ROS scavenger.
(3) Naturally occurring antioxidants such as lipophilic carotenoids, alpha-tocopherol (vitamin E), glutathione and ascorbic acid (vitamin C) react with and neutralize a variety of free radicals/ROS.
(4) Antioxidant enzymes such as catalase, superoxide dismutase and gluthatione peroxidase/reductase deactivate ROS.
(5) DNA repair mechanisms repair UV-induced DNA damage.
(6) Epidermal hyperplasia and hyperkeratosis increase the physical barrier to UV.

Skin color and SPT, sunburn reaction and photoaging

Skin color is the result of a blend of colors due to various chromophores present in the skin: melanin (brown), oxyhaemoglobin (red), deoxygenated haemoglobin (blue) and carotene (yellow-orange). Melanin, however, is the most important contributor to skin color.[108]

SPT is a concept that was introduced in 1975 to establish one's sensitivity to UV.[107;109] It was used to determine a safe starting dose for UV therapy. SPT is based on the history of reactions of a person's skin to sun exposure. To determine the SPT the following questions need to be answered:[109] (1) How does the skin respond to 45-60 minutes of sun exposure at noon, in the northern latitudes (20° to 45°), in the early summer (+/- 90-120 mJ/cm² of UVB)? (2) How intense is the sunburn reaction (pain, erythema, oedema)? (3) How much tan has developed after 7 days? Based on this history and on the constitutive skin color, people are categorized into six phototypes (originally 4 later 6 skin phototypes - Table 3). *Constitutive* skin color is the degree of pigmentation of non-stimulated, sun-protected skin. It is genetically determined and reflects the inherent content and distribution of melanin in the epidermis.[107;108] Tan or *facultative* skin color is the inducible skin pigmentation. The degree of pigmentation raised above the constitutional level varies according to genetically determined tanning ability and history of UV exposure.[107;108;110] Whereas hair and eye color are unreliable guides to sunburn sensitivity, SPT is a good predictor of a person's susceptibility to the acute and chronic damaging effects of UV. The lower SPTs (SPT I-III, 'white' skin) are at far greater risk

Table 3. Skin phototypes according to Fitzpatrick[109;112]

SPT	Constitutive skin color	MED (UVB) in mJ/cm²	Sunburn sensitivity	Facultative skin color	Photoaging
I	white	15-30	always burns	never tans	very sensitive, early onset
II	white	25-40	burns easily	tans minimally	strong, early onset
III	white	30-50	burns moderately	tans moderately	moderate to strong
IV	olive	40-60	burns minimally	tans well	moderate to low
V	brown	60-90	rarely burns	tans profusely	slow and low
VI	dark brown or black	90-150	never burns	tans profusely	slow and minimal

of developing sunburn, photoaging and skin cancer including melanoma, basal cell and squamous cell carcinoma.[109;111;112] In contrast, 'black' skin (SPT VI) is much more resistant to the damaging effects of solar radiation. Since there is no significant difference in epidermal thickness or stratum corneum thickness between black and white skin (although the stratum corneum is possibly more compact in black skin) the difference in pigmentation is believed to be primarily responsible for the increased resistance of black skin.[112;113] Melanin is synthesized in melanosomes and transferred from melanocytes to surrounding keratinocytes.[114] One melanocyte supplies approximately 36 keratinocytes with melanin. As keratinocytes differentiate, melanosomes are degraded and melanin is released in the cytoplasm, ultimately appearing as non-degradable, amorphous melanin in the stratum corneum. The number of melanocytes does not differ between black and white skin. However, the total amount of melanin is far greater in black skin. Moreover, melanosomes in black skin are larger, non-aggregated and more stable, and the amount of melanin per melanosome is much greater.[112;115;116] Melanin efficiently absorbs and scatters UV, and is a powerful free radical/ROS scavenger.[117] Thus melanin shields and protects cellular and extracellular components in both the epidermis and the dermis.

The importance of the skin's natural defenses against the damaging effects of UV is illustrated by several clinical syndromes. The importance of DNA global genome nucleotide excision-repair mechanism is exemplified by Xeroderma Pigmentosum (XP, a group of inherited skin disorders). Due to a reduced ability or inability to repair UV-induced DNA damage, these patients are extremely sensitive to UV, and, despite measures to limit UV-exposure, rapidly develop (pre)malignant skin lesions.[118] In XP the UV-induced skin damage is mostly limited to the epidermal compartment. Patients with Oculocutaneous Albinism have an inherited pigmentary disorder characterized by a normal number of melanocytes but a defect in melanin synthesis (reduced or absent tyrosinase activity). These patients are highly susceptible to sunburn, photoaging and skin carcinogenesis.[119] Vitiligo is an acquired skin condition characterized by depigmented skin lesions in which

melanocytes are lost from the epidermis.[120] Persons with SPT VI and vitiligo offer a natural disease model to further substantiate the photoprotective properties of melanin.

In summary, different SPTs show a different susceptibility to UV-induced skin changes including photoaging. Melanin directly absorbs UV photons and scavenges UV-induced ROS, and appears to play a major role in the skin's defense against UV-induced skin changes.

IV. Prevention and treatment of sunburn reaction and photoaging

Prevention and treatment methods for sunburned skin and/or photoaged skin described in literature include the following measures, products and interventions: behavioral changes, clothing and hats, sunscreens, DNA-damage repair enzymes, antioxidants, dietary lipids, osmolytes, hydroxy acids, fluorouracil, imiquimod, retinoids, botulinum toxin, chemical peels, dermabrasion, photodynamic therapy, laser therapy, injectables, and surgical procedures.[28;121;122]

A Cochrane review[123] entitled 'interventions for photodamaged skin' assessed the effects of topically applied treatments, systemic drug treatments, laser therapy and surgical procedures on photodamaged skin. The selection criteria for this systemic review were: (1) randomized controlled trials which compared drug or surgical interventions with no treatment or with placebo or with another drug, and (2) studies must include adults with mild, moderate or severe photodamaged facial or forearm skin. Thirty studies of variable quality were included. Based on these thirty studies the author's conclusions were: (1) Topical tretinoin cream (a retinoid) improves skin changes (fine and coarse wrinkles, roughness, freckles and pigmentation) associated with prolonged sun exposure. The greatest improvements occurred with higher concentrations of tretinoin, but at the expense of greater local irritation. (2) More evidence is needed before any recommendations can be made on oral or topical polysaccharide- or hydroxy- acids. (3) It is unclear how useful surgical, laser or chemical peels are in the absence of suitable control groups. Both techniques lead to pain and redness after the procedure. The authors did not find any randomized controlled trials on the effectiveness of application of sunscreens or wearing of protective clothing in preventing photoaging of the skin.

Retinoids, corticosteroids, vitamin E and sunscreens

Because retinoids, corticosteroids, vitamin E and sunscreens are often mentioned and/or have been studied extensively with respect to prevention and management of sunburn reactions and/or photoaging they are discussed in more detail.

Retinoids

There are a significant number of studies investigating the effects of retinoids on photoaging. The improvement of human photoaged skin following treatment with topical retinoids, although modest, has been shown to be highly statistically significant.[28;124-126] The described (long term) effects of topical retinoids on the skin are: (1) reduction and redistribution of epidermal melanin, (2) improved ultrastructural characteristics of the epidermis, (3) increased anchoring fibrils, (4) increased deposition of papillary dermal collagen, and (5) increased vascularity in the papillary dermis. Kossard *et al.*[127] investigated the effect of daily application of 0.05% tretinoin for 6 months on photoaged skin. They

found that tretinoin treatment particularly affected the epidermis and had no effect on the degree of solar elastosis.

The effects of retinoids are probably mediated through binding to nuclear retinoic acid receptors and subsequent binding of the ligand/receptor complex to specific sites on particular genes. Several effects are proposed to result from an inhibition of the induction of activator protein-1 (AP-1). Inhibition of AP-1 activity and reduced TGF-β signaling in keratinocytes and fibroblasts leads to a reduced expression of MMPs and increased expression of collagen types I and III.[128] This mechanism (i.e., inhibition of AP-1 activity by retinoids), together with the hypothesis that keratinocyte- and fibroblast-derived MMPs are responsible for ECM damage leading to solar elastosis, are often quoted as the basis of the effectiveness of retinoids in the treatment of photoaging. Application of retinoids does not inhibit infiltration of neutrophils following skin exposure to erythemogenic doses of UVB and SSR.[77]

Corticosteroids

(Gluco)corticosteroids are cholesterol derivatives which are normally synthesized in the adrenal cortex. Corticosteroids are involved in a wide range of physiologic systems including carbohydrate metabolism, protein catabolism, lipid catabolism, electrolyte and water homeostatis, stress response, immune response and regulation of inflammation.[129;130] Corticosteroids are fat soluble and easily diffuse across lipid membranes into the cytoplasmic compartment of cells where they bind to receptor proteins. The corticosteroid-binding receptor is activated when two hormone molecules are bound. Subsequently, the hormone-receptor complex is transferred to the nucleus where it exerts its functions by influencing transcription.[129;130]

Chronic use of corticosteroids induces atrophy of the skin.[129;131] Considering this catabolic effect, it is logical that topical or systemic steroids are not used to treat photoaged skin. Corticosteroids have, however, been used to treat sunburned skin and several studies have shown that UV-induced erythema can be suppressed by topical corticosteroids.[132;133] Other authors failed to show an effect of topical and systemic corticosteroids on UV-induced erythema.[134;135] The consensus of opinion with respect to the management of an acute sunburn is not to administer corticosteroids.[136] Topical corticosteroids are widely used for different inflammatory skin conditions, including those where infiltration of neutrophils is involved.[137] Fisher *et al.*[77] investigated the effect of a powerful topical steroid on UV-induced MMP upregulation and on infiltration of neutrophils and found that both were inhibited. The effect of (topical) steroids on UV-induced ROS has, to our knowledge, not been studied.

Vitamin E

As mentioned earlier, the skin contains both antioxidant enzymes and non-enzymatic antioxidants. Vitamin E or tocopherol is an example of the latter. It is an essential nutrient and a major lipophilic antioxidant in plasma, membranes and tissues.[138;139] There are eight naturally occurring tocopherols of which α-tocopherol is the most important, both in quantity and activity.[139] α-Tocopherol is easily oxidized and thus inactivated. The acetate derivative of α-tocopherol is, however, more stable. Relative vitamin E activity of specific tocopherols depends on biochemical reactivity, resorption from the gut, cellular uptake and metabolic turnover. Vitamin E scavenges free radicals/ROS and plays a particularly important role in protecting polyunsaturated fatty acids against oxidation.[138;139] Polyunsaturated fatty acids are major constituents of cytoplasmic- and mitochondrial-lipid membranes. In the presence of iron or toxins, ROS can be generated from polyunsaturated fatty acids and initiate a chain reaction. Vitamin E can neutralize ROS and the chain reaction may thus be prevented.[138;139]

The effect of topical and systemic vitamin E on sunburn reaction and photoaging has been studied quite extensively in both animals and humans.[140] Numerous studies have demonstrated that topical and/or systemic vitamin E administration prior to UV exposure is capable of reducing acute skin responses such as erythema and oedema (particularly when combined with vitamin C).[141-144] Conversely, other studies have shown little or no effect of vitamin E on UV-induced erythema.[140;145] Several murine studies have shown a protective effect against UV-induced wrinkle formation.[140] In general, the acetate derivative of α-tocopherol, although more stable, is less protective.[140]

Both topical and systemic vitamin E products are being marketed as anti-aging products. Although there are *in vitro* and *in vivo*, animal and human data supporting a positive effect, randomized controlled trials are lacking.

Sunscreens

Sunscreens prevent UV from penetrating the skin. Sunscreens contain UV filters that can be grouped into two broad categories: *organic* filters (or 'chemical' filters) which absorb UV photons and *inorganic* filters (or 'physical' filters) which reflect UV photons.[24;28] Organic filters are usually 'invisible' and thus cosmetically appealing. Absorption of UV-photons by organic filters releases heat and may lead to the formation of active products capable of interacting with cutaneous molecules. Although first generation organic filters tended to be unstable, current filters are photostable. There is a long list of safe organic filters presently available, which absorb UV along a wide range of wavelengths such that a combination of filters can achieve adequate UVA and UVB protection.[24;28] Zinc oxide and titanium dioxide are examples of inorganic filters. In addition to reflecting UV, these compounds also reflect visible light, making them cosmetically less attractive. Their advantage is, however, that they are chemically inert and protect against UVA, UVB and vis-

ible light.[24;28] Modern sunscreens contain both organic and inorganic filters. Nanoparticle technology has facilitated the production of cosmetically more acceptable sunscreens that also contain inorganic filters. Skin protection factor (SPF) is a widely used concept that refers to a sunscreen's protection against UV-induced (mostly UVB) erythema.

Sunscreens have been shown to effectively prevent or reduce UV-induced erythema.[146] Also, sunscreens can prevent UV-induced neutrophil infiltration. Murine studies have demonstrated that sunscreens can prevent solar elastosis.[147] As mentioned earlier there are few if any randomized controlled trials investigating the efficacy of sunscreens in preventing or slowing down photoaging of human skin.

V. Outline of this thesis

The primary objectives of this thesis are to determine the role of neutrophils in the pathophysiology of photoaging, and to critically examine the current hypothetical model for the pathophysiology of photoaging.
Secondary objectives are:

(i) To determine the expression, activity and origin of photoaging-associated proteolytic enzymes in 'young' and 'elderly' skin, different skin phototypes ('black' and 'white'), sun-exposed and sun-protected skin, and SSR-exposed skin.
(ii) To show that in chronically sun-exposed skin elastic fiber breakdown is more important than collagen breakdown.
(iii) To provide further evidence for the role of ROS in the pathophysiology of photoaging.
(iv) To determine the protective effect of skin pigmentation, sunscreens, topical vitamin E, and betamethasone against SSR-induced erythema and neutrophil influx.

The current hypothetical model for the pathophysiology of photoaging focuses on collagen degradation and states that keratinocyte- and fibroblast-derived MMPs play a crucial role in the dermal ECM damage seen in photoaged skin. However, solar elastosis, the histopathologic hallmark of photoaged skin, is characterized by deposition of elastotic material in the mid and upper dermis. This elastotic material is most likely derived from degraded elastic fibers. Neutrophils infiltrate the skin following UV exposure but have not received much attention with respect to the pathophysiology of photoaging. Neutrophils, however, are capable of generating and releasing proteolytic enzymes which can damage both collagen and elastic fibers. Skin pigmentation, melanin, appears to be a major protective factor against the acute and chronic damaging effects of UV on the skin. The mechanisms by which skin pigmentation protects against photoaging have not been fully elucidated. A possible mechanism could be protection against UV-induced neutrophil influx. Multiple commercially available products claim that they are effective in slowing down or even reversing the photoaging process. These products include sunscreens and ROS scavengers such as topical vitamin E.

In chapters 2 and 4 we compare black and white skin with respect to the expression of photoaging-associated proteolytic enzymes and other SSR-induced responses. In chapters 2, 3 and 4 data is shown supporting an important role for neutrophils in the pathophysiology of photoaging. The same data poses questions regarding the current hypothetical model. In chapter 5 we analyze collagen types and elastic fiber components in different skin phototypes. In addition, we investigate the effect of neutrophils, neutrophil elastase, ROS, and direct UV exposure on elastic fiber staining. In chapter 6 we review the probable role of neutrophils in photoaging, and we discuss preventive measures and

potential pharmacological targets in this respect. In chapter 7 we investigate the effect of sunscreens, topical betamethasone and topical vitamin E on SSR-induced erythema and neutrophil influx.

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Chapter 2

Responses of black and white skin to solar-simulating radiation: differences in DNA photodamage, infiltrating neutrophils, proteolytic enzymes induced, keratinocyte activation and IL-10 expression

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Abstract

Black skin is more resistant to the deleterious effects of ultraviolet radiation than white skin. A higher melanin content and a different melanosomal dispersion pattern in the epidermis are thought to be responsible for this. Our purpose was to compare skin responses in black and white skin following exposure to solar-simulating radiation (SSR) to further investigate the photoprotective properties of black skin. Six volunteers of skin phototype I-III (white) were exposed to (doses measured directly with a Waldmann UV detector device) 12 000-18 000 mJ per cm^2 (2 MED) of SSR and compared with six volunteers of skin phototype VI (black) exposed to 18 000 mJ per cm^2 (<1 MED) of SSR. The presence and distribution of skin pigment, DNA photodamage, infiltrating neutrophils, photoaging associated proteolytic enzymes, keratinocyte activation, and the source of interleukin 10 (IL-10) in skin biopsies taken before and after exposure were studied. In all white skinned subjects, 12 000-18 000 mJ per cm^2 of SSR induced DNA damage in epidermal and dermal cells, an influx of neutrophils, active proteolytic enzymes, and diffuse keratinocyte activation. Additionally, in three of the white skinned volunteers IL-10 positive neutrophils were found to infiltrate the epidermis. Except for DNA damage in the suprabasal epidermis, none of these changes was found in black skinned subjects. Increased skin pigmentation appears to be primarily responsible for the observed differences in skin responses. Our data could provide an explanation as to why black skin is less susceptible to sunburn, photoaging, and skin carcinogenesis.

Introduction

Black skin is better protected against the damaging effects of ultraviolet radiation (UV) than white skin. These effects include sunburn (acute effect), photoaging, and skin carcinogenesis (chronic effects).[1] The mechanisms underlying the photoprotective properties of black skin are still poorly understood. A higher melanin content, however, and a different melanosomal dispersion pattern in the epidermis ensuring a greater barrier to UV, are thought to be responsible.[1-3]

Documented effects of UV on the skin, which have, or may be compared between persons of different skin color are as follows: DNA photodamage, infiltration of inflammatory cells, induction of photoaging-associated proteolytic enzymes, keratinocyte proliferation, and immune suppression.

A previous investigation has studied the protective effect of skin pigment with respect to UV-induced DNA damage.[4] Persons with skin phototypes (SPT) IV and II were exposed to repeated suberythemogenic doses of solar-simulating radiation (SSR) and the outcome was compared. It appeared that the levels of DNA damage induced were dependent on the physical (in Joules per cm^2 rather than the erythema effective dose (in MED or minimal erythemal dose) of SSR. Although the two groups differed in terms of the mean minimal erythemal dose (IV>II), the amount of epidermal DNA damage induced was similar in both groups after exposure to equal physical doses of SSR. A more recent study has described a difference in the distribution of UV-induced DNA damage between subjects with SPT I/II and SPT V/VI.[5] In the former, DNA photoproducts were found in both the epidermis and the upper dermis. In the latter, however, DNA photoproducts were restricted to post-mitotic cells in the upper epidermis.

Inflammatory cells infiltrate the skin following UV exposure, and neutrophilic granulocytes are known to be present in sunburned skin.[6] Neutrophils are potent producers of a wide array of proteolytic enzymes, including neutrophil elastase and matrix metalloproteinase 9 (MMP-9).[7] These products are potentially capable of inflicting serious extracellular matrix damage.[8-10] MMP-1, produced by keratinocytes and fibroblasts, is the matrix metalloproteinase most often associated with extracellular matrix damage in the pathogenesis of photoaging of the skin.[11;12]

UV radiation can activate keratinocytes and induce (hyper) proliferation *in vivo* as demonstrated by increased epidermal thickness following UV exposure.[13] This may be regarded as an adaptive process since a thicker epidermis serves as a more effective physical barrier. Activated, hyperproliferative keratinocytes express a different set of cytokeratins (K6, K16, and K17) as compared with basal (K5, K14) and differentiating keratinocytes (K1, K2, and K10).[14] Immunohistochemical staining of these proteins thus enables the possibility of distinguishing between different activation/proliferation states of keratinocytes.

Selgrade *et al.*[15] have previously investigated UV-induced immunosuppression in subjects with different SPT. They demonstrated that the degree of immunosuppression induced with a solar simulator was dependent on the erythemal reactivity. Subjects with a weak or no erythemal response (mainly SPT IV-VI) showed a flat dose-response curve, i.e., no erythema and no immunosuppression.

The cytokine associated with UV-induced immunosuppression is interleukin 10 (IL-10).[16] IL-10-producing inflammatory cells infiltrate the epidermis following exposure to erythemogenic doses of UV.[17] IL-10-producing cells may be involved in skin carcinogenesis[16;18;19] and thus play a role in the difference found between black and white skinned persons in (UV associated) skin cancer incidence.

The purpose of this study was to further compare skin responses in black and white skin after UV exposure. A solar simulator was used as a UV source because it best approaches the emission spectrum of natural sunlight. The following parameters were investigated: SSR-induced DNA photodamage, infiltrating neutrophils, the presence and enzymatic activity of (selected) proteolytic enzymes, keratinocyte activation markers, and the presence and source of IL-10.

Materials and Methods

Volunteers, irradiation procedures, and biopsy processing

The skin characteristics of six healthy white- (SPT I-III: mean age 24.5 y, SEM 1.06) and six healthy black-(SPT VI: mean age 24.3 y, SEM 0.99) skinned volunteers are summarized in (Table 1). Six other white skinned volunteers (SPT I-III: mean age 25.3, SEM 3.21) were additionally recruited to study skin responses after exposure to different erythema effective doses of SSR (Table I, volunteers 13-18). All white subjects were of Dutch origin. Black subjects were of West-African and Afro-(South) American descent. The medical ethical committee of the University Medical Center Utrecht approved the study. Informed consents were obtained from all volunteers prior to commencing the experimental procedures.

For the irradiation protocols, CLEO Natural lamps (Philips, Eindhoven, the Netherlands) were used. This solar simulator emits predominantly UVA and 3% UVB. The ratio of UVB/UVA is comparable to mid-day sunlight during summer at 52°N, Amsterdam. The spectral energy distribution shows a continuum ranging from 290 to 420 nm, with a maximum around 355 nm. Relative spectral distribution measurements were performed with a calibrated standard UV-visible spectrometer (model 742, Optronic Laboratories, Orlando, FL, USA).

Throughout the experiments irradiation of the skin was monitored with a UV detector device (Waldmann, Schwenningen, Germany). The output at the surface of the skin was kept at 6.0 mW per cm2 by adjusting the distance of the source to the exposed skin

Table 1. Volunteer characteristics

No.	SPT	Skin color	Gender	Age (years)	MED (mJ per cm²-Waldmann)	MED (mJ per cm²-CIE eff)*	PD[a] (mJ per cm²-Waldmann)
1	II	White	Female	21	8700	50.8	17 400
2	II	White	Male	26	6300	36.8	12 600
3	I	White	Female	22	6000	35.0	12 000
4	II	White	Male	24	9000	52.8	18 000
5	II	White	Male	26	6300	36.8	12 600
6	III	White	Male	28	9000	52.8	18 000
7	VI	Black	Male	28	-	-	18 000
8	VI	Black	Male	22	-	-	18 000
9	VI	Black	Male	26	-	-	18 000
10	VI	Black	Male	22	-	-	18 000
11	VI	Black	Male	24	-	-	18 000
12	VI	Black	Female	24	-	-	18 000
13	II	White	Female	30	6300	36.8	[b]
14	II	White	Female	19	6300	36.8	[b]
15	I	White	Female	39	4500	26.3	[b]
16	II	White	Female	21	6300	36.8	[b]
17	III	White	Male	24	9000	52.8	[b]
18	II	White	Male	19	6300	36.8	[b]

White skinned volunteers (nos. 1-6) were exposed to 2 MED of SSR. This was equivalent to 12 000-18 000 mJ per cm²-Waltmann. All black skinned volunteers (nos. 7-12) were exposed to 18 000 mJ per cm²-Waltmann. Volunteers 13-18 participated in a dose-response study.

*calculated wavelength weighted UV doses using the CIE erythema action spectrum.

[a] PD: applied physical dose: irradiance measured with a Waldmann UV detector device multiplied by the time of exposure.

[b]Sun-protected buttock skin was exposed to 0, 0.5, 1, and 2 MED.

SPT, skin phototype; MED, minimal erythemal dose; SSR, solar simulating radiation.

area. Applied physical doses were calculated by multiplying the irradiance measured with the time of exposure.

The same experienced investigator determined the minimal erythemal doses (MED) of the white skinned volunteers. A specially constructed test device[20] with nine windows (each 3 x 10 mm), which open consecutively in a geometrical time series (up to 50 min exposure time) exposing the underlying skin, was used for this purpose. The MED was taken as the 3 x 10 mm patch of irradiated buttock skin which showed only just perceptible erythema and defined borders. To allow inter-study comparisons, the wavelengths adjusted erythema effective doses were calculated using the CIE erythema action spectrum.[21] These values are given in Table 1.

Following MED determination, the first group of white skinned volunteers (1-6, Table 1) was irradiated with a dose of 2 MED. Three areas of approximately 2 x 2 cm on the buttock were exposed and 4 mm punch biopsies were taken from the irradiated sites after 6, 24, and 48 h. A control biopsy was taken from a patch of non-irradiated buttock skin. The six other white skinned volunteers were irradiated with increasing doses of SSR (0, 0.5, 1, and 2 MED) and biopsies were taken after 24 h.

Black skinned volunteers were irradiated with a fixed physical dose of SSR (18 000 mJ per cm^2-Walmann) corresponding to 2-3 MED of the white skinned volunteers. As with the first group of white skinned volunteers, three 2 x 2 cm areas on the buttock were exposed, and biopsies were taken after 6, 24, and 48 h including a control biopsy.

The biopsies were snap-frozen in liquid nitrogen, embedded in Tissuetek (Sakura, Torrance, CA, USA), and stored at -80°C. In order to stain the skin immunohistochemically and evaluate enzymatic activity *in situ*, biopsies were cut into 6 µm thick skin sections with a freezing microtome and mounted on 3-aminopropyl tri-ethoxy silane (A3648, Sigma, St Louis, Missouri)-coated slides. Subsequently, the slides were left to dry and either put on Silicagel (MERCK, Darmstadt, Germany) to be used the following day or temporarily stored at -80°C.

Immunohistochemistry

To investigate UV-induced DNA photodamage thymine dimers were analyzed. The following steps (1-8) summarize the immunohistochemical staining procedure: skin sections were fixed, respectively, in (1) acetone containing 0.06% hydrogen peroxide, (2) 25% acetic acid-PBS solution, and (3) 0.07 M sodium hydroxide-70% ethanol-phosphate-buffered saline (PBS) solution. Next, the sections were consecutively incubated with (4) 10% normal rabbit serum (NRS), (5) monoclonal mouse anti-thymine dimers (Kamiya Biomedical Company, Seattle, Washington; diluted 1:2000 in 1% NRS), (6) biotinylated rabbit anti-mouse IgG1 (ZYMED, San Francisco, California; diluted 1:200 in 1% NRS), and (7) alkaline phosphatase (AP)-conjugated avidin-biotin complex (DAKO A/S, Glostrup, Denmark; diluted 1:50 in PBS). (8) AP activity was demonstrated using naphthol ASBI (Sigma) as a substrate and new fuchsine (Sigma) 10 mg per 100 ml as chromogen dissolved in 0.1 M Tris-HCL + 0.05 M $MgCl_2$ (pH 8.5) resulting in a pink staining. Endogenous AP activity was inhibited by adding levamisole (35 mg per 100 mL) to the above reaction mixture.

The presence of neutrophils in skin sections was demonstrated by staining cells using monoclonal mouse anti-human neutrophil elastase (NP57, DAKO A/S; diluted 1:50 in 1% normal human serum (NhuS)/1% normal horse serum (NhoS)). Sections were fixed in dry acetone and pre-incubated with 10% NhuS/10% NhoS. Subsequently, the sections were incubated with mouse anti-human neutrophil elastase (primary antibody). This was followed by incubation with biotinylated horse anti-mouse immunoglobulin (secondary

antibody obtained from Vector, Burlingame, California; diluted 1:800) and AP-labeled streptavidine (Boehringer Mannheim GmbH, Mannheim, Germany; diluted 1:300). Both primary and secondary antibodies were diluted with 1% NhuS/1% NhoS. AP activity was detected as described above. Sections were counterstained with Mayer's hematoxylin.

MMP-9 and -1, cytokeratins 6, 16, and 17 were detected by performing practically identical staining procedures as described above for neutrophil elastase, differing only in the primary antibodies used. These were (all monoclonal antibodies): mouse anti-human MMP-9 (Neomarkers, Fremont, California; diluted 1:50), mouse anti-human MMP-1 (Oncogene, Boston, Massachusetts; diluted 1:50) and mouse anti-human cytokeratin 6, 16, and 17 (all three purchased from Novocastra, Newcastle, UK; diluted 1:50).

IL-10 expression was analyzed using monoclonal mouse anti-human IL-10 (Instruchemie, Delfzijl, the Netherlands; diluted 1:300). Frozen skin sections were dried in air and fixed in 100 mL dry acetone containing 0.06% hydrogen peroxide. After preincubating with 0.1% bovine serum albumin (BSA)-PBS, the sections were incubated overnight at 4°C with the IL-10 primary antibodies (diluted 1:300 in PBS-0.1% BSA). The following day, the sections were incubated with (step 1) biotinylated horse anti-mouse immunoglobulin (diluted 1:800 in 1% BSA-PBS) and (step 2) horseradish peroxidase-conjugated avidin-biotin complex (DAKO A/S; diluted 1:50 in 1% BSA-PBS). Staining was executed with 3-amino-ethyl-carbazole (AEC) purchased from Sigma (20 mg AEC in 5 ml DMF added to 95 mL of acetate buffer and 100 µL hydrogen peroxide). Finally, counterstaining was performed with Mayer's hematoxylin.

To stain both IL-10 and neutrophil elastase on the same slide, skin sections were first incubated with anti IL-10 antibodies as described above. Following incubation with biotinylated horse anti-mouse immunoglobulin (see above: step 1 of the second day in the IL-10 single staining protocol), sections were incubated with alkaline phosphatase-conjugated avidin-biotin complex (diluted 1:50 in 1% BSA–PBS). Next, the skin sections were incubated with 10% normal mouse serum (NMS) followed by incubation with horseradish peroxidase-conjugated neutrophil elastase NP57 (DAKO A/S; diluted 1:4 in 1% NMS) and ultimately staining was performed with Fast Bleu BB salt (Sigma) and AEC. Fast Bleu BB salt staining solution was prepared by adding 35 mg of levamisol, 25 mg of fast blue BB Salt, 12,5 mg of naphthol-ASMX (Sigma) dissolved in 1 mL of DMF to 100 mL of 0,05 M magnesiumchloride Tris-HCl.

(Note: The times of incubation and washing steps with PBS-Tween (0.05%) have been omitted in the descriptions for the purpose of clarity. New fuchsine, AEC, and fast bleuBB salt enzymatic activity was halted with distilled water.)

Microscopic evaluation

Immunohistochemically stained skin sections were examined by light microscopy at x200 and x400 magnification. For quantification of cell numbers, stained cells in an area

below and parallel to the dermal–epidermal junction (band-form; 0.445 mm broad) were counted. Results are expressed as mean number of positive cells per mm^2.

In situ zymography[22]

In situ zymography was performed using the following products: EnzChek Elastase Assay Kit (E-12056), DQ-collagen (D-12060), and DQ-gelatin (D-12054) all purchased from Molecular Probes, Eugene, Oregon.

To determine elastase enzyme activity, skin sections stored at -80°C were left to dry at room temperature. Thereafter, a reaction buffer (0.05 M TRIS-HCl, 0.15 M NaCl, 5 mM CaCl2, and 0.2 mM NaN3, pH 7.6) containing 40 μg per mL DQ-Elastin was transferred onto the skin sections and allowed to incubate for a short period of time. Enzyme activity was subsequently detected using fluorescence microscopy (FITC-channel, ex/em 505/515) and microphotographs were promptly taken. Skin sections incubated with a specific elastase inhibitor (supplied with the elastase assay kit) were used as negative controls.

To determine collagenase and gelatinase activity a similar procedure was followed: the same reaction buffer contained 40 μg per mL DQ-collagen type I or DQ-gelatin (both fluorescein conjugated). After transferring the solution onto the skin sections and covering them with coverslips, a short incubation period followed. Enzyme activity was detected by fluorescence microscopy using a different wavelength (FITC channel, ex/em 495/515).

Statistical analysis

After logarithmic transformation of cell numbers (as the distribution of cell numbers per stained cross-sectional area is skewed to the right), a Student's t test was performed to compare stained cell numbers in non-irradiated and irradiated skin. Correlations were calculated with Pearson's test. Statistical analyses were performed using GraphPad Prism software (GraphPad Software, V.2.04a, San Diego, CA). P values <0.05 were considered significant.

Results

DNA photodamage

Irradiating black and white skin with the same physical dose of SSR induced thymine dimers throughout the suprabasal epidermis in both skin types (Figure 1). Numbers of positive staining cells in these layers of the epidermis did not show great inter-individual variance and also seemed independent of SPT. In contrast, thymine dimers in basal keratinocytes were SPT dependent. Although they could scarcely be detected in basal keratinocytes of black skinned persons, they were abundantly present in basal keratinocytes of white skinned persons (particularly in the supra-papillary region). Furthermore,

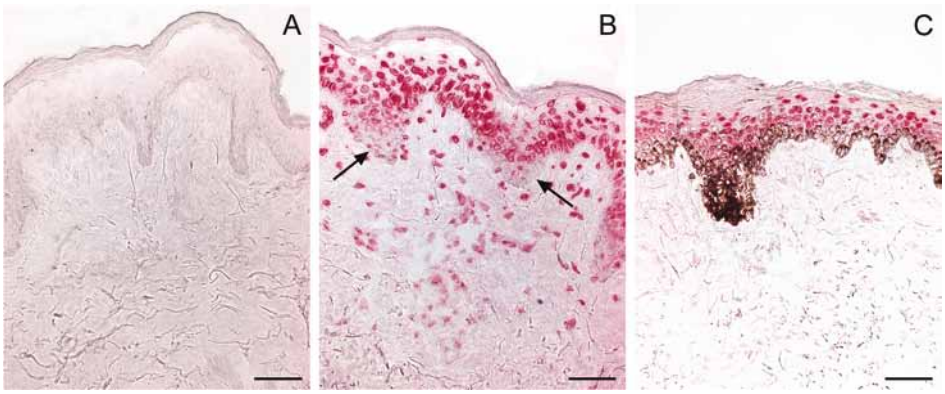

Figure 1. Distribution of SSR-induced DNA photodamage in black and white skin. Thymine dimer staining before exposure (white skin) (A) and 6 h after exposure to 18 000 mJ per cm^2-Waldmann of SSR in white (B) and black skin (C). Basal keratinocytes situated in the depths of the epidermal folds in white skin are relatively protected (→). In the upper dermis, thymine dimers are abundantly present in white but not in black skin. *Scale bars: 50 µm.*

thymine dimer positive cells were effectively absent in the dermis of irradiated black skin, although they were present in large numbers in the dermis of irradiated white skin. These thymine dimers (superficial- to mid-dermal regions) were also induced when exposing white skinned individuals to suberythemogenic doses of SSR (0.5 MED, data not shown). To exclude the possibility that dermal thymine dimer positive cells had in fact migrated from the epidermis, additional biopsies were taken immediately following 2 MED of SSR exposure. Localization of thymine dimer positive cells at this time point proved to be similar to that in the biopsies taken 6 h after exposure (data not shown).

Neutrophil elastase, MMP-9, and MMP-1 positive cells

Large numbers of neutrophil elastase, MMP-9, and MMP-1 positive cells were detected in white skin that had been irradiated with 12 000-18 000 mJ per cm^2-Waldmann of SSR. None of these cells was detected in irradiated black skin (Figure 2 and Table 2a). In the dose-response study conducted with a separate group of white skinned volunteers, neutrophil elastase, MMP-9, and MMP-1 positive cells were only detected in skin that had been exposed to equal or more than 1 MED of SSR (Table 2b).

In situ elastase, gelatinase, and collagenase enzyme activity

Elastase, gelatinase, and collagenase activity was prominent in white skin that had been irradiated with 12 000-18 000 mJ per cm^2-Waldmann (Figure 3). No such enzyme activity was detected in irradiated black skin. In the dose-response study, elastase, gelatinase, and collagenase activity was only detected in skin that had been irradiated with at least 1 MED of SSR (data not shown).

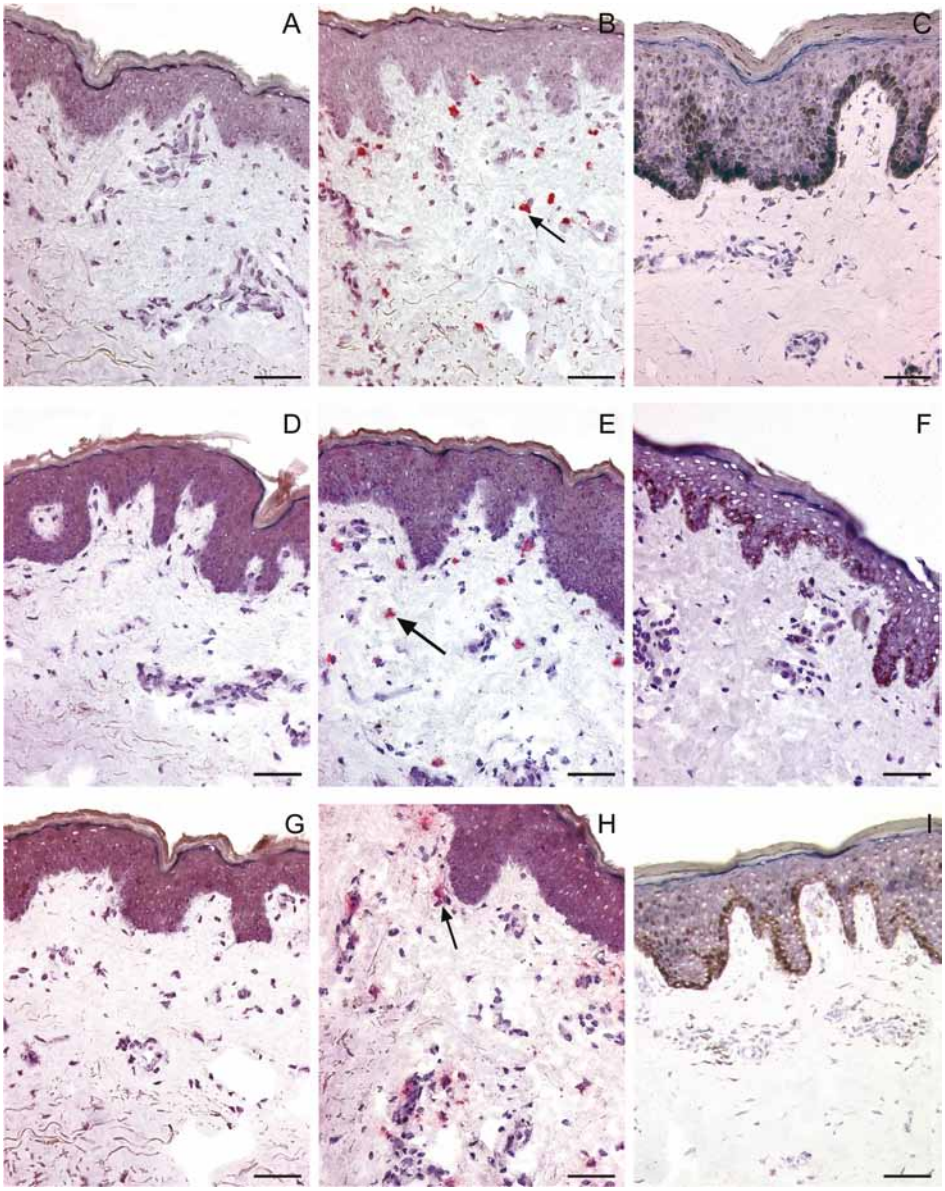

Figure 2. Detection of neutrophil elastase, MMP-9 and MMP-1 in white but not in black skin following exposure to an equal physical dose of SSR. Neutrophil elastase (B), MMP-9 (E), and MMP-1 (H) positive cells in white skin six hours after exposure to 18 000 mJ per cm^2-Waldmann of SSR. No such staining is found in black skin exposed to an equal physical dose of SSR, as shown in C (neutrophil elastase), F (MMP-9), and I (MMP-1), respectively. A, D, and G are the white skin controls before exposure. *Scale bars: 50μm.*

Table 2. Staining pattern of neutrophil elastase, MMP-9 and MMP-1

(a) 6, 24, and 48 h after exposing sun-protected white and black skin to 12 000-18 000 mJ per cm^2-Waldmann of SSR (1)

	Mean number of positive cells per mm^{2*}					
	Neutrophil elastase[a]		MMP-9[b]		MMP-1[c]	
Hours after exposure	**SPT I–III**	**SPT VI**	**SPT I–III**	**SPT VI**	**SPT I–III**	**SPT VI**
Control	0	0	0	0	0	0
6	132 (23)	0	55 (18)	0	79 (18)	0
24	196 (23)	0	102 (32)	0	111 (29)	0
48	141 (30)	0	40 (19)	0	65 (12)	0

(b) 24 h after exposing white skin to increasing MED doses of SSR. 2 MED is equivalent to 12 000-18 000 mJ per cm^2-Waldmann of SSR (2)

	Mean number of positive cells per mm^2 †		
MED dose[d]	**Neutrophil elastase**	**MMP-9**	**MMP-1**
0	0	0	0
0.5	0	0	0
1	77 (18)	13 (8)	12 (4)
2	248 (46)	87 (32)	103 (25)

Note: (1) * SEM values in between brackets (SPT I-III n=6, SPT VI n=6). [abc] When comparing the number of positive cells in black and white skin after 6, 24, and 48 h, a statistical difference was found ($P<0.05$). (2) † SEM values in between brackets (SPT I-III n=6). [d] Differences in numbers of positive staining cells between suberythemogenic and erythemogenic doses were statistically significant ($P<0.05$). This also accounted for cell numbers in skin irradiated with 1 MED compared to skin irradiated with 2 MED ($P<0.05$).
MMP, matrix metalloproteinase; SSR, solar simulating radiation; SPT, skin phototype; MED, minimal erythemal dose.

Keratinocyte activation

The cytokeratins 6, 16, and 17 were upregulated after 24 and 48 h in the epidermis of white skinned volunteers. Since these three cytokeratins showed identical staining patterns, only the results for cytokeratin 16 are shown here (Figure 4). The staining intensity (maximal after 48 h) varied between the white skinned volunteers, but increased expression following irradiation was consistent. Black skinned volunteers showed no upregulation of these cytokeratins.

IL-10 producing neutrophils

In three of the six white skinned volunteers, IL-10 positive cells were found to infiltrate the epidermis 24 and 48 h after irradiation. Double staining for neutrophil elastase and IL-10 showed double staining positive cells (Figure 5). These cells were further characterized to be CD11b positive and CD36 negative (data not shown). No IL-10 positive/

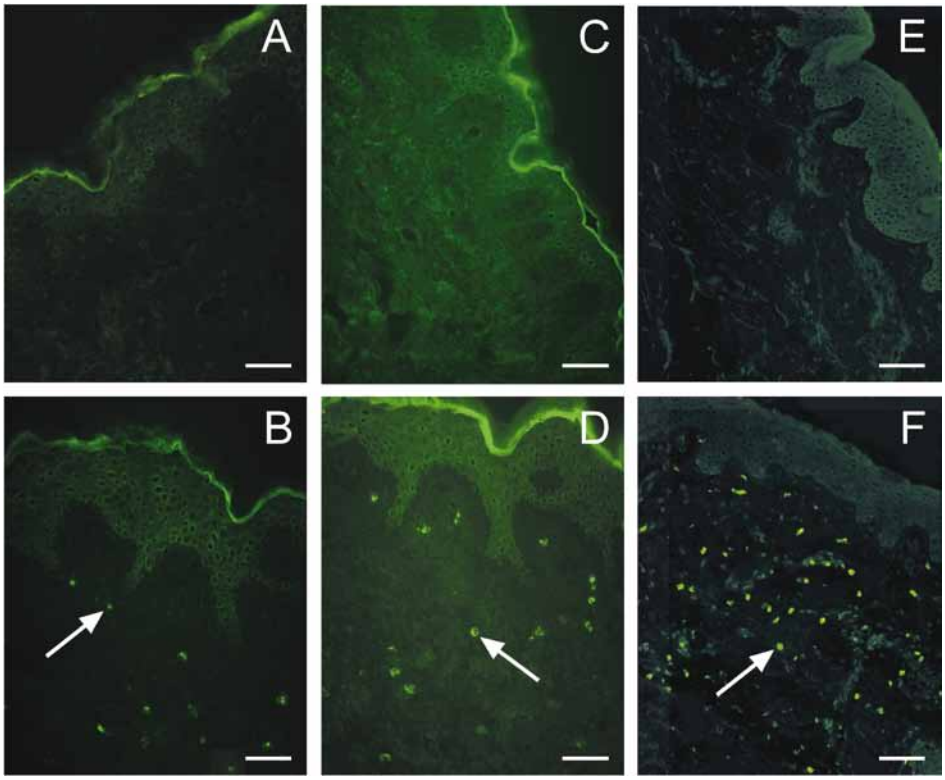

Figure 3. *In situ* elastase, gelatinase, and collagenase enzyme activity in white skin where neutrophil elastase, MMP-9, and MMP-1 positive cells had been immunohistochemically detected. Enzyme activity was located in the upper dermis in a spot-like manner. A and B show collagenase activity, respectively, before and 6 h after SSR exposure. C and D show gelatinase activity before and after SSR exposure. E and F show elastase activity before and after SSR exposure. Compared with collagenase and gelatinase activity, elastase activity was most conspicuous. Elastase most likely reflects neutrophil elastase enzyme activity. Gelatinase probably reflects MMP-9 enzyme activity. Collagenase activity could reflect MMP-1 activity. *Scale bars: A-D: 50 µm, scale bars: E and F: 80 µm.*

neutrophil elastase positive/CD11b positive/CD36 negative cells were found to infiltrate the epidermis of the black skinned volunteers at any time point.

Discussion

We compared skin responses in black and white skinned individuals after exposure to a fixed physical dose of SSR. Additionally, we performed a dose-response study with solar simulating radiation on white skinned subjects. Our results suggest that the observed differences found in black and white skin after SSR exposure are due to a difference in skin pigmentation. Figures 1, 2, and 4 demonstrate the presence of abundant skin pig-

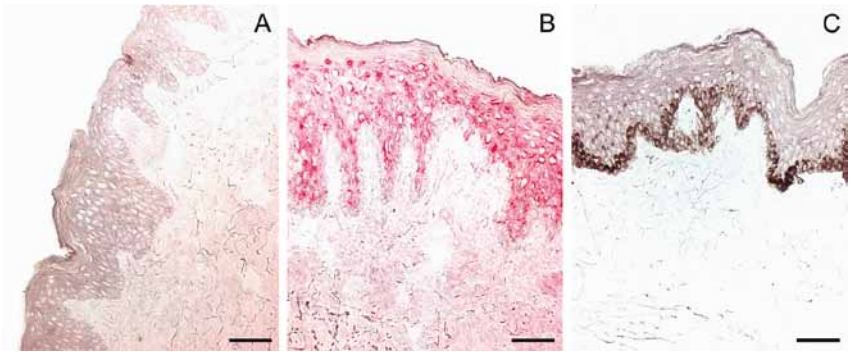

Figure 4. Cytokeratin 16 expression in irradiated white skin. The keratinocyte activation marker cytokeratin 16 was expressed in the epidermis of white skinned individuals 24 and 48 h after exposure to 18 000 mJ per cm^2-Waldmann of SSR. This cytokeratin was not upregulated in black skin after exposure to 18 000 mJ per cm^2-Waldmann of SSR. (A) White skin control before exposure. (B) White skin 48 h after exposure. (C) Black skin 48 h after exposure. *Scale bars: 50 µm.*

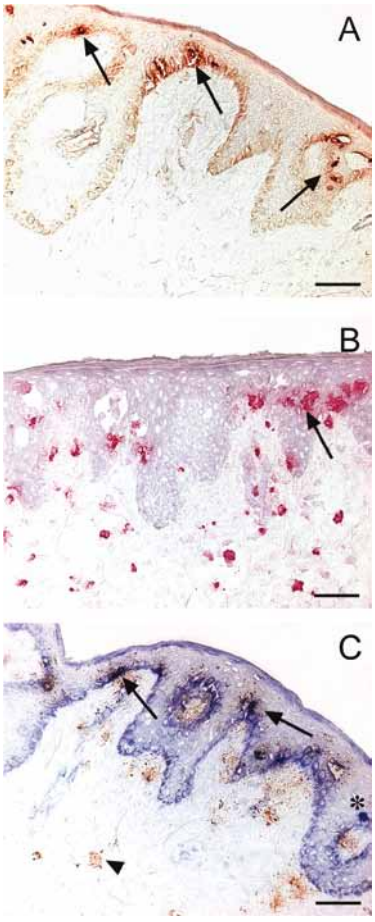

Figure 5. IL-10 positive neutrophils appear to infiltrate the epidermis of irradiated white skin. In three of the six white skinned volunteers, neutrophil elastase positive cells infiltrated the epidermis (B: →). In the same three individuals IL-10 positive cells were found in the epidermis (A →). Double staining revealed mostly neutrophil elastase/ IL-10 double positive cells in the epidermis (C: →), a sporadic neutrophil elastase negative/ IL-10 positive cell in the epidermis (C: *) and neutrophil elastase positive/ IL-10 negative cells in the dermis (C: ►). Note: In the single staining procedure Il-10 was visualized using AEC and stains brown (A). In the double staining procedure, Fast bleu BB salt was used to visualize IL-10 and therefore stains bleu (C). *Scale bars: 50 µm.*

ment (melanin) in black skin as compared with white skin. Melanin is concentrated at the apical pole of basal keratinocytes. Melanin appears to function as a barrier to SSR and protect basal keratinocytes and the dermis beneath from its damaging effects.

Histological evidence to support a barrier function of melanin is provided by the distribution of SSR-induced DNA photodamage in black and white skin: suprabasal epidermal cells were equally damaged, whereas basal keratinocytes and cells in the dermis were relatively protected in black skin (Figure 1). In this way, proliferating cells in black skin appear to be better protected against the mutagenic effects of SSR and against malignant transformation.

Another indication that black skin is better protected against the damaging effects of SSR is the absence of a neutrophilic infiltrate and the absence of MMP-9 and MMP-1 positive cells in irradiated black skin. These cells were abundantly present in white skin irradiated with an equal, or even a lower physical dose of SSR (Table 2a and Figure 2).

Neutrophil elastase was used as a marker to detect the presence of neutrophil infiltration, since neutrophils cannot be detected morphologically in frozen skin sections. Neutrophil elastase is a potent proteolytic enzyme[8;10] and *in situ* zymography strongly suggests that where it is immunohistochemically detected in our study, it is also enzymatically active. The same appears to apply to MMP-9 and MMP-1 detected in our study (Figure 3). In the skin, MMP-9 has generally been associated with keratinocytes and fibroblasts. Our data, however, suggests that MMP-9 is in fact neutrophil derived. In an ongoing study (data not shown), an influx of neutrophils following SSR exposure was confirmed using paraffin-embedded skin sections (in which neutrophils can be detected morphologically). In the same study, additional frozen skin sections showed immunohistochemical double staining of neutrophil elastase and MMP-9. Our data are supported by studies that also show enzymatically active MMP-9 as a product of neutrophils.[23;24] Although neutrophils and MMP-9 and MMP-1 positive cells were not detected in black skin, it is likely that these cells will appear when black skin is irradiated with higher doses of SSR. In the dose-response study, we have shown that erythemogenic doses are required to induce these cells (Table IIb) and 18 000 mJ per cm^2-Waldmann was not sufficient to induce erythema in black skin. This may explain why black skin is more resistant to photoaging. Higher absolute doses of SSR (and thus sunlight exposure) are necessary to induce infiltrating neutrophils, which, by releasing active proteolytic enzymes, can contribute to photoaging.

A third hallmark is the expression of the cytokeratins 6, 16, and 17, which was observed only in white skin (Figure 4), more specifically in white skin irradiated with ≥1 MED of SSR (data not shown). The expression of these activation markers is likely to precede epidermal hyperplasia that could serve as a protective mechanism by increasing the physical barrier to solar radiation. Whether an interaction between neutrophil elastase and keratinocytes, as recently described *in vitro*,[25] plays a role in this activation

process remains to be elucidated. Increased mitosis of basal keratinocytes may precede epidermal hyperplasia. The expression of Ki67 (a mitosis marker) was measured in our skin samples. A statistical difference in Ki67 positive cell numbers between black and white skin after SSR exposure was not detected. The expression of cytokeratins, however, was maximal after 48 h and expression of mitotic markers should therefore also be investigated at later time points.

An interesting finding was that in three of the six white skinned volunteers, IL-10/neutrophil elastase double-positive cells infiltrated the epidermis (Figure 5). Sporadic IL-10 positive/neutrophil elastase negative cells were also found in the epidermis, but the majority were double positive. Whether IL-10 positive neutrophils appear in the skin prior to IL-10 positive macrophages[17] still needs further investigation. Importantly, keratinocytes have also been shown to produce IL-10 following UV exposure.[26] IL-10 positive cells were not detected in the dermis of white skinned volunteers, nor were they detected in irradiated black skin. A variety of studies have reported that IL-10 is a product of neutrophils.[27-30] Besides their destructive capabilities, neutrophils that have infiltrated the skin may thus also have an immunosuppressive function. This concept is supported by data describing an increased incidence of skin cancer in dermatological conditions, where (chronic) infiltration of neutrophils is involved.[31;32]

In summary, the following skin changes were detected in white skin exposed to 12 000-18 000 mJ per cm^2-Waldmann of SSR: DNA photodamage in the epidermis and dermis; infiltrating neutrophils; the presence of enzymatically active neutrophil elastase, MMP-9 or MMP-1; activation of keratinocytes; and IL-10 positive cells in the epidermis. In black skin irradiated with 18 000 mJ per cm^2-Waldmann of SSR, DNA damage was limited to the suprabasal epidermis and none of the other skin changes was observed. Although other genetic factors may be involved, skin pigmentation appears to be primarily responsible for the observed differences. Skin pigment appears to increase the threshold dose at which SSR induces an inflammatory response in the skin. This is confirmed by data that show that the MED of black skinned individuals is a factor 2-10 higher than that of white skinned individuals (SPT VI compared with SPT I-III).[33] Our findings provide an explanation as to why black skinned individuals are less susceptible to sunburn, photoaging, and skin carcinogenesis.

Acknowledgements

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Chapter 2b

UV-protection by melanin and hyperkeratosis in vitiligo patients with skin phototype VI: 2 case reports

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Study to be completed

Abstract

Black skin [skin phototype (SPT) VI] is more resistant to photoaging than white skin (SPT I-III). Furthermore, it has been shown that black skin is more resistant to UV-induced neutrophil influx than white skin. Neutrophils are potent cells capable of generating and releasing proteolytic enzymes which can damage the extracellular matrix. Thereby, neutrophils could contribute to photoaging.
Vitiligo is an acquired skin disease characterized by depigmentation of the skin caused by loss of melanocytes from the epidermis. Melanocytes synthesize melanin which is transferred to surrounding keratinocytes and protects the skin against the damaging effects of UV. To show that melanin does indeed have a photoprotective effect and can prevent UV-induced neutrophil influx, vitiligo in patients with SPT VI could be a helpful natural disease model. Here we present two case reports that indicate that heavy melanization of the epidermis but also strong hyperkeratosis can prevent UV-induced neutrophil influx.

Introduction

Vitiligo is an acquired skin disease characterized by depigmented (white) macular lesions in which melanocytes are lost from the epidermis. The disease is classified according to the extent of skin involvement and the distribution of depigmentation. Several hypotheses with respect to the pathophysiology of vitiligo have been proposed:[1-4] (1) the autoimmune hypothesis, whereby cytotoxic T-cells or antibody-mediated cytotoxicity cause destruction of melanocytes; (2) the autocytotoxicity hypothesis, in which cytotoxic precursors or metabolites of melanin induce melanocytic cell death; (3) the neural hypothesis, wherein neural activity plays a role in the destruction of melanocytes; (4) the genetic hypothesis, in which melanocytes have a genetic defect that is triggered at some point and impedes their growth and differentiation. Considering the different physiologic and pathologic findings in vitiligo, multiple mechanisms may be involved (the heterogenous hypothesis).

Light microscopic examination of 'active' vitiligo shows absence of/or degenerative melanocytes, and may show focal vacuolar degeneration and some perivascular inflammatory changes in the dermis. Longstanding vitiligo, however, only shows an absence of melanocytes and is often only recognized by a pathologist when specific immunohistochemical stains (to demonstrate the absence of melanocytes) are performed.[1;5]

There is conflicting data with respect to vitiligo and sunburn sensitivity. A number of studies have shown that the minimal erythema dose (MED) of vitiligenous skin is not significantly lower than the MED of normal skin. One study showed that this was due to relative hyperkeratosis in lesional skin as compared to adjacent normal skin.[6] Studies investigating the MED of vitiligenous skin, however, have mainly included patients with fair skin (SPT I-IV) in whom the difference in pigmentation between lesional and normal skin is often quite subtle, particularly when the areas of skin investigated are sun-protected (i.e., not tanned). Certainly patients with black skin (SPT VI) and vitiligo claim that their vitiligenous skin 'burns' more easily than their normally pigmented skin. Thus, vitiligenous skin could be more susceptible to a sunburn reaction.

Literature is also not entirely clear on whether vitiligenous skin is more susceptible to photocarcinogenesis and photoaging.[7-9] Generally, data suggests no increased risk of skin cancer and photoaging. This is rather surprising considering the fact that one of the most important barriers against UV (i.e., melanin) is lost. There are a number of possible explanations as to why signs of chronic UV damage may not be(come) obvious in vitiligenous skin: (1) Vitiligo can be a temporary condition, hence there is not enough time to reveal a difference in susceptibility to photocarcinogenesis and photoaging between lesional and normal skin. (2) As touched on above, differences in skin pigmentation between lesional and normal skin can be subtle, therefore increased sensitivity to UV is marginal. (3) Compensatory protective mechanisms, such as hyperkeratosis, may occur. (4) Persons with vitiligo avoid sun exposure to reduce increased contrast between

lesional and normal skin. (5) Social stigma encourages patients to cover or camouflage the affected areas, thus unwittingly protecting vitiligenous skin against UV. On the other hand, different modalities of UV-therapy are used to treat vitiligo, thus increasing UV exposure.[1] The duration of UV-treatment is often extended due to the therapy-resistant and chronic character of the disease. However, normally therapy will be seized following a cumulative treatment of ±2 years and thus the total UV dose is still relatively low.

It has been shown that black skin (SPT VI) is more resistant to UV-induced neutrophil influx than white skin (SPT I-III).[10] Neutrophils are capable of generating powerful proteolytic enzymes which can damage the extracellular matrix[11] and thereby could contribute to photoaging of the skin. To show that melanin does indeed have a photoprotective effect and can prevent UV-induced neutrophil influx, vitiligo in patients with SPT VI could be a helpful natural disease model.

Vitiligo and photoaging: 2 case reports

To further investigate the photoprotective properties of melanin and the role of skin-infiltrating neutrophils in photoaging, a study involving vitiligo patients was set up. Recruitment of two groups of vitiligo patients was foreseen.

Group 1: inclusion of elderly patients with SPT VI and stable, longstanding (>10 years) vitiligo on sun-exposed skin to study the occurrence of solar elastosis, the hallmark of photoaged skin. During a single visit punch biopsies would be taken from sun-exposed lesional and adjacent normal skin.

Group 2: inclusion of vitiligo patients with SPT VI to study solar-simulating radiation (SSR)-induced neutrophil influx and DNA damage. Lesional and adjacent normal skin would be exposed to 18 000 mJ/cm^2 of SSR (equivalent to 2-3 MED for persons with SPT I-III). 24 hours after SSR exposure punch biopsies would be taken from unexposed lesional and adjacent normal skin (controls) and from SSR-exposed lesional and adjacent normal skin.

After the study was approved by the medical ethical committee of the University Medical Center Utrecht patient recruitment was started.

Mainly due to a lack of patients with vitiligo and SPT VI in the region, we finally recruited only 2 patients. Both patients were included in group 2. Despite the fact that the study has not yet been completed, the preliminary data are worth reporting here.

The materials and methods with respect to staining of neutrophil elastase (as a marker of neutrophils) and staining of thymine dimers (as a marker for UV-induced DNA damage) are described in chapter 2.

Case reports

Patient 1 (female, age 34 yrs) had stable vitiligo for 20 years and was tested on the abdomen. Exposure of depigmented skin and adjacent normal black skin to 18 000 mJ/cm^2 of SSR induced erythema and neutrophil influx in depigmented skin only (Figure 1a). SSR-induced thymine dimers were distributed throughout the epidermis and upper dermis in depigmented skin, but were limited to suprabasal keratinocytes in adjacent normal black skin (Figure 1b).

Patient 2 (male, age 32 yrs) had vitiligo for 8 years on the dorsum of his hands and fingers. The inclusion of this patient was during a hot period in the summer (July 2008) and a few months earlier he had received UVB therapy. Exposure to 18 000 mJ/cm^2 of SSR was not sufficient to induce erythema or neutrophil influx in either depigmented or adjacent normal black skin. Interestingly, the control biopsy of his depigmented skin already showed thymine dimers which were particularly prominent in the upper dermis (Figure 2). Furthermore, his depigmented skin showed an extremely thickened stratum corneum. In accordance with the skin localization (i.e., dorsal hand) the stratum corneum of adjacent normal black skin was also thickened but not as much as his depigmented skin. Exposure to 18 000 mJ/cm^2 of SSR increased the intensity of thymine dimers in depigmented skin and induced thymine dimers in suprabasal keratinocytes of adjacent normal black skin. However, compared to patient 1 (Figure 1b) staining of thymine dimers in the epidermis of both lesional and normal skin was much less intense (Figure 2).

Discussion

In chapter 2 we compared black and white skin and showed that strong pigmentation of the epidermis protects basal keratinocytes and the dermis beneath against directly induced DNA damage, and increases the damage threshold at which SSR induces neutrophil influx. The results from patient 1 are in line with and further substantiate the results in chapter 2. The results from patient 2 are more surprising. It appeared that due to thickening of the stratum corneum (most likely caused by UVB therapy and/or the fact that is was mid-summer) the threshold for SSR-induced erythema and neutrophil influx was increased. The reactive thickening of the stratum corneum, however, did not fully protect against thymine dimer induction by SSR. Interestingly, thymine dimer induction in depigmented skin was particularly prominent in the dermal compartment. Thymine dimer induction in the epidermis of patient 2 in both depigmented and normal skin was increased following SSR exposure, but staining of thymine dimers in this compartment was not very intense. This could be explained by a preferential absorption of shorter UV wavelengths (UVB) by keratin,[12] whereby the epidermal cells are relatively spared of

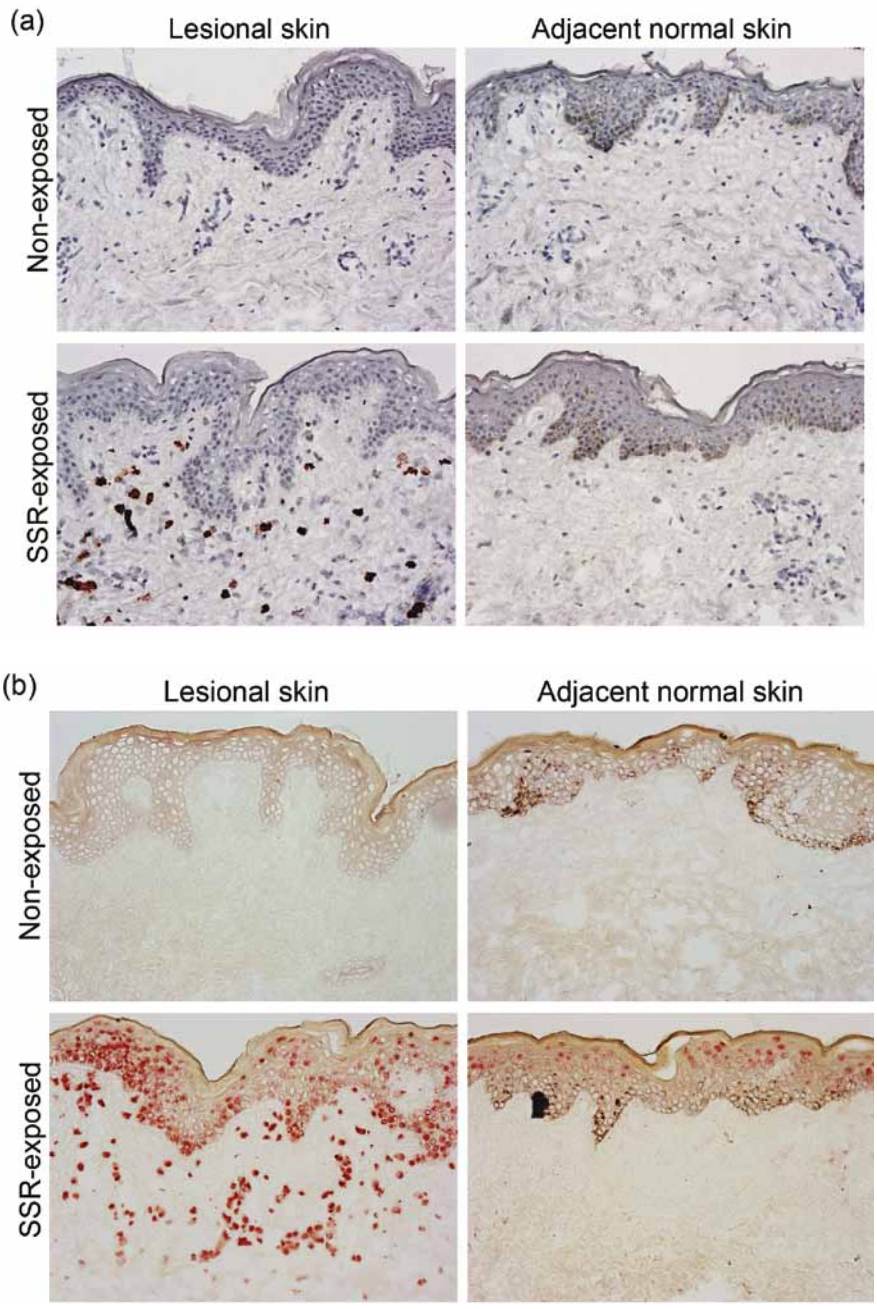

Figure 1. Patient 1 with vitiligo on the sun-protected abdomen. Depigmented and adjacent normal black skin were exposed to 18 000 mJ/cm^2 of SSR. Neutrophil influx was only observed in the depigmented skin **(a)**. SSR-induced thymine dimers were distributed throughout the epidermis and upper dermis in depigmented skin, but were limited to suprabasal keratinocytes in adjacent normal black skin **(b)**.
Original magnification: x200.

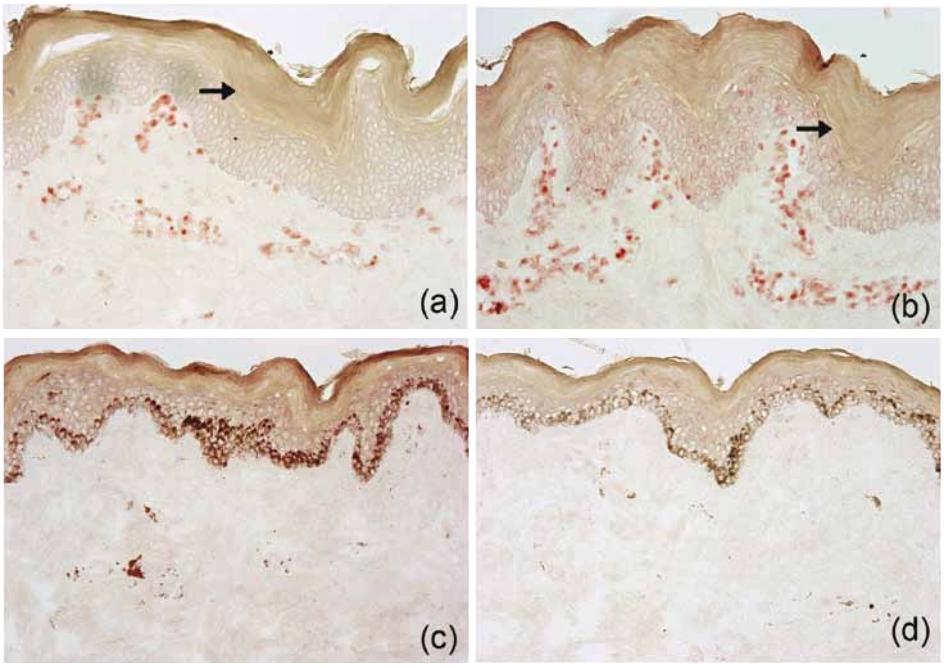

Figure 2. Patient 2 with vitiligo on sun-exposed hands. Depigmented and adjacent normal black skin were exposed to 18 000 mJ/cm^2 of SSR. Neutrophil influx was not observed in either depigmented or normal black skin (data not shown). Depigmented skin, in particular, showed strong hyperkeratosis (arrows, panels a and b). The control biopsy of his depigmented skin already showed thymine dimers which were particularly prominent in the upper dermis (panel a). SSR exposure increased the intensity of thymine dimers in both the epidermis and dermis of depigmented skin (panel b), and induced thymine dimers in suprabasal keratinocytes of normal black skin (panel d). Compared to patient 1 (Figure 1b), however, staining of thymine dimers in the epidermis of patient 2 was notably less intense in both depigmented skin and adjacent normal black skin. *Original magnification: x200.*

thymine dimer induction. Longer UV wavelengths (e.g., UVA2) penetrate deeper into the skin and induce thymine dimers in the upper dermis. Since skin biopsies were taken 24 hours after SSR exposure, another explanation could be that dermal cells repair UV-induced DNA damage at a slower rate than keratinocytes. However, in patient 1 skin biopsies were also taken 24 hours after SSR exposure and here we did observe intense staining of thymine dimers in the epidermis of both lesional and normal skin. These observations would suggest that shorter UV wavelengths and damage to basal keratinocytes, are particularly important in SSR-induced erythema and neutrophil influx.

In summary, patient 1 demonstrates that vitiligenous skin is indeed more susceptible to UV-induced erythema and neutrophil influx. Patient 2 demonstrates that this can at least be partly compensated by reactive hyperkeratosis. Due to a lack of patients we were not able to study susceptibility to photoaging. Clearly it is necessary to recruit more patients into this study and our findings so far need further confirmation.

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Chapter 3

Skin-infiltrating neutrophils following exposure to solar-simulating radiation could play an important role in photoaging of human skin

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Abstract

Background The pathophysiology of photoaging of the skin has been studied extensively. Matrix metalloproteinases (MMPs) originating from keratinocytes and fibroblasts are thought to play a primary role in this process. Although neutrophils are potent producers of a wide array of proteolytic substances and are present in sunburned skin, their contribution to the pathophysiology of photoaging has been described only in murine studies.
Objectives To determine the role of neutrophils in photoaging of human skin.
Methods Healthy white-skinned volunteers were recruited and their sun-protected buttock skin was exposed to solar-simulating radiation (SSR) in dose-response and time-course studies. Punch biopsies were taken and the influx of neutrophils and the expression of neutrophil elastase and MMPs was studied using immunohistochemical techniques and *in situ* zymography.
Results Neutrophil elastase and MMPs were detected only in skin irradiated with erythemogenic doses (≥ 1 minimal erythema doses) of SSR. Immunohistochemical double staining demonstrated neutrophils to be the major source of MMP-1, MMP-8 and MMP-9. *In situ* zymography showed elastase, collagenase and gelatinase enzyme activity in those cells.
Conclusions Our study suggests that neutrophils participate in the process of photoaging of human skin as they infiltrate the skin and release enzymatically active elastase (neutrophil elastase), collagenase (MMP-1) and gelatinase (MMP-9).

Introduction

Solar radiation can damage human skin. Acute overexposure to solar radiation causes a clinical sunburn.[1] Chronic exposure can lead to skin changes such as plaque-like thickening, loss of skin tone, deep furrowing and fine wrinkle formation. Collectively, these changes may be termed 'photoaged skin' or a 'solar scar'.[2]

The pathophysiology of photoaging has been investigated extensively. A hypothetical model was proposed by Fisher *et al.*[3;4] They postulated that imperfect repair following skin matrix damage after exposure to sunlight accumulates and ultimately leads to the formation of a solar scar. It was demonstrated that suberythemal exposure(s) to ultraviolet (UV) B radiation induces three matrix metalloproteinases (MMPs) in human skin *in vivo*: collagenase (MMP-1), gelatinase B (MMP-9) and stromelysin-1 (MMP-3). The combined actions of these enzymes can fully degrade skin collagen. Based on the localization of *in situ* hybridized MMP mRNA, immunohistochemical staining of MMP protein and *in situ* determination of enzyme activity, it was concluded that the MMPs mainly originated from keratinocytes and fibroblasts.

Another cell that may contribute to sun-induced skin damage is the neutrophilic granulocyte. Neutrophils are present in sunburned skin.[1] Moreover, they are potent producers of a wide array of proteolytic substances,[5] including the pluripotent neutrophil elastase,[6;7] MMP-8 and MMP-9. Nevertheless, their contribution to photoaging in humans has been somewhat neglected. The role of neutrophil elastase was investigated in two murine studies[8;9] and neutrophil elastase was recently studied as a product of fibroblasts.[10] In humans, only MMP-8 has been studied as a product of neutrophils.[11] It was found that following a single exposure to UVB, skin-infiltrating neutrophils contained enzymatically inactive MMP-8 protein. MMP-8 mRNA could not be detected and it was concluded that MMP-8 does not contribute significantly to UV-induced skin collagen degradation. Similar to MMP-8, neutrophils isolated from human donor blood contained MMP-9 in an inactive latent form.[12] However, the presence and enzymatic activity of MMP-9 within skin-infiltrating neutrophils following exposure to solar radiation or solar-simulating radiation (SSR) has not yet been studied. MMP-1 has, to our knowledge, not been convincingly demonstrated as a product of neutrophils.

To investigate the role of neutrophils in the pathophysiology of photoaging further, healthy volunteers were exposed to SSR in a dose-response and a time-course study. SSR was chosen because it approaches the emission spectrum of natural sunlight in the UV range. To study the presence and enzymatic activity of neutrophil elastase and MMPs, biopsies were taken, processed and subjected to immunohistochemical staining and *in situ* zymography.

Materials and methods

Irradiation procedure and tissue sample collection

Twelve healthy white-skinned volunteers (six men and six women) aged 19-39 years (mean ± SEM 23.8 ± 1.6) were recruited. Volunteer characteristics including their Fitzpatrick skin phototype are shown in Table 1. The study protocol was approved by the medical ethics committee of the University Medical Center in Utrecht and all volunteers gave informed consent.

Table 1. Volunteer characteristics: volunteers 1-6 participated in the dose-response study, and volunteers 7-12 were recruited for the time-course study

No.	Gender	Age	SPT	Symbol[a]
1	Female	30	II	■
2	Female	19	II	◊
3	Female	39	I	▼
4	Female	21	II	+
5	Male	24	III	▲
6	Male	19	II	o
7	Female	21	II	♦
8	Male	26	II	•
9	Female	22	I	□
10	Male	24	II	×
11	Male	26	II	*
12	Male	28	III	Δ

SPT, Skin phototype according to Fitzpatrick. [a]Symbols used in figures 2a,b, representing individual volunteers.

CLEO Natural lamps (Philips, Eindhoven, the Netherlands) were used throughout the irradiation procedures. This solar simulator emits predominantly UVA and 3% UVB. The UVB/UVA ratio is comparable to midday sunlight during the summer in Amsterdam (52 °N). The spectral energy distribution ranges from 290 to 420 nm, with a peak around 355 nm. Relative spectral distribution measurements were performed with a calibrated standard UV-visible spectrometer (model 742; Optronics Laboratories, Orlando, FL, U.S.A.). During the experiments the output at the surface of the skin was measured at regular intervals and kept at a constant of 6.0 mW cm^2. These measurements were carried out with a Waldmann UV detector (Waldmann, Schwenningen, Germany).

Minimal erythema doses (MED) at sun-protected buttock skin were determined for all 12 volunteers by an experienced investigator using a specially constructed electronic device.[13] Following MED determination six volunteers (dose-response study) were ir-

radiated with different doses of SSR. Three areas of approximately 2 x 2 cm on the buttock were exposed to 0.5, 1 and 2 MED. Punch biopsies (4 mm) were taken from the irradiated sites after 24 hours and one control biopsy was taken from non-irradiated buttock skin. Three 2 x 2 cm areas on the buttock of the remaining six volunteers (time-course study) were exposed to 2 MED of SSR. Punch biopsies were taken before SSR exposure and 6, 24 and 48 hours after irradiation. Biopsies were snap-frozen in liquid nitrogen, embedded in Tissuetek (Sakura, Torrance, CA, U.S.A.) and stored at -80 °C. To obtain paraffin-embedded skin sections, additional biopsies were taken from the volunteers participating in the time-course study before and 6 hours after SSR exposure.

Hematoxylin and eosin staining

Both frozen skin sections and paraffin-embedded skin sections were stained with hematoxylin and eosin according to standard procedures.

Immunohistochemistry

Six-micrometer thick skin sections were cut with a freezing microtome and mounted on slides coated with 3-aminopropyl triethoxy silane (A3648; Sigma, St Louis, MO, U.S.A.). Single- and double-staining procedures were carried out using the following monoclonal antibodies: horseradish peroxidase (HRP)-conjugated mouse anti-human neutrophil elastase (NP57; Dako, Glostrup, Denmark; diluted 1:4 and 1:40); mouse anti-human MMP-1 (Oncogene, Boston, MA, U.S.A.; diluted 1:50); mouse anti-human MMP-8 (Oncogene; diluted 1:20); mouse anti-human MMP-9 (Neomarkers, Fremont, CA, U.S.A.; diluted 1:50). Different steps in the staining procedures were followed by rinsing the slides in phosphate-buffered saline (PBS) containing 0.05% Tween. PBS was also used as diluent. Final staining was done with new fuchsin or fast blue BB salt or 3-aminoethylcarbazole (AEC), all purchased from Sigma.

Neutrophil elastase, matrix metalloproteinase (MMP)-1, MMP-8 and MMP-9 single-staining procedures

Sections were fixed in dry acetone (7 min) and preincubated with 10% normal human serum (NhuS)/10% normal horse serum (NhoS). Subsequently, the slides were incubated with the respective primary antibodies (step 1). Incubation with biotinylated horse anti-mouse immunoglobulin (Vector, Burlingame, CA, U.S.A.; diluted 1:800) and alkaline phosphatase (AP)-labelled streptavidin (Boehringer Mannheim, Mannheim, Germany; diluted 1:300) comprised steps 2 and 3 of the staining protocol. Both primary and secondary antibodies were diluted in 1% NhuS/1% NhoS.

AP activity was demonstrated using naphthol ASBI as substrate and new fuchsin (10 mg in 100 mL) as chromogen dissolved in 0.1 M Tris-HCl + 0.05 M $MgCl_2$ (pH 8.5), resulting in a pink staining. Endogenous AP activity was inhibited by adding levamisole

(35 mg in 100 mL) to the reaction mixture above. The enzymatic reaction was halted in distilled water and the slides were counterstained with Mayer's hematoxylin.

Double-staining procedures for neutrophil elastase/matrix metalloproteinases

Slides fixed in dry acetone containing 150 µL 30% hydrogen peroxide (7 min) were preincubated with 10% NhuS/NhoS and incubated with: (i) MMP primary antibodies in 1% Nhus/NhoS, (ii) horse anti-mouse-biotin in 1% NhuS/NhoS, and (iii) AP-conjugated avidin-biotin complex (Dako; diluted 1:50). Next, the slides were preincubated with 10% normal mouse serum and incubated with anti-elastase-HRP. The first staining solution was prepared by adding 35 mg of levamisole, 25 mg of fast blue BB salt and 12.5 mg of naphthol-ASMX (Sigma) dissolved in 1 mL of dimethylformamide (DMF) to 100 mL of 0.05 M $MgCl_2$ in Tris-HCl. After transferring this solution on to the slides and allowing the enzymatic reaction to take place, staining was terminated in distilled water. Following pretreatment with 0.1 M acetate buffer (pH 5) the double stain was executed with 100 µL 30% hydrogen peroxide added to a filtered solution containing 20 mg of AEC dissolved in 5 mL of DMF mixed with 95 mL of 0.1 M acetate buffer (pH 5). Finally, distilled water was used to stop the enzymatic reaction.

Microscopic evaluation

Stained skin sections were examined with a light microscope at x200, x400 and x1000 magnification. Stained cells were quantified by carefully counting their numbers in a 0.445-mm broad, band-like area below and parallel with the dermal-epidermal junction. The results are expressed as mean number of positive cells per mm^2.

In situ zymography

EnzChek Elastase Assay Kit (E-12056), DQ-collagen (D-12060) and DQ-gelatin (D-12054), all purchased from Molecular Probes (Eugene, OR, U.S.A.), were used to determine *in situ* elastase, collagenase and gelatinase enzyme activity.[14] Based on the use of specific inhibitors, these enzyme activities are considered to reflect neutrophil elastase, MMP-1 and MMP-9 proteolytic activity, respectively.

Skin sections stored at -80 °C were left to dry at room temperature. Reaction buffer (0.05 M Tris-HCl, 0.15 M NaCl, 5 M $CaCl_2$ and 0.2 mM NaN_3, pH 7.6) containing 40 µg per mL DQ-elastin was transferred on to the skin sections and allowed to incubate for a short period of time. Elastase enzyme activity was subsequently detected using a fluorescence microscope [fluorescein isothiocyanate (FITC) channel, excitation/emission wavelengths 505/515 nm]. Skin sections incubated with a specific elastase inhibitor (supplied with the elastase assay kit) were used as negative controls. Collagenase and gelatinase enzyme activities were detected similarly. Here the above-described reaction buffer contained 40 µg per mL DQ-collagen type I or DQ-gelatin, both fluorescein-

conjugated. A short incubation period followed the transferral of the solution on to the skin sections. Enzyme activity was detected by fluorescence microscopy using a different wavelength (FITC channel, excitation/emission wavelengths 495/515 nm).

Statistical analysis

A Student's t-test was performed to compare stained cell numbers in non-irradiated and irradiated skin. As the distribution of cell numbers per stained cross-sectional area is skewed to the right, cell numbers were logarithmically transformed. Correlations were computed using Pearson's test. Statistical analyses were executed using GraphPad Prism software (GraphPad Software, V. 2.04a). $P < 0.05$ was considered significant.

Results

Infiltration of neutrophils after exposure to different doses of solar-simulating radiation

An influx of neutrophils was observed in skin irradiated with ≥1 MED of SSR (Figure 1). Morphologically recognizable neutrophils could be detected only in paraffin-embedded tissue sections and not in snap-frozen sections. Neutrophils were visualized in frozen skin sections by staining for neutrophil elastase.

Staining of neutrophil elastase, matrix metalloproteinase (MMP)-1, MMP-8 and MMP-9 following exposure to different doses of solar-simulating radiation

As mentioned above, neutrophil elastase-positive cells were exclusively detected in skin that had been irradiated with an SSR dose of ≥1 MED. Equally, MMP-1, MMP-8 and MMP-9 proteins were detected only in skin irradiated with ≥1 MED of SSR (Figure 2a). MMP staining occurred in a smudged spot-like manner throughout the upper dermis, suggesting that the MMPs are present in or secreted by particular cells. Peak numbers of stained cells in our dose series were seen after exposure to 2 MED of SSR. This dose was therefore used in the time-course study.

Time course study of elastase- and matrix metalloproteinase (MMP)-1-, MMP-8 and MMP-9- positive cells after exposure to 2 minimal erythemal doses of solar-simulating radiation

Neutrophil elastase-positive cells were detected at 6 hours after exposure (Figures 2b and 3). In our time series neutrophil elastase-positive cell numbers reached a maximum after 24 h. At 48 hours both the number of neutrophil elastase-positive cells and the staining intensity declined. A similar pattern was observed for staining of MMP-1, MMP-8 and MMP-9. Both neutrophil elastase- and MMP-positive cells were mainly located in the upper dermis.

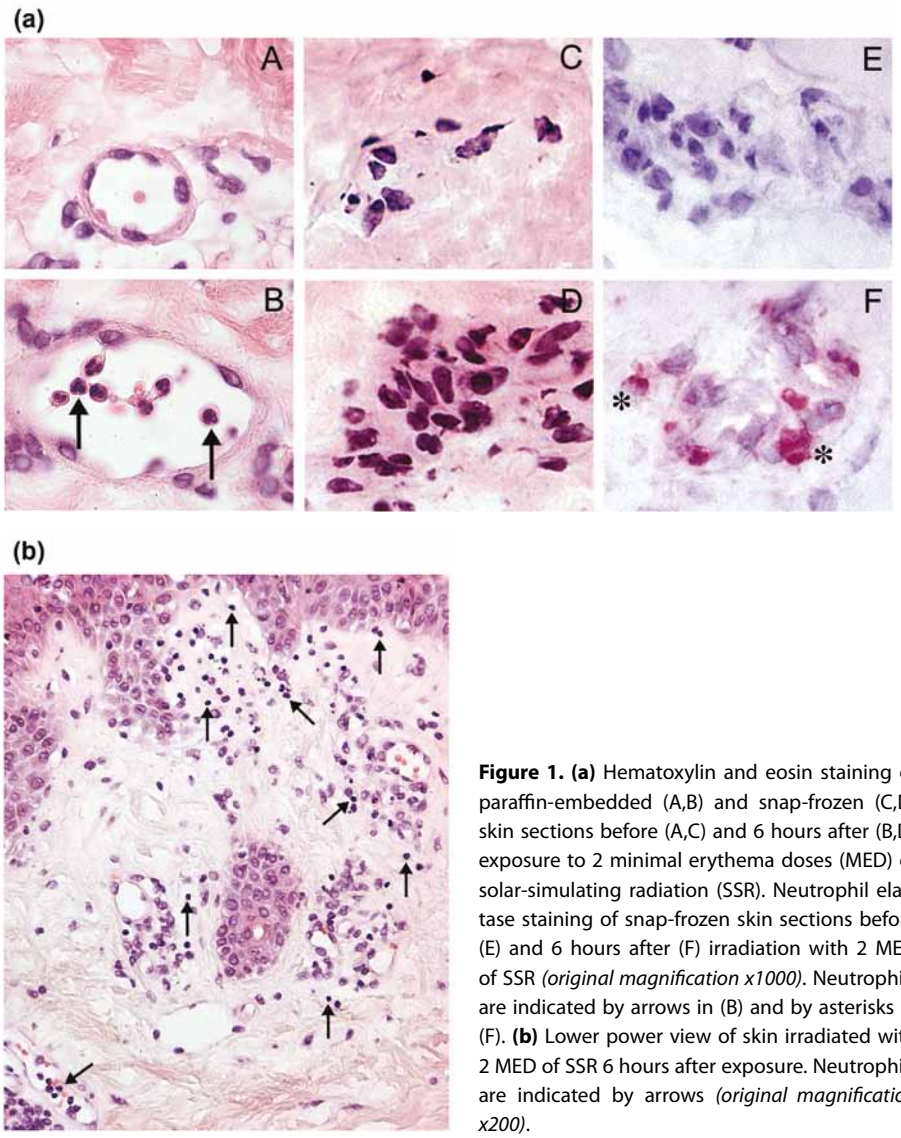

Figure 1. (a) Hematoxylin and eosin staining of paraffin-embedded (A,B) and snap-frozen (C,D) skin sections before (A,C) and 6 hours after (B,D) exposure to 2 minimal erythema doses (MED) of solar-simulating radiation (SSR). Neutrophil elastase staining of snap-frozen skin sections before (E) and 6 hours after (F) irradiation with 2 MED of SSR *(original magnification x1000)*. Neutrophils are indicated by arrows in (B) and by asterisks in (F). **(b)** Lower power view of skin irradiated with 2 MED of SSR 6 hours after exposure. Neutrophils are indicated by arrows *(original magnification x200)*.

Although the interindividual variation in the number of positive cells for each marker at different time points was considerable, intraindividually the number of neutrophil elastase-positive cells correlated well with the number of MMP-1-, MMP-8- and MMP-9-positive cells [Pearson correlation coefficients after log transformation: R elastase/MMP-1 = 0.74 ($P < 0.0001$); R elastase/MMP-8 = 0.80 ($P < 0.0001$); R elastase/MMP-9 = 0.67 ($P = 0.0002$)].

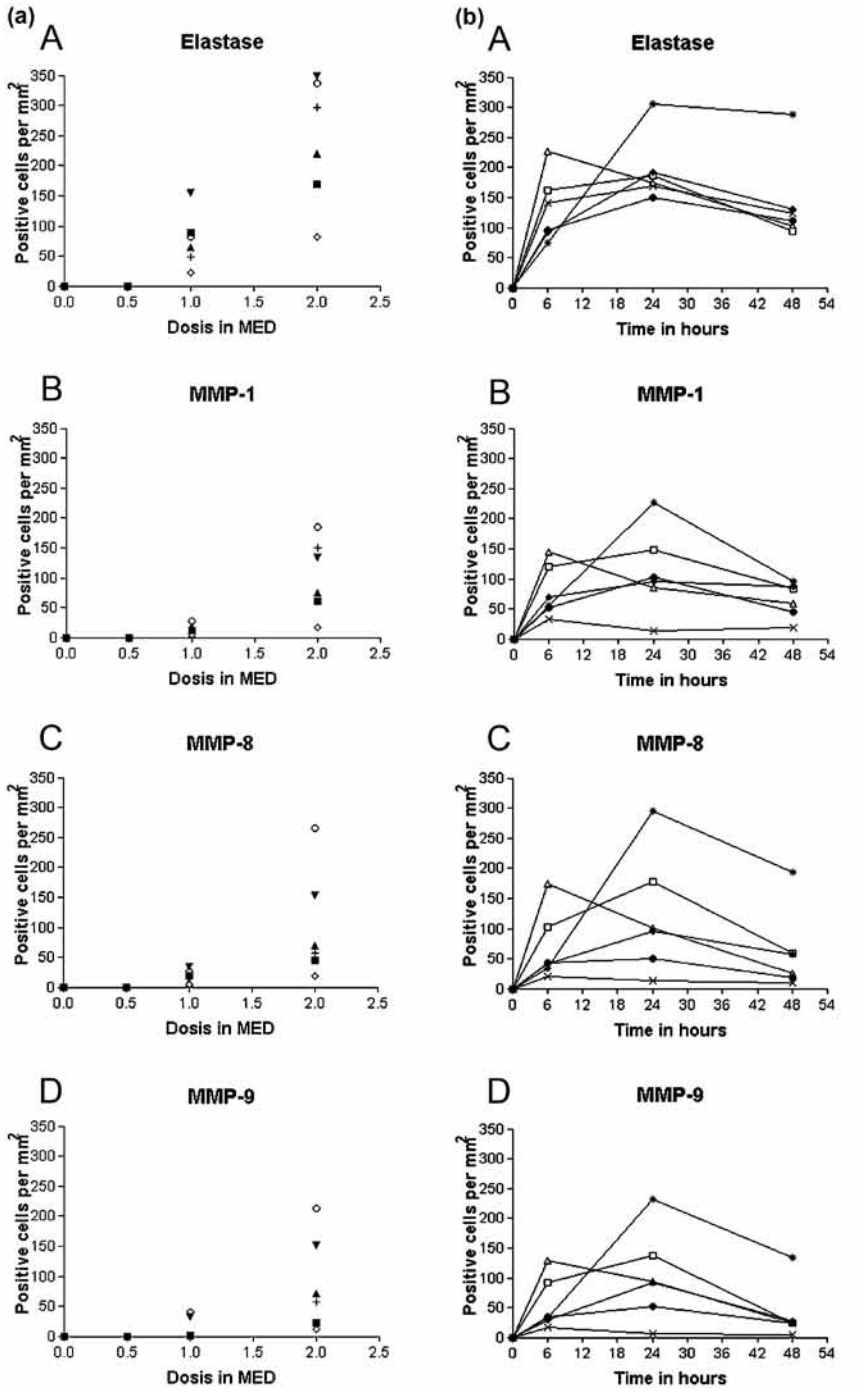
(a)
A
Elastase
Positive cells per mm2
Dosis in MED
B
MMP-1
C
MMP-8
D
MMP-9
(b)
A
Elastase
Time in hours
B
MMP-1
C
MMP-8
D
MMP-9

Figure 2

Figure 2. (a) Number of (A) neutrophil elastase-, (B) matrix metalloproteinase (MMP)-1-, (C) MMP-8- and (D) MMP-9-positive cells detected in the skin of the six volunteers (Table 1: volunteers 1-6) participating in the dose-response study after exposure to different doses of solar-simulating radiation (SSR). Note that no positive staining cells (symbols depicting each volunteer superimpose on the zero mark of the y-axis) were detected in non-irradiated skin and skin irradiated with 0.5 minimal erythema doses (MED) of SSR. Statistical analysis revealed significant differences in cell numbers in non-irradiated vs. ≥1 MED irradiated skin ($P < 0.05$) and in skin irradiated with 1 MED vs. skin irradiated with 2 MED ($P < 0.05$). **(b)** Number of (A) neutrophil elastase-, (B) MMP-1-, (C) MMP-8- and (D) MMP-9-positive cells before and 6, 24 and 48 hours after SSR exposure (2 MED) in six individuals (Table 1: volunteers 7-12) taking part in the time-course study. For each marker, the number of positive cells at a particular time point differs considerably between individuals. Within all but one (symbol x) of the individuals the number of neutrophil elastase-positive cells correlates well with the number of MMP-1-, MMP-8- and MMP-9-positive cells.

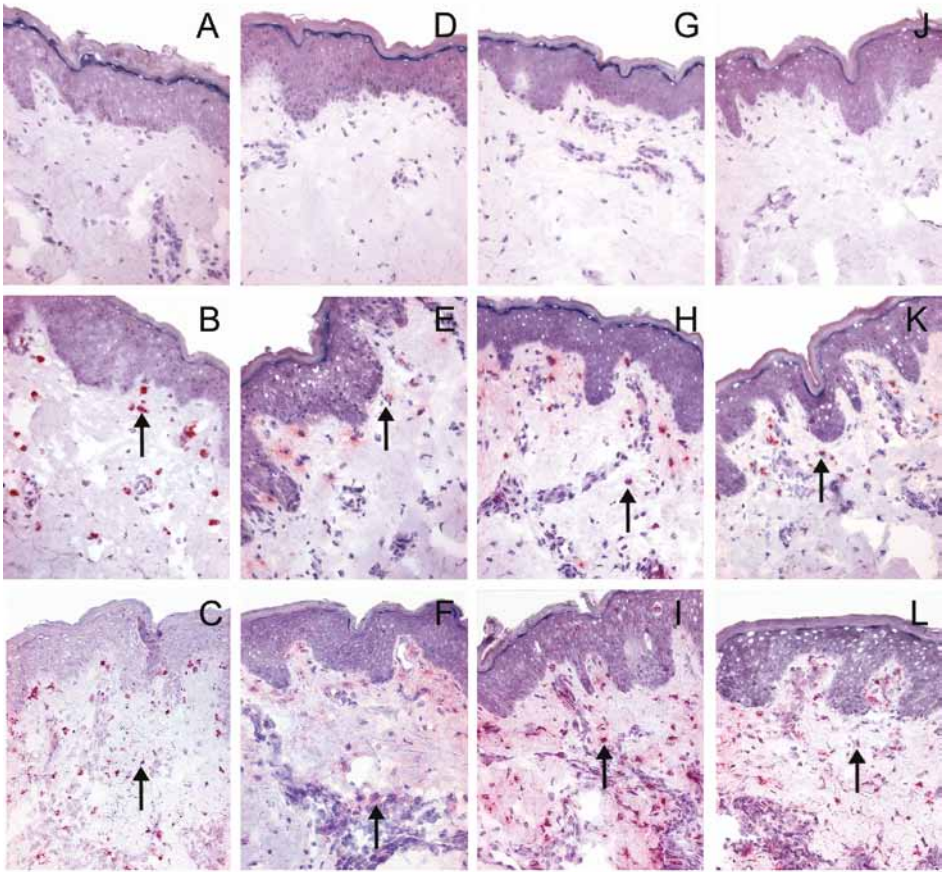

Figure 3. Immunohistochemical staining with monoclonal antibodies directed against neutrophil elastase (A-C), matrix metalloproteinase (MMP)-1 (D-F), MMP-8 (G-I) and MMP-9 (J-L). The upper row of photographs shows absence of staining in the unexposed skin. The middle and bottom rows show neutrophil elastase- and MMP-1-, MMP-8- and MMP-9-positive staining cells (arrows) in the skin, respectively, 6 and 24 hours after exposure to 2 MED of solar-simulating radiation *(original magnification x200)*.

Double staining of neutrophil elastase with matrix metalloproteinase (MMP)-1, MMP-8 and MMP-9 in skin exposed to solar-simulating radiation

In order to characterize MMP-1-, MMP-8- and MMP-9-positive cells, double staining was carried out with neutrophil elastase. Almost all MMP-1-, MMP-8- and MMP-9-positive cells in the dermis double stained with neutrophil elastase (Figure 4). Using cell

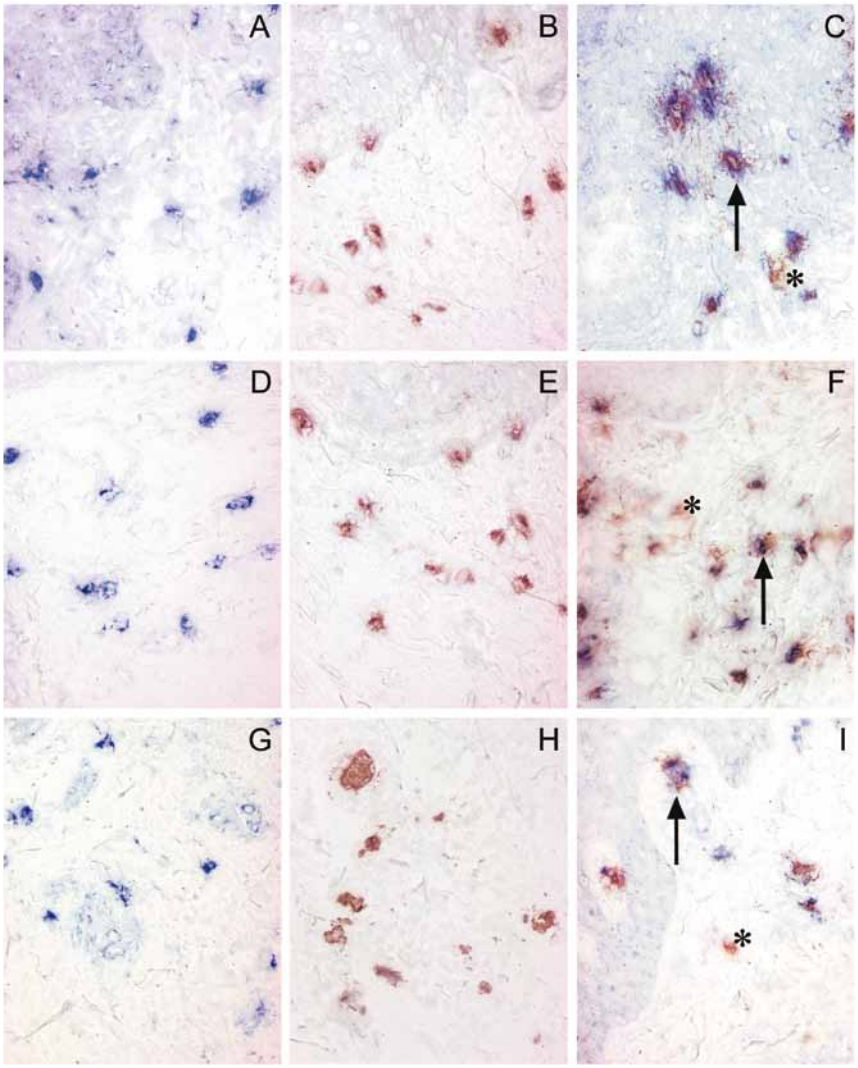

Figure 4. Double staining of neutrophil elastase-positive cells with matrix metalloproteinase (MMP)-1 (A-C), MMP-8 (D-F) and MMP-9 (G-I). The left and middle columns of photographs are controls in the double-staining procedure showing sections stained only with MMPs and neutrophil elastase, respectively. Double-staining cells (arrows) are seen in the right column of photographs. Some neutrophil elastase-positive/MMP-negative cells (asterisks) are also seen *(original magnification x400)*.

numbers from the single staining procedures it was calculated that a mean of 55% of neutrophil elastase-positive cells double stained with MMP-1, 55% with MMP-8 and 44% with MMP-9.

Neutrophil elastase, matrix metalloproteinase (MMP)-1 and MMP-9 enzyme activity is located mainly in the upper dermis

MMP-1, MMP-9 and neutrophil elastase enzyme activity was detected in a spot-like manner mainly in the upper dermis in SSR-exposed skin (Figure 5). Enzyme activity was detected only in skin irradiated with ≥1 MED of SSR. Numbers of fluorescent spots reflecting enzyme activity were similar to the corresponding numbers of stained cells in the immunohistochemical staining of the protein.

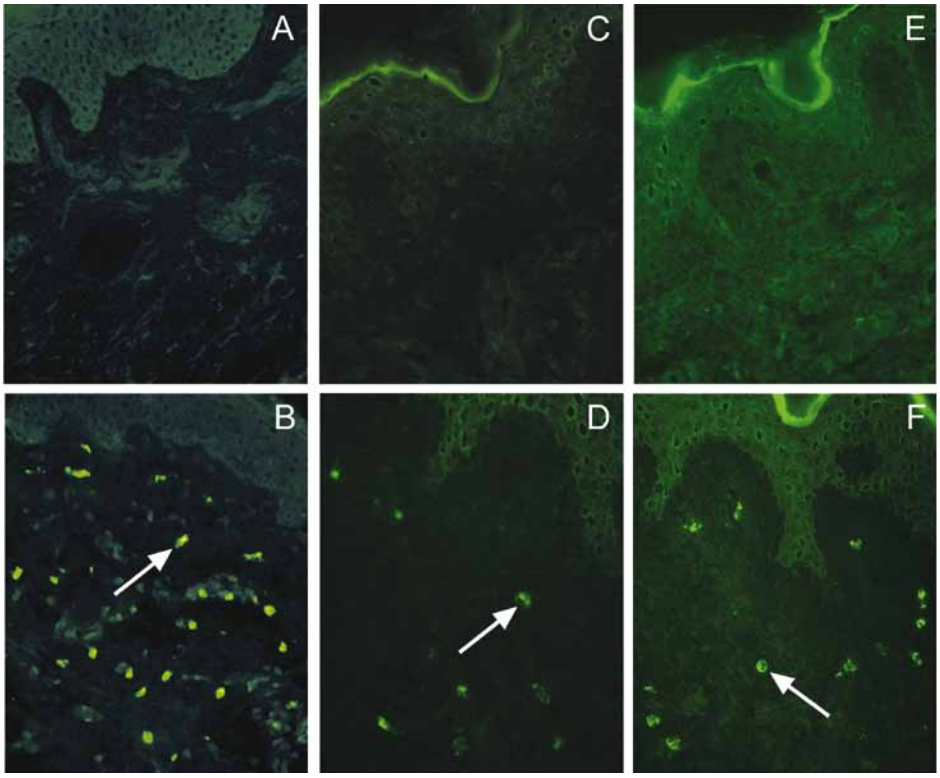

Figure 5. Neutrophil elastase (A,B), matrix metalloproteinase (MMP-1) (C,D) and MMP-9 (E,F) *in situ* zymography. The upper panel of photographs shows no enzyme activity present in unexposed skin. The lower panel shows neutrophil elastase, MMP-1 and MMP-9 enzyme activity in skin 6 hours after exposure to 2 minimal erythema doses of solar-simulating radiation. Enzyme activity was also detected after 24 and 48 hours (data not shown). The numbers and distribution of enzyme activity (fluorescent spots) were similar to those of corresponding protein staining (type and time point). *Original magnification: (A,B) x125, (C-F) x200.*

Discussion

When investigating the possible involvement of neutrophilic granulocytes in SSR-induced skin damage we made some unexpected observations. Although neutrophils are known to be present in sunburned skin, their morphology was completely absent in frozen skin sections. They could be recognized morphologically only in paraffin-embedded skin sections. In frozen skin sections the presence of neutrophils is generally demonstrated by immunohistochemical staining of neutrophil elastase. Thus neutrophils were shown to be abundant in skin that had been exposed to a single dose of 2 MED of SSR (representing a radiation dose equivalent to sunburn) in both the paraffin-embedded skin sections and the frozen skin sections. The cells primarily infiltrated the dermis but also the epidermis. Neutrophils were not detected in skin irradiated with <1 MED of SSR. The fact that these cells lose their conspicuous morphology in frozen skin sections and the fact that they do not infiltrate suberythematous irradiated skin is probably the reason why they have received little attention with respect to the pathogenesis of photoaging of human skin.

Another surprising observation was that, contrary to the findings of Fisher *et al*.,[3;4] we observed prominent MMP-1, MMP-8 and MMP-9 immunohistochemical staining only in skin that had been irradiated with ≥1 MED of SSR. Immunohistochemical double staining with neutrophil elastase suggested that these MMPs were neutrophil derived and not keratinocyte or fibroblast derived. Even though small amounts of neutrophil elastase may be present in macrophages and fibroblasts, together with the paraffin-embedded skin sections showing the presence of morphologically distinct neutrophils, our results strongly suggest that the MMPs are indeed neutrophil derived.

In all skin sections analyzed, the number of neutrophil elastase-positive cells exceeded that of MMP-1-, MMP-8- and MMP-9-positive cells. One explanation is that not all neutrophils produce MMPs. Furthermore, we found that 12-20% of the neutrophil elastase-positive cells double stained with CD36, a marker for macrophages. CD36+ cells did not, however, double stain with the MMPs under study (data not shown).

Our *in situ* zymography results provide additional evidence that erythemogenic doses of SSR are required to induce MMPs *in vivo*. Elastase, collagenase and gelatinase activity was detected only in skin that had been irradiated with ≥1 MED. This finding bypasses the necessity to demonstrate mRNA for these enzymes, as the detection of mRNA in itself is no proof of the presence of enzymatically active protein.

MMP-9 as an extracellular matrix-damaging agent has previously been connected to infiltrating neutrophils in cryptogenic organizing pneumonia[15] and adult periodontitis.[16] In adult periodontitis investigators proclaimed MMP-9 to originate primarily from infiltrating neutrophils rather than resident fibroblasts, epithelial cells or macrophages. Cryptogenic organizing pneumonia is an interstitial lung disease characterized by the production, deposition and proteolysis of extracellular matrix components.

MMP-1 as a product of neutrophils has, to our knowledge, not been shown convincingly in previous studies. In an ongoing study we also detected MMP-1 protein in freshly isolated neutrophils. Many neutrophil products are stored in preformed granules[5] and are probably not synthesized de novo as these cells infiltrate their target organ. Nielsen *et al.*[17] investigated MMP-9 expression in human breast cancer and found that infiltrating neutrophils were an important source of MMP-9 protein. However, MMP-9 mRNA could not be detected in these cells. Similarly, studies at mRNA level may not show MMP-1 mRNA in neutrophils.

In contrast to MMP-8 and MMP-9, staining for MMP-1 showed subtle staining of the epidermis. This was particularly apparent in the double-stained skin sections where no counterstaining was performed. MMP-1 epidermal staining was present in all skin sections, including the controls (taken from skin not exposed to UV radiation), and ended abruptly at the epidermal-dermal junction. MMP-1 is most likely to be constitutionally expressed in keratinocytes of the epidermis as *in situ* zymography showed a similar faint fluorescent pattern in the epidermis. Again, prominent MMP-1 enzyme activity and MMP-1 protein staining were detected only in skin exposed to ≥1 MED of SSR, and were particularly located in the dermis.

MMP-8 as a product of neutrophils was studied by Fisher *et al.*[11] As mentioned in the introduction, it was not considered an important contributor to the photoaging process.

In another ongoing study we irradiated healthy freshly obtained breast reduction skin (similarly treated and incubated as human skin explants used in the investigations conducted by Boisnic *et al.*[18]) with equal and higher physical doses of SSR (doses applied in the *in vivo* experiments). Irradiated and incubated *ex vivo* skin was processed and skin sections were stained for the presence of MMP-1, MMP-8, MMP-9 and neutrophil elastase. None of these enzymes could be detected. As neutrophils are not present in breast reduction skin samples, these findings support our *in vivo* findings and support a possible role of infiltrating neutrophils in photoaging of the skin.

Fisher *et al.*[3;4] proposed that elevated levels of keratinocyte- and fibroblast-derived MMPs following brief exposure to the sun every other day is the mechanism that leads to persistent breakdown of skin connective tissue and accelerated skin ageing. Yet, severe wrinkle formation and plaque-like thickening of the skin is particularly seen in outdoor workers and sun-worshippers: persons whose skin has frequently been burned (i.e., exposed to erythemogenic doses of SSR). Our results suggest a different mechanism in which exposure to erythemogenic doses of sunlight and possibly infiltrating neutrophils play an important role. However, further investigations with repeated exposures to erythemogenic doses of SSR are required to confirm this hypothesis.

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Chapter 4

Photoaging-associated matrix metalloproteinases: how relevant are they to the photoaging process?

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Abstract

Background Sunlight-induced matrix metalloproteinases (MMPs) are believed to play an important role in the pathophysiology of photoaging. Keratinocyte- and fibroblast-derived MMP-1 has particularly been studied in this context. Erythemogenic doses of solar-simulating radiation (SSR) induce skin-infiltrating neutrophils which express MMPs, neutrophil elastase and other proteinases capable of contributing to photoaging.
Objectives To reevaluate the role of MMP-1,-2,-3,-8,-9 and -12 and neutrophil elastase in photoaging.
Methods Different skin samples were investigated: young and elderly, black and white, sun-protected and sun-exposed, and SSR-exposed. Peripheral blood neutrophilic cells were isolated from healthy donor blood. Immunohistochemistry and immunofluorescence were used to demonstrate MMPs and neutrophil elastase expression. MMP-specific monoclonal antibodies were tested for specificity prior to use. To show functional activity of MMPs and neutrophil elastase *in situ* zymography was performed.
Results Testing the specificity of the MMP monoclonal antibodies revealed monospecificity for all monoclonal antibody batches except for one anti-MMP-1 batch. This batch appeared to be contaminated with anti-MMP-8 and was not used in further experiments. This finding implicates rectification of previously reported MMP-1 expression by infiltrating neutrophils (Rijken *et al*, Br.J.Dermatol 2005 Feb; 152(2): 321-8). MMP-1 and MMP-3 staining and collagenase activity were mostly detected in the epidermis and showed a sharp demarcation between the epidermis and the dermis. Furthermore, MMP-1 and MMP-3 staining and collagenase activity appeared to be consistent in all skin samples and not increased in SSR-exposed skin. MMP-2 and MMP-12 could not be detected in any of the skin samples. Epidermal MMP-9 staining and gelatinase activity appeared consistent in all skin samples. However, prominent spot-like MMP-9 staining and related gelatinase activity were only detected in the dermis of white skin exposed to erythemogenic doses of SSR. These skin samples also showed coinciding spot-like MMP-8 and neutrophil elastase staining and elastase activity. Although neutrophil smears showed cytoplasmic collagenase activity, SSR-induced neutrophil-derived MMP-8 (*in vivo* detected) appeared enzymatically inactive.
Conclusions Our study suggests that most MMPs, including MMP-1, are less relevant to the photoaging process than previously thought. Only neutrophil-derived MMP-9, together with neutrophil elastase, are likely important contributors to the dermal damage observed in photoaged skin.

Introduction

Sunlight-induced matrix metalloproteinases (MMPs) are believed to play an important role in the pathophysiology of photoaging.[1;2] Interstitial collagenase, MMP-1, has been most studied in this context. Photoaged skin is characterized by damaged elastic and collagen fiber networks[3;4] and MMPs are capable of degrading elastic and collagen fibers.[5;6] MMPs have been shown to be induced in the skin by different electromagnetic wavelength spectra: natural sunlight, solar-simulating radiation (SSR), UVB, UVA, and infrared radiation (IR).

Fisher *et al.*,[2;7] whose studies primarily focus on collagen degradation, showed that MMP-1, MMP-3 and MMP-9 can be induced in human skin following exposure to suberythemogenic doses of UVB. Induction of MMP-1, -3 and -9 by UVA and SSR has also been shown.[1;8-10] Collectively MMP-1, -3 and -9 are capable of breaking down skin collagens and thereby could play a role in the pathophysiology of photoaging. Based on the localization of immunohistochemically stained MMPs, *in situ* hybridisation of mRNA, laser capture microdissecation coupled with real-time reverse transcriptase PCR, *in situ* zymography and literature data, Fisher *et al.*[1;2] concluded that the UV-induced MMPs were fibroblast- and, predominantly, keratinocyte-derived. In their *in vivo* studies, MMP mRNA was mostly located in the epidermis and MMP protein was also mainly induced in this compartment[2]. The expression of MMP protein by fibroblasts was based on the morphology of MMP positive cells in the dermis and on *in vitro* data. Fisher *et al.*[11] further showed that MMP-8 is induced following exposure of human skin to erythemogenic doses of UV. MMP-8 co-localized with UVB- and SSR-induced skin-infiltrating neutrophils. They suggested that MMP-8 does not contribute to photoaging since in their studies UV-induced MMP-8 appeared to be enzymatically inactive.

Based on our experimental findings we have previously suggested that SSR-induced, neutrophil-derived proteolytic enzymes could be important contributors to the photoaging process.[12] Neutrophil elastase, in particular, may contribute to photoaging of the skin: neutrophil elastase is an important immunohistochemical marker of neutrophils[13] and is a potent proteolytic enzyme capable of damaging the extracellular matrix.[14] With respect to MMPs, our results differed from the results of Fisher *et al.* in that suberythemogenic doses of UV did not induce significant amounts of MMPs. Neutrophils infiltrate the skin following exposure to erythemogenic doses of natural sunlight, UVA, UVB and SSR.[15-17] Neutrophils have been shown to express MMP-8, MMP-9, MMP-12[18;19] and in our studies MMP-1 also co-localized with skin-infiltrating neutrophils.[12] Although these proteolytic enzymes are normally stored in vesicles in an inactive form, our *in situ* zymogaphy studies suggested that neutrophil-derived MMPs are enzymatically active in the SSR-exposed skin.[12;17;20]

MMP-2 mRNA has been shown to be induced in the skin by PUVA, UVB and SSR.[9;21] MMP-2 induction by SSR has also been shown by immunohistchemistry.[22] MMP-12 induc-

tion following exposure to UVB/UVA2 has been reported.[23] MMP-2 and MMP-12 should be taken into account with respect to photoaging since they have elastolytic properties.[6]

Cho *et al.*[15] showed that significant amounts of MMP-1 and MMP-9 mRNA and protein are induced in sun-exposed, cloth-covered skin. Since clothes block the penetration of UV but do not block the penetration of IR, this indicates that IR induces MMP-1 and MMP-9. Based on the hypothesis of Fisher *et al.* concerning the pathophysiology of photoaging where MMP-1 and -9 play an important role, the authors concluded that IR must contribute to the photoaging process. In their experiments, exposure of cloth-covered skin to natural sunlight did not induce any skin-erythema and consequently neutrophils were not detected.

Taken together, different wavelength-spectra appear to be capable of inducing different MMPs in both resident and skin-infiltrating cells. SSR best approaches the UV spectrum of natural sunlight. Therefore, next to natural sunlight which has the disadvantage that the dosimetry is difficult to control, SSR appears to be the radiation of choice when studying photoaging *in vivo*. Basal gene expression and induction by SSR of MMP-1, -2, -3, -8, -9, -12 mRNA has been studied.[1] This particular study, however, did not show data on MMP protein expression and induction. In the present study we examined the expression of the aforementioned MMPs and neutrophil elastase by immunohistochemistry in young and elderly skin, black and white skin, sun-protected (buttock) and sun-exposed (forearm) skin, and SSR-exposed skin. Black skin photoages at a much slower rate than white skin.[24] Thus, a comparison of black and white skin is extremely useful in studying SSR-induced skin changes as differences observed herein may also be relevant to photoaging. For the detection of MMP-1 we used monoclonal anti-MMP-1 antibodies from different companies and of different IgG isotypes. In addition, purified MMP proteins were used as controls for testing of staining specificity. Gelatinase, collagenase and elastase activity was studied by *in situ* zymography in both the collected skin samples and in neutrophils isolated from peripheral blood.

Materials and Methods

Volunteer inclusion, irradiation procedures, and skin sample collection and processing

Skin samples from 40 healthy volunteers were used in the experimental procedures. Volunteers from different age-groups (18 - 28yrs and 56 - 63yrs) and different skin phototypes (white skin: SPT I, II, III and black skin: SPT VI) were included in the study. 4mm skin biopsies were taken from sun-protected buttock skin, sun-exposed forearm skin, and SSR-exposed buttock skin. Additionally, mamma reduction skin was obtained from the department of Plastic Surgery and healthy donor blood was obtained from the donor

service of the University Medical Center Utrecht. The study was approved by the medical ethical committee of the University Medical Center Utrecht and informed consent was obtained from all volunteers.

Sun-protected buttock skin of 18/40 volunteers was exposed to SSR. 12/18 (6 black- and 6 white-skinned subjects) of these volunteers participated in a time-course study (biopsies taken before, 6, 24 and 48 hours after irradiation of the skin with 2 MED of SSR). 6/18 volunteers participated in a dose-response study (biopsies taken 24 hours after irradiation with 0.5, 1 and 2 MED of SSR). CLEO Natural lamps (Philips, Eindhoven, the Netherlands) were used as a source of SSR. CLEO Natural lamps emit mostly UV-A and 3% UV-B. The spectral energy distribution of CLEO Natural lamps ranges from 290 nm to 420 nm, with a maximum around 355 nm. The MED of white-skinned (SPT I-III) subjects was determined using a specifically designed test device with nine 3 by 10mm windows that open consecutively exposing the underlying skin. Black-skinned subjects were exposed to 18 000 mJ/cm^2 which is equivalent to 2-3 MEDs of white-skinned subjects.

Skin biopsies collected were snap frozen in liquid nitrogen and embedded in Tissuetek (Sakura,Torrance, CA,USA). Biopsies were cut into 6µm thick skin sections with a freezing microtome, mounted on Superfrost® Plus coated slides (Thermo scientific, Waltham, MA, USA), and left to dry on Silicagel (MERCK, Darmstadt, Germany).

Immunohistochemical and immunofluorescence analyses

The antibodies used to detect different MMPs and neutrophil elastase by immunohistomchemistry are listed in Table 1a. Isotype (IgG_1, IgG_{2a}, IgG_{2b}) control experiments were performed (obtained from 'the binding site group ltd', Birmingham, UK). Most of the immunohistochemically stained skin sections were not counterstained with hematoxylin because hematoxylin can obscure the stained MMPs.

To test for any cross-reactivity of the MMP-targeting-antibodies, the following control experiments using purified MMPs (listed in Table 1b) were performed (Figure 1a): (i) two, 8mm diameter, sun-protected, mamma-reduction skin sections were placed on a glass slide; (ii) undiluted, purified MMPs were applied onto one of the skin sections and directly onto the glass slide, and left to dry in air; (iii) immunohistochemical staining procedures (described below) with all the MMP-targeting-antibodies used were performed to test whether these antibodies react specifically with their respective MMP proteins coated onto the glass slides.

Immunohistochemical staining procedures were as follows: (i) fixation of skin sections in dry acetone containing 200µl hydrogen peroxide; (ii) pre-incubation with 10% normal human serum (NhuS)/normal horse serum (NhoS)/bovine serum albumin (BSA); (iii) incubation with mouse anti-human MMP (and anti-human neutrophil elastase)-specific immunoglobulin (diluted in 1% NhuS/BSA, see Table 1a for specific dilutions); (iv) washing of skin sections in phosphate-buffered saline (PBS)/Tween 0.05%;

Table 1a. Antibodies used to detect MMPs and neutrophil elastase

Product	Isotype	Dilution	Obtained from	Manufacturers Comment
Anti-MMP-1 clone III7	Mouse monoclonal IgG_1	1:50	Oncogene (now Calbiochem), San Diego, CA, USA	Recognizes latent and active forms of MMP-1 Immunogen: within AA 82-250 of pro-MMP-1
Anti-MMP-1 clone III7	Mouse monoclonal IgG_1	1:50	Calbiochem, San Diego, CA, USA	Recognizes latent and active forms of MMP-1 Immunogen: within AA 82-250 of pro-MMP-1
Anti-MMP-1 clone SB12e	Mouse monoclonal IgG_{2b}	1:50	Abcam, Cambridge, UK	Recognizes MMP-1 Immunogen: recombinant full length human MMP-1
Anti-MMP-1 clone 41-1E5	Mouse monoclonal IgG_{2a}	1:4	Calbiochem, Darmstadt, Germany	Recognizes latent and active forms of MMP-1 Immunogen: synthetic peptide AA 332-350 of human MMP-1
Anti-MMP-2 clone SB13a	Mouse monoclonal IgG_1	1:500	Abcam, Cambridge, UK	Recognizes MMP-2
Anti-MMP-3 clone SB14d	Mouse monoclonal IgG_{2a}	1:50	Abcam, Cambridge, UK	Recognizes MMP-3
Anti-MMP-8 clone 100608	Mouse monoclonal IgG_{2a}	1:20	R&D Systems, Minneapolis, MN, USA	Recognizes latent and active forms of MMP-8
Anti-MMP-9 clone GE-213	Mouse monoclonal IgG_1	1:50	Neomarkers, Fremont, CA, USA	Recognizes latent and active forms of MMP-9
Anti-MMP-12 Clone 4D2	Mouse monoclonal IgG_1	1:20	R&D Systems, Minneapolis, MN, USA	Recognizes latent MMP-12 only
Anti-neutrophil elastase	Mouse monoclonal IgG_1	1:100	DAKO A/S, Glostrup, Denmark	Recognizes the three isoforms of neutrophil elastase

Table 1b. Purified human MMP proteins used to determine the sensitivity and specificity of the anti-MMP antibodies listed in Table 1a

Product	Obtained from
Purified MMP-1 protein	Abcam, Cambridge, UK
Purified MMP-2 protein	Abcam, Cambridge, UK
Purified MMP-3 protein	Abcam, Cambridge, UK
Purified MMP-8 protein	Biomol Int, Plymouth Meeting, PA, USA
Purified MMP-9 protein	Abcam, Cambridge, UK
Purified MMP-12 protein	Abcam, Cambridge, UK

(v) incubation with biotinylated horse anti-mouse immunoglobulin (Vector, Burlingame, CA; diluted 1:300 in 1% NhuS/BSA); (vi) washing step in PBS/Tween 0.05%; (vii) incubation with horseradish peroxidase (HRP)-conjugated avidin-biotin complex (Vectastain ABC-HRP, Vector; diluted 1:1000 in 1% NhuS/BSA); (viii) washing step in PBS/Tween 0.05%; (ix) staining with 3-amino-ethyl-carbazole chromogen (Scytec, Logan, Utah, USA); (x) staining halted with distilled water. Stained skin sections were examined by light microscopy at 200x and 400x magnification.

Next to immunohistochemistry, MMP-1 expression was visualized using FITC-labeled rabbit anti-mouse antibodies (DAKO A/S, Glostrup, Denmark firm; diluted 1:20): steps (i) to (iv) as described above were followed by incubation with FITC-labeled antibodies, a washing step, and finally MMP-1detection by fluorescence microscopy (FITC channel, ex/em 495/515).

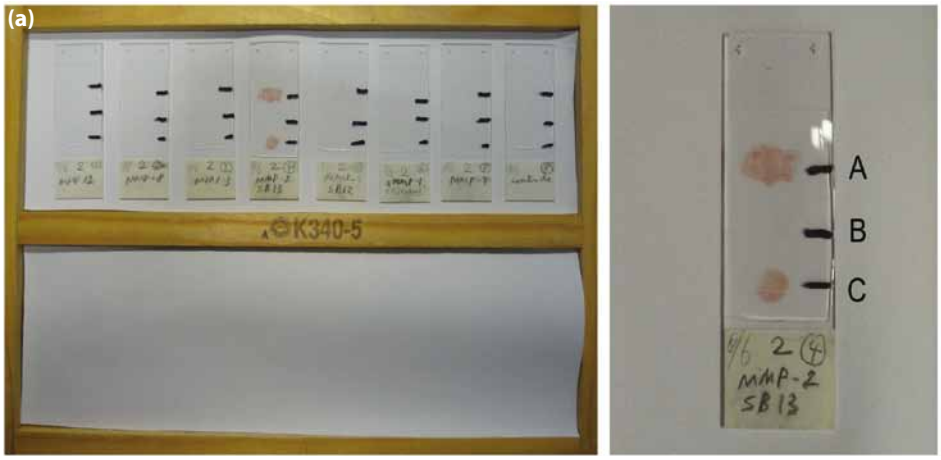

Figure 1. Specificity testing of monoclonal antibodies. (a) Shows an illustrative example of the experimental set-up. Two skin sections were mounted on a glass slide (lane A and B, right panel). Purified MMP protein was applied directly onto the glass slide (lane C) and onto one of the skin sections (lane A), and left to dry. Subsequently, immunohistochemical staining with various anti-MMP-antibodies was carried out. The left panel shows the results of MMP-2 protein applied onto the glass slides and immunohistochemical staining with anti-MMP-1, -2, -3, -8, -9 and -12 antibodies. In this example, as expected, only anti-MMP-2 antibodies reacted with MMP-2 protein.
(b) Shows that one of the anti-MMP-1 antibodies used cross-reacted with MMP-8. Purified MMP-1 and MMP-8 protein were applied onto the left two glass slides (as described above) and allowed to incubate with clone III7 anti-MMP-1 antibody, obtained from Oncogene. Both glass slides show positive staining, indicating that the antibody binds to MMP-1 but also to MMP-8. The right two glass slides show that the same clone purchased from Calbiochem at a later stage did not cross-react with MMP-8.

Neutrophil isolation and *in situ* zymography

The following procedure was carried out to isolate neutrophils from healthy donor blood: (i) addition of 0.4% (w/v) trisodium citrate (pH 7.4) to anticoagulate the blood; (ii) centrifugation over isotonic Ficoll (Pharmacia, Uppsala, Sweden) to remove mononuclear cells; (iii) lysis of erythrocytes in an isotonic NH_4Cl solution; (iv) washing and resuspension of neutrophils in incubation buffer (20 mM Hepes, 132 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1.2 mM KH_2PO_4, 5 mM glucose, 1 mM $CaCl_2$ and 0.5% human serum albumin); (v) spinning down of neutropils (1000 RPM for 5 minutes).

Neutrophils were smeared onto Superfrost® Plus coated slides and left to dry on Silicagel.

EnzChek Elastase Assay Kit (E-12056), DQ-collagen (D-12060) and DQ-gelatin (D-12054) all purchased from Molecular Probes (Eugene, OR, USA) were used to determine *in situ* elastase, collagenase and gelatinase enzyme activity. Slides with mounted skin sections or isolated neutrophil smears were used in these experiments. Reaction buffer (0.05 M TRIS-HCl, 0.15 M NaCl, 5 mM $CaCl_2$, and 0.2 mM NaN_3, pH 7.6) containing 40 μg/ml DQ-Elastin was transferred onto the slides and allowed to incubate for a short period of time. Elastase enzyme activity was detected using a fluorescence microscope (FITC-channel, ex/em 505/515). 0.01-1 mM N-methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone supplied with the elastase assay kit was used as a specific neutrophil elastase inhibitor. Collagenase and gelatinase enzyme activity were detected in a similar fashion. Here the above described reaction buffer contained 40μg/ml DQ-collagen type I or DQ-gelatin, both fluorescein conjugated. The solutions were transferred onto the skin sections and, following a short incubation time, enzyme activity was detected by fluorescence microscopy using a slightly different wavelength (FITC channel, ex/em 495/515). 0.1-10 mM of 1,10-phenanthroline was used as a general metalloproteinase inhibitor. To inhibit collagenase, gelatinase and elastase activity, skin sections were incubated with the MMP and/or neutrophil elastase inhibitors for one hour before applying DQ-collagen, DQ-gelatin and DQ-elastin.

Results

Specificity of MMP-targeting-antibodies, isotype controls and non-specific staining of sebaceous glands

Prior to immunohistochemical staining of MMPs, the monoclonal antibodies listed in Table 1a were tested for specificity. All monoclonals listed reacted with their respective purified MMP proteins. Purified MMP proteins (Table 1b) clearly adhered to the glass slides both when applied directly and when applied onto skin sections that were already mounted on the glass slides. Figure 1a shows an illustrative example of the experimen-

tal set-up. In this example, only anti-MMP-2 antibodies reacted with purified MMP-2 protein. The other anti-MMP antibodies (anti MMP-1, -3, 8, -9, -12) did not bind to the purified MMP-2 protein adhered to the glass slide.

Of all tested antibodies, one anti-MMP antibody showed cross-reactivity with another purified MMP protein: anti-MMP-1 antibody, obtained from Oncogene and used in previous experiments[12;17], reacted with purified MMP-1 protein but also reacted with purified MMP-8 protein (see Figure 1b). The same clone, purchased from Calbiochem at a later date, only reacted with purified MMP-1 protein and showed no cross-reactivity with purified MMP-8 protein (Figure 1b).

Isotype controls showed no staining of the epidermis or dermal cells.

Non-specific staining of sebaceous glands occurred in all stained skin sections (i.e., in those where a sebaceous gland was present). Control experiments, where different steps in the staining procedure were omitted, showed that this was not due to endogenous peroxidase activity or 'sticking' of antibodies, but was due to 'sticking' of avidin-biotin HRP complex to the sebaceous glands.

MMP-1 staining using different anti-MMP-1 antibodies

Since the staining pattern of MMP-1 in the skin is crucial to its role in photoaging different anti-MMP-1 antibodies were evaluated (Figure 2). Clone III7 anti-MMP-1 antibodies, purchased initially from Oncogene and later from Calbiochem, showed a similar staining pattern of the epidermis. The Oncogene batch, however, showed prominently stained cells in the dermis of skin that had been exposed to erythemogenic doses of SSR. No such prominently stained MMP-1 positive cells in the dermis were observed with any of the other anti-MMP-1 antibodies investigated. As indicated above, the Oncogene batch showed cross-reactivity with MMP-8.

Staining with clone SB12e (Abcam) showed that MMP-1 staining was concentrated in the epidermis. When examined closely, staining intensity appeared highest perinuclearly and slightly reduced in between the keratinocytes. The stratum corneum was devoid of staining. As with clone III7, MMP-1 staining showed a sharp demarcation between the epidermis and the dermis. Dermal staining of MMP-1 was more subtle and appeared to involve most nucleated cells. In the dermis the MMP-1 staining pattern of many cells appeared to be eccentric (inset, Figure 2, panel d).

Staining with clone 41-1E5 (Calbiochem) showed a different pattern in that epidermal MMP-1 staining appeared to coincide with the nuclei of keratinocytes (Figure 2). Most dermal cells also showed subtle MMP-1 staining but this was less prominent compared to MMP-1 staining of keratinocytes. The former cells showed more eccentric MMP-1 staining, i.e., not obviously bound to nuclei.

Visualization of MMP-1 by immunofluorescence showed a similar pattern to that seen with immunohistochemistry (Figure 2).

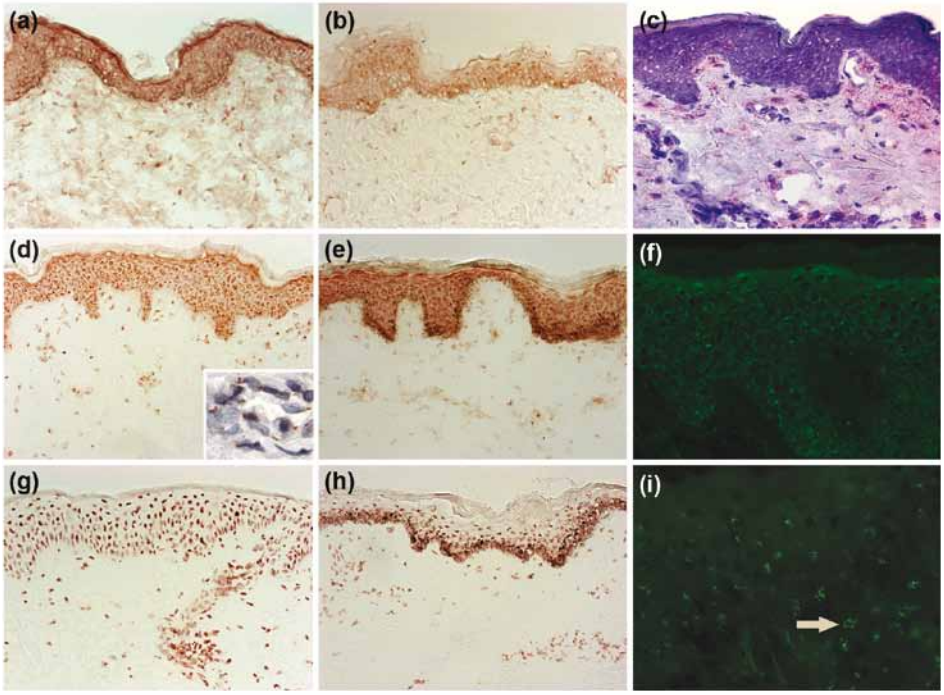

Figure 2. Staining pattern of MMP-1 using different monoclonal antibodies. Clone III7 purchased from Oncogene **(a)** and clone III7 purchased from Calbiochem **(b)** show a similar staining pattern in non-irradiated skin. Of all the anti-MMP-1 antibodies tested only clone III7 (Oncogene) showed a clear induction of 'MMP-1' (i.e., MMP-8) positive cells in the dermis following exposure to erythemogenic doses of SSR **(c)***. Panel c also demonstrates how powerful counterstaining can obscure real MMP-1 staining in the epidermis. The other anti-MMP-1 antibodies also demonstrate MMP-1 expression in the dermis but this expression was more subtle and was not significantly increased by SSR exposure. MMP-1 expression, as demonstrated by clone SB12e, is similar in white **(d)** and black **(e)** skin. Equally, MMP-1 expression is the same in white **(g)** and black **(h)** skin when staining with clone 41-1E5. The exact staining pattern of clone 41-1E5, however, is different from clone SB12e (and clone III7). MMP-1 staining with clone 41-1E5 appears to coincide with the nuclei of keratinocytes. Panels **(f)** and **(i)** confirm this difference in staining pattern between clones SB12e and 41-E5 by immunofluorescent 'staining' of MMP-1: diffuse and perinuclear fluorescence of the epidermis with clone SB12e and nuclear fluoresence (→) with clone 41-E5. In the dermis MMP-1 staining of many cells appeared to be eccentric (inset). *Images a-e, g and h: original magnification x200; images f, i and inset: original magnification x400.*

* image previously published by Rijken et al[12]

Skin color, skin localization and SSR exposure did not strongly (see further) influence the MMP-1 staining pattern except, as discussed above, in the oncogene batch where erythemogenic doses of SSR induced prominently stained cells in the dermis.

Neutrophil smears appeared negative for MMP-1 (SB12e) by immunofluorescence.

Immunohistochemical staining of MMPs and neutrophil elastase in young and elderly, black and white, sun-exposed forearm and sun-protected buttock, and SSR-exposed skin

MMP-1 and MMP-3 staining patterns and intensities appeared consistent in all skin samples examined: young and elderly, black and white, sun-exposed and sun-protected, and SSR-exposed skin. MMP-1 and MMP-3 staining was mostly concentrated in the epidermis. However, most nucleated dermal cells appeared to be positive as well. In skin samples where there was increased cellularity in the dermis this subtle dermal staining was increased due to increased cell numbers (e.g., inflammatory cells in skin exposed to erythemogenic doses of SSR).

MMP-2 and MMP-12 staining was not detected in any of the skin samples examined.

MMP-8 was only detected in skin exposed to erythemogenic doses of SSR: prominent spot-like stains were observed in the dermis.

MMP-9 staining of the epidermis was detected in all skin samples. A clear difference (increase or decrease) between the skin samples in epidermal MMP-9 staining was not observed. Similar to MMP-8 staining, intense MMP-9 staining in the dermis was only observed in skin exposed to erythemogenic doses of SSR.

Neutrophil elastase staining was only detected in skin exposed to erythemogenic doses of SSR.

The staining patterns of MMP-1 (clone SB12e), MMP-2, MMP-3, MMP-8, MMP-9 and MMP-12 in SSR-protected and SSR-exposed white skin are presented in Figure 3. The only difference between black and white skin was the absence, in all black skin samples, of prominent spot-like staining in the dermis of MMP-8, MMP-9 and neutrophil elastase. Even exposing black skin to 18 000 mJ/cm^2 (equivalent to 2-3 MED of white skin) failed to induce prominent MMP-8, MMP-9 and neutrophil elastase staining in the dermis.

In situ zymography

Collagenase activity was detected in all skin samples collected: young and elderly, black and white, sun-protected and sun-exposed, and SSR-exposed skin. Collagenase activity was most prominent in the epidermal compartment (Figure 4). There was a clear demarcation between the epidermis and the dermis. Increased cellularity in the dermis (e.g., following exposure of the skin to erythemogenic doses of SSR) appeared to increase spot-like fluorescence in the dermis, although fluorescence in the epidermis remained more prominent. A neutrophil smear showed cytoplasmic collagenase activity (Figure 4).

In both skin sections and neutrophil smears, collagenase activity could not be fully inhibited by 1,10-phenanthroline (a general MMP inhibitor). Nor could collagenase activity be fully inhibited by a combination of 1,10-phenanthroline and N-methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone (an inhibitor of neutrophil elastase), although there appeared to be an additional inhibitory effect.

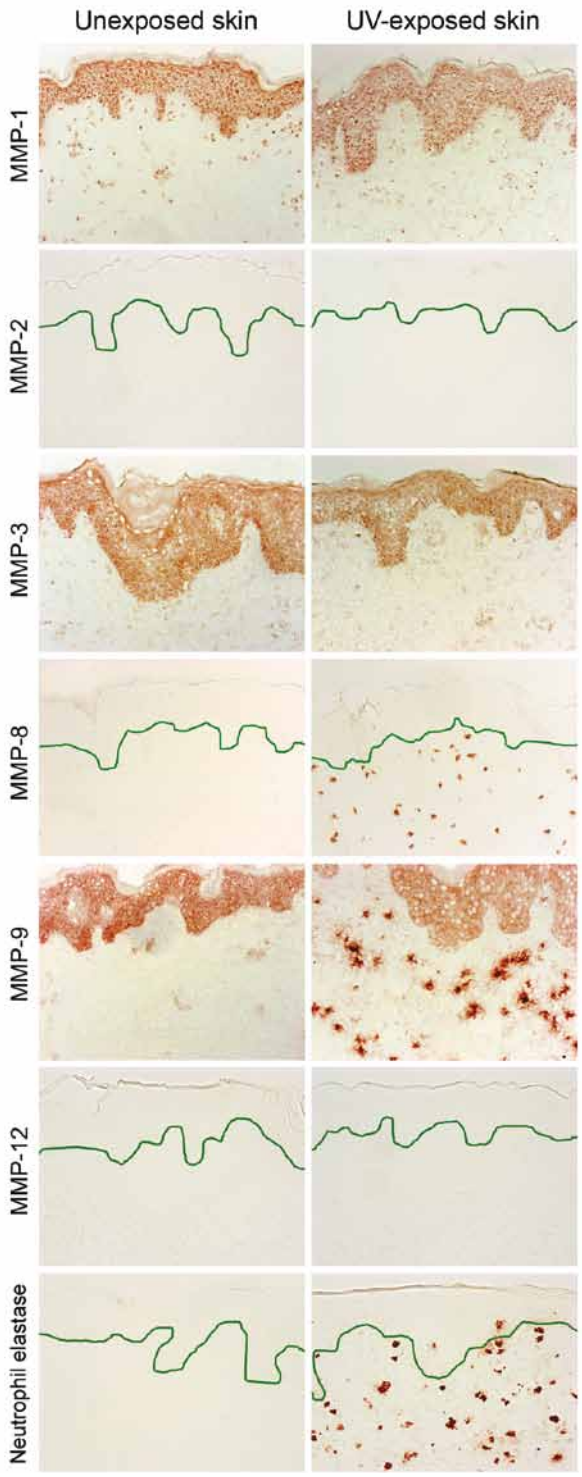

Figure 3. Expression of photoaging-associated MMPs and neutrophil elastase in unexposed and SSR-exposed (2 MED) white buttock skin. MMP-1 (stained with clone SB12e), MMP-3 and MMP-9 was clearly expressed in the epidermis of all skin samples. Exposure to SSR did not clearly influence the pattern or intensity of MMP-1, -3 and -9 staining in this compartment. MMP-1, -3 and -9 staining showed a sharp demarcation between the epidermis and dermis in all skin samples. Subtle dermal MMP-1 positive cells were detected in both unexposed and irradiated skin, and, apart from some increased cellularity, irradiation did not significantly influence the dermal MMP-1 staining pattern. Dermal MMP-3 staining showed a similar pattern to dermal MMP-1 staining. Prominent, spot-like, dermal MMP-9 staining was only detected in irradiated skin. Staining of MMP-8 and neutrophil elastase showed a similar spot-like pattern in irradiated skin (erythemogenic doses).
MMP-2 and MMP-12 staining could not be detected.
All images: original magnification x200.

Similar to collagenase activity, gelatinase activity was detected in all skin samples. Overall, gelatinase activity was more prominent than collagenase activity. Gelatinase activity was detected in the epidermis with increasing activity moving up from the basal keratinocytes and very prominent in the subcorneal area (Figure 4). The stratum

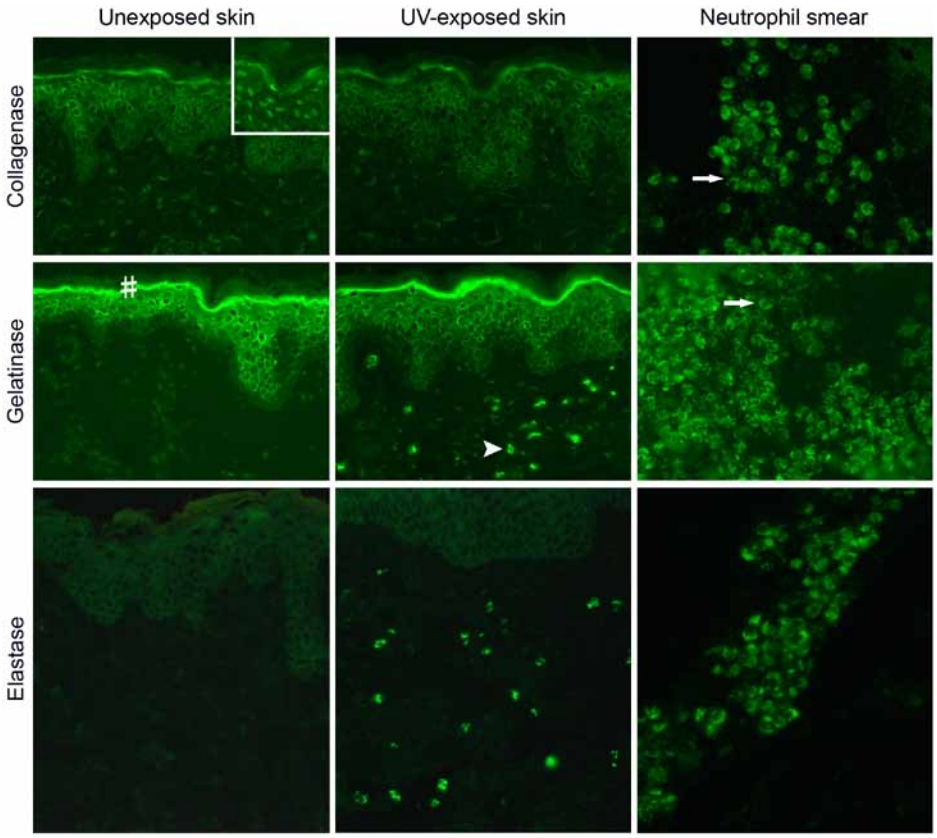

Figure 4. Collagenase, gelatinase and elastase *in situ* zymography. Collagenase activity was mainly detected in the epidermis and appeared to coincide with the MMP-1 and MMP-3 immunostaining. Collagenase activity was not influenced by SSR-exposure or skin color. Collagenase activity appeared to be located perinuclearly in most skin samples although numerous skin samples also showed intranuclear activity (top left, inset). Gelatinase activity was also clearly detected in the epidermis of all skin samples, with intense fluorescence in the subcorneal region (#). Elastase activity was not detected in unexposed skin or in any of the black skin samples. When comparing all skin samples, the most striking difference was the presence of prominent spot-like gelatinase (►) and elastase activity in skin that had been exposed to erythemogenic doses of SSR.
Neutropil smears also showed collagenase, gelatinase and elastase activity. Due to apparent cytoplasmic enzyme-activity, neutrophils could be recognized morphologically (→). Elastase activity was not influenced by a general MMP inhibitor, but could be completely inhibited by a specific inhibitor of neutrophil elastase in both skin sections and neutrophil smears. Collagenase and gelatinase activity could not be completely inhibited by the general MMP inhibitor and/or the neutrophil elastase inhibitor.
Original magnification x200 and x400.

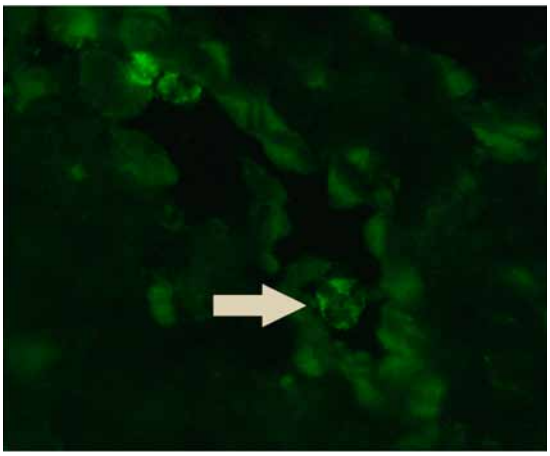

Figure 5. *x400* magnification of spot-like gelatinase activity in the dermis (see also Figure 4). The morphology of an intravascular neutrophilic granulcyte can be clearly recognized.

corneum itself, similar to collagenase, did not show gelatinase activity. Again, there was a clear demarcation between the epidermis and the dermis. In white skin exposed to erythemogenic doses of SSR, prominent spot-like gelatinase activity was detected in the mid and upper dermis. Greater magnification of the some of the spot-like centers of gelatinase activity in the dermis showed morphologically recognizable neutrophils, mostly when they were located intravascularly (Figure 5). A neutrophil smear also showed prominent gelatinase activity (Figure 4). Even more so than collagenase activity, gelatinase activity was not fully inhibited by 0.1 to 10 mM of 1,10-phenanthroline or by a combination of 1,10-phenanthroline and N-methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone.

Elastase activity was only detected in white skin exposed to erythemogenic doses of SSR. In these skin samples, spots of elastase activity were observed in the mid and upper dermis (Figure 4). As observed with gelatinase *in situ* zymography, some of these spots could be recognized as being neutrophils. Elastase activity appeared to be completely inhibited by N-methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone. Elastase activity was not inhibited by 1,10-phenanthroline.

Discussion

Immunohistochemistry, immunofluorescence and *in situ* zymography are extremely valuable tools to examine proteolytic enzymes *in vivo*. However, there are numerous potential confounding factors with respect to the execution of these techniques and the interpretation of their results.[25;26] For example, immunohistochemistry is prone to artefacts such as: folding of skin sections, loss of skin sections from the glass slides they are mounted on, loss of nuclei from the epidermis, variable staining, background staining, and contamination of antibodies. These confounding factors necessitate that the techniques are carried out

and interpreted by experienced laboratory personnel and researchers. A further potential confounding factor is obscuring of immunohistochemical staining by counterstaining. To create optimal conditions for immunohistochemical detection of MMPs in the present study, we omitted counterstaining with hematoxylin. Particularly when examining the epidermis, powerful counterstaining will obscure MMPs expressed in that compartment. Also, MMPs expressed in nuclei will be obscured by counterstaining.

Prior to immunostaining of skin samples we applied solubilized purified human MMPs directly onto glass slides and onto skin sections already mounted on the glass slides. This procedure allowed us to test the specificity of the purchased anti-MMP antibodies. The 'sticking' of externally applied purified MMP protein onto skin sections and glass slides suggests that MMPs generated *in vivo* by cells in the epidermis and dermis also remain *in situ* during the experimental procedures.

We have previously reported that MMP-1 positive cells infiltrate the skin following exposure to erythemogenic doses of SSR,[12;17] and we showed that these MMP-1 positive cells double-stained with neutrophil elastase.[12] This led us to conclude that neutrophils express MMP-1. In these experiments the anti-MMP-1 antibody batch obtained from Oncogene was used.[12;17] However, all experiments carried out thereafter with different anti-MMP-1 antibody batches failed to reproduce the staining of MMP-1 in association with skin-infiltrating neutrophils. Control experiments described in the present study indicate that the Oncogene batch used at the time cross-reacts with MMP-8 protein. Since neutrophils are known to express MMP-8[19] and infiltrate the skin following exposure to erythemogenic doses of SSR,[17] this explains our earlier inaccurate conclusion with respect to MMP-1 expression by neutrophils. The same anti-MMP-1 clone purchased from Calbiochem at a later stage did not cross-react with MMP-8 protein. For this reason, we believe that contamination of the anti-MMP-1 Oncogene batch with anti-MMP-8 antibodies must have occurred at our own laboratory.

Other immunohistochemical studies show upregulation of MMP-1 protein in the epidermis following exposure of the skin to UV.[2;27;28] In the present study we examined MMP-1 staining in young and elderly skin, black and white skin, sun-protected and sun-exposed skin, and SSR-exposed skin. As discussed above, we first confirmed that the anti-MMP-1 antibodies of different (newly purchased) clones actually react with MMP-1 protein and do not react with other MMP proteins. We observed MMP-1 staining of the epidermis in all skin samples and were not able to detect a consistent difference in staining intensity between the skin samples. Even exposure to erythemogenic doses of SSR appeared to have little effect on MMP-1 staining pattern or intensity. Similarly, collagenase activity of equal intensity was observed in the epidermis of all skin samples. Both MMP-1 protein-staining and collagenase activity showed a sharp demarcation between the epidermis and the dermis indicating that these proteins do not diffuse across this barrier. These findings question the hypothesis that keratinocyte-derived MMP-1

plays a major role in the pathophysiology of photoaging: i.e., question the hypothesis that keratinocyte-derived MMP-1 is primarily responsible for dermal changes observed in photoaged skin. Curiously enough, the data on which this hypothesis is originally based show that UV mainly induced MMP-1 protein in the epidermis while at exactly the same time-point collagenase activity (i.e., MMP-1 enzyme activity) was mostly detected in the dermis.[2]

The MMP-1 staining pattern of clone 41-1E5 differed from that of the other clones tested. Although all clones specifically reacted with MMP-1 protein, Clone 41-1E5 mainly stained the nuclei of epidermal cells. Nuclear staining of MMP-1 has in fact been reported by other authors who proposed that 'intracellular association of MMP-1 to nuclei and mitochondria confers resistance to apoptosis of cells'.[29] According to the manufacturers' product characteristics sheet, the MMP-1 epitopes of clone 41-1E5 and clone III7 differ and the epitope of clone SB12e is not specified. The manufacturers claim that clones 41-1E5 and III7 both recognize latent and active forms of MMP-1. The manufacturer of clone SB12e only mentions that it 'recognizes MMP-1'.

Another interesting observation is the eccentric staining of MMP-1 positive dermal cells. Perhaps it indicates the direction of travel of cells. Importantly, this pattern (and intensity) of MMP-1 staining was seen in all skin samples: young and elderly, black and white, sun-exposed and sun-protected, and SSR-exposed.

Parallel to MMP-1 we investigated the expression of MMP-2, -3, 8, 9 and 12.
Our control experiments clearly showed that anti-MMP-2 and MMP-12 antibodies were sensitive and specific for their respective proteins, but we were unable to detect MMP-2 or MMP-12 in any of the skin samples. The published images of UV-induced immunohistochemical staining of MMP-12 protein (an *in vivo* study) show very faint staining, and the data was not supported by positive control experiments.[23] Our data suggest a limited, if any, role for MMP-2 and MMP-12 in the pathophysiology of photoaging.
Similar to MMP-1, MMP-3 appeared to be constitutionally expressed in the skin and staining intensity appeared consistent in all investigated skin samples and independent of exposure to SSR. MMP-3 was mostly detected in the epidermis and there was a clear demarcation between the epidermis and the dermis. Therefore, it seems unlikely that MMP-3 is an important contributor to SSR-induced dermal damage.
The most striking observation with respect to changes in staining of MMPs in the different skin samples, was the presence of spot-like staining of MMP-8 and MMP-9 in the dermis of white skin exposed to erythemogenic doses of SSR. This spot-like staining was not observed in skin exposed to suberythemogenic doses of SSR or in any of the other skin samples. Neutrophil elastase, the immunohistochemical marker for neutrophils[13], was also only detected in white skin exposed to erythemogenic doses of SSR. Neutrophils are known to express both MMP-8 and MMP-9[19] and we have previously shown that SSR-induced MMP-8 and MMP-9 positive cells double-stain with neutrophil elastase.[12]

We therefore conclude that the above described spot-like staining of MMP-8 and MMP-9 co-localizes with neutrophils.

Although neutrophil smears demonstrated both collagenase and gelatinase activity, only striking gelatinase activity was observed in the dermis of skin sections obtained from white skin exposed to erythemogenic doses of SSR. This suggests that MMP-8 present in skin-infiltrating neutrophils is enzymatically inactive or less active. Neutrophil smears also demonstrated elastase activity and elastase activity was clearly observed in the dermis obtained from white skin exposed to erythemogenic doses of SSR: i.e., dermal elastase activity was detected in the same skin sections that showed prominent dermal gelatinase activity. Elastase activity in these skin sections appeared to be completely inhibited by N-methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone (an inhibitor of neutrophil elastase). These findings suggests that neutrophil elastase is solely responsible for the observed elastase enzyme activity, and is not caused by MMPs with elastolytic properties. Collagenase activity and particularly gelatinase activity could not be fully inhibited by 1,10-phenanthroline (a general MMP inhibitor) or by a combination of 1,10-phenanthroline and N-methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone. This demonstrates the drawback of these assays when performed *in vivo*: i.e., next to MMPs and neutrophil elastase[30] other proteolytic enzymes can demonstrate collagenase and/or gelatinase activity.[31] For example, dermal gelatinase activity observed in SSR-exposed skin may partly be due to neutrophil-derived Cathepsin G, a proteinase with gelatinolytic properties that is not inhibited by 1,10-phenanthroline or N-methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone.[32]

Taken together, our findings imply that the contributing role of MMPs to the extracellular matrix damage observed in photoaged skin may actually be quite limited. We would argue that: (1) even if MMP-1, -3 and -9 are upregulated in the epidermis following UV exposure as demonstrated by other authors, it is highly unlikely that these MMPs massively cross the dermal-epidermal junction; (2) since MMP-1 was similarly expressed in black and white skin, and black skin is much less prone to photoaging, a major role of MMP-1 in photoaging is questionable; (3) since MMP-2 and MMP-12 were not detected in any of the investigated skin samples, their role in photoaging cannot be very important; and (4) since MMP-8 appears to be enzymatically inactive in skin-infiltrating neutrophils its role in photoaging is doubtful. Only MMP-9 and neutrophil elastase and their related enzyme activities were clearly induced by SSR in the mid and upper dermis: i.e., the compartment where damaged collagen and elastic fibers are observed in photoaged skin.

In summary, with respect to the investigated MMPs, only neutrophil-derived MMP-9, is likely to be a major contributor to SSR-induced collagen damage. Neutrophil elastase, and not MMPs with elastolytic properties, is likely to be a major contributor to SSR-induced elastic fiber damage.

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Conflict of interest

The authors state no conflict of interest.

After the study was initiated Dr. Piet L.B. Bruijnzeel became employed at Organon now Merck, Sharp & Dohme, Department of Translational Medicine, ECREM, Oss, the Netherlands. Recently he has accepted a position at AstraZeneca as Medical Science Director, Loughborough (Charnwood), United Kingdom. Organon nor Merck, Sharp & Dohme nor AstraZeneca have contributed to this manuscript, either financially or otherwise. Dr. Piet L.B. Bruijnzeel holds an honorary research position at the Department of Dermatology of the University Medical Center Utrecht.

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Chapter 5

Solar elastosis results from elastic fiber degradation and can be induced by neutrophil elastase and reactive oxygen species: an immunohistochemical study of black and white skin

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Submitted

Abstract

Background Solar elastosis is the hallmark of photoaged skin. Most authors will agree that the elastotic material observed in photoaged skin is mainly derived from degraded elastic fibers. Nevertheless, a widely accepted hypothesis regarding the pathophysiology of photoaging focuses on collagen fiber degradation. This appears to be contradictory. Black skin is less prone to solar elastosis and photoaging than white skin.
Objectives (a) To investigate the extracellular matrix (ECM) changes in photoaged skin (*in vivo*), and (b) to investigate the effect of neutrophils, neutrophil elastase, hydrogen peroxide and various ultraviolet radiation (UV) sources on the ECM *ex vivo.*
Methods (a) We compared ECM components in sun-exposed and sun-protected, young and elderly, black and white skin. Biopsies taken were immunohistochemically stained for elastin, fibrillin-1 and collagen types I, III, IV and VII. (b) We exposed skin sections to neutrophils isolated from peripheral blood, pus, neutrophil elastase, hydrogen peroxide (a source of reactive oxygen species), and various sources of ultraviolet radiation. Skin sections thus exposed were immunohistochemically stained for elastin.
Results (a) The most striking observation was intense elastin staining in the mid and upper dermis of sun-exposed, elderly, white skin. Elastin staining of sun-exposed, elderly, black skin was slightly increased. Elastin staining of all other skin samples was minimal and showed a characteristic pattern confirming the concept that normal elastic fibers consist of a core of elastin surrounded by a mantle of fibrillin-rich microfibrils. (b) Exposing skin sections to neutrophils isolated from peripheral blood, pus, neutrophil elastase, hydrogen peroxide, and various sources of UV: to different degrees all but the last induced an elastin staining pattern similar or identical to that observed in photoaged skin.
Conclusions Our results suggest that solar elastosis is indeed an end-product of elastic fiber degradation, and that neutrophil-elastase and reactive oxygen species are capable of inducing solar elastosis. We hypothesize that during photoaging part of the fibrillin-rich mantle of elastic fibers is degraded, thereby exposing insoluble elastin. Since elastin is highly resistant to proteolysis, it accumulates, leading to solar elastosis.

Introduction

'Solar elastosis' is the hallmark of photoaged skin and is often used synonymously with 'photoaging'. Photoaging is the process whereby natural sunlight or artificial ultraviolet radiation (UV) sources gradually induce skin changes. When examining the literature on the histopathology and pathofysiology of photoaging, there appears to be a contradiction: although histologically elastic fiber changes are most conspicuous, the pathophysiology focuses mainly on collagen fiber degradation.

Photoaged skin is characterized by a loss of elasticity, rough texture, irregular pigmentation, telangiectasia, plaque-like thickening and coarse wrinkles.[1] The rough texture and irregular pigmentation of photoaged skin are mostly due to changes in the epidermis. Histologically, the epidermis may show the following features: hyperplasia or atrophy; disappearance of dermal papillae; thickening of the basement-membrane; focally increased numbers and irregular distribution of melanocytes and melanosomes; atypical keratinocytes; parakeratosis; and thickening of the stratum corneum.[1]

The most conspicuous defect in the dermis is the presence of elastotic material in the mid and upper dermis, so-called 'solar elastosis' or 'basophilic degeneration'. Fully developed photoaged skin shows alternating areas of fibrous, granular and homogenous elastotic material.[2-4] Its origin has been subject of much debate.[5] Hypotheses include: (1) that the elastotic material is derived from elastic fibres;[3] (2) that it is a degradation product of collagen fibres;[6] (3) that it is a degradation product of both collagen and elastic fibres;[7] and (4) that it is an abnormal product of UV-stimulated fibroblasts.[2;8;9] Braverman and Fonferko[2] observed decreased amounts of collagen in areas of elastosis, but found no morphological evidence that collagen degeneration participated in the production of the elastotic material. Based on electron-microscopic studies and on immunohistochemical staining patterns of multiple markers of the extracellular matrix, several groups have concluded that the elastotic material in photoaged skin must be derived primarily from degenerated elastic fibres.[3;10]

Other observations in the dermis of photoaged skin include a decrease in the total amount of collagen, disorganized collagen fibers, increased amounts of ground substance and dilated blood vessels.[11]

A widely accepted hypothesis regarding the pathophysiology of photoaging focuses on collagen fiber degradation. Herein, UV-induced, fibroblast- and keratinocyte- derived matrix metalloproteinases (MMPs) are responsible for the clinical and histological signs of photoaged skin.[12;13] More recently, we have proposed that, together with directly induced damage to the extracellular matrix (ECM) by UV-induced reactive oxygen species (ROS), infiltrating neutrophils could play an important role in the pathophysiology of photoaging.[14-17] Neutrophils infiltrate the skin following a certain threshold of UV-damage to the skin. This threshold is equivalent to the minimal erythema dose (MED).[14;15] For

Table 1*. Skin phototypes (SPT) I-VI according to Fitzpatrick, constitutive skin color, UVB minimal erythema dose (MED), and susceptibility to sunburn and photoaging

SPT	Constitutive skin color	MED (mJ/cm²)	Sunburn susceptibility	Photoaging
I	white	15-30	high	early onset; strong
II	white	25-40	high	early onset; strong
III	white	30-50	moderate	moderate to strong
IV	olive	40-60	low	moderate to low
V	brown	60-90	very low	slow; low
VI	black	90-150	very low	slow; minimal

*Adapted from (1) Fitzpatrick TB. Editorial: The validity and practicality of sun-reactive skin types I through VI. Arch Dermatol 1988, 124: 869–871, and (2) Taylor SC. Skin of color: Biology, structure, function, and implications for dermatologic disease. J Am Acad Dermatol 2002, 46: S41–S62.

persons with skin phototypes (SPT) I-II, who are particularly susceptible to sunburn and photoaging, this threshold can be reached in less than 10 minutes of sun exposure (Table 1). Skin-infiltrating neutrophils are packed with potent proteolytic enzymes capable of damaging both collagen and elastic fibers.[18-21] Neutrophil-derived proteolytic enzymes are associated with the ECM damage observed in lung emphysema,[22] rheumatoid arthritis,[23] wound infection[24] and other conditions.[25;26] Neutrophil elastase, an important immunohistochemical marker, is a particularly potent protease that has been described as a major contributor to solar elastosis in a mouse study.[27] The above-mentioned ROS can be UV-induced,[28] but can also be neutrophil-derived.[21] ROS generated by neutrophils are involved in neutralizing plasma-derived anti-proteinases and activating neutrophil-derived proteases.[21]

Most studies that have investigated skin changes in photoaged skin involve Caucasian subjects (SPT I-IV). Black skin (SPT VI) is better protected against sunburn and photoaging than white skin.[29] We have previously shown that a significantly higher dose of UV is necessary to induce a neutrophilic infiltrate in black skin.[14] This finding supports our hypothesis that neutrophils may be important contributors to the pathophysiology of photoaging.

In the present study we compared sun-exposed and sun-protected, young and elderly, black and white skin, focusing on elastic- and collagen-fiber components. We further investigated whether neutrophils, neutrophil elastase, hydrogen peroxide (a source of ROS), and various UV-sources can induce solar elastosis-like defects *ex vivo*.

Materials and Methods

Volunteer inclusion

Four groups of healthy volunteers were recruited: young white-skinned (aged 18-26 years), young black-skinned (aged 20-28 years), elderly white-skinned (aged 56-63 years) and elderly black-skinned (aged 57-63). In total, twenty-seven persons were recruited. Volunteer characteristics are summarized in Table 2. The study protocol was approved by the medical ethics committee of the University Medical Center Utrecht. All volunteers gave their written informed consent.

Table 2. Volunteer characteristics

Group	No. of volunteers	Gender M/F	Age group	Skin color	SPT	Mean age (years)	SEM
I	7	5M/2F	young	white	I-III	21.3	0.92
II	5 + 3*	6M/2F	young	black	VI	22.9	0.64
III	7**	4M/3F	elderly	white	I-III	59.0	0.87
IV	5	3M/2F	elderly	black	VI	60.5	1.23

*dorsal forearm skin- and buttock skin-biopsies were obtained from respectively three and five separate volunteers in this group. **additional biopsies were obtained from neck skin from three volunteers.
M, male; F, female; SPT, skin phototype; SEM, standard error of the mean.

Skin-biopsy collection and processing

Two 4 mm biopsies were taken from sun-exposed dorsal forearm skin and sun-protected buttock skin of each volunteer, except from volunteers in the young black-skinned group where dorsal forearm skin and buttock skin biopsies were obtained from respectively three and five separate volunteers. Two additional 3 mm biopsies were taken from three of the elderly white-skinned volunteers: from the centre of a wrinkle and adjacent skin in the neck region. Mamma reduction skin was obtained from the plastic surgery department. Skin samples obtained were snap frozen in liquid nitrogen, embedded in Tissuetek (Sakura Finetek Europe, Zoeterwoude, the Netherlands) and stored at -80°C. Six-micrometer skin sections were cut using a freezing microtome and mounted on Superfrost® Plus coated slides (Thermo scientific, Waltham, MA, USA).

Neutrophil isolation

Neutrophils were isolated from healthy donor blood obtained from the donor service of the University Medical Center Utrecht. The following steps were carried out: addition of 0.4% (w/v) trisodium citrate (pH 7.4) to anticoagulate the blood; centrifugation over isotonic Ficoll (Pharmacia, Uppsala, Sweden) to remove mononuclear cells; lysis of erythrocytes in an isotonic NH_4Cl solution; and, finally, washing and resuspension of neutrophils in incubation buffer (20 mM Hepes, 132 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1.2 mM

KH_2PO_4, 5 mM glucose, 1 mM $CaCl_2$ and 0.5% human serum albumin). Subsequently, neutrophils were spinned down (1000 RPM for 5 minutes). A highly concentrated suspension of 100 µl of buffer containing $5x10^6$ neutrophils was used in the experiments. Neutrophils were activated with 0.001 mM N-formyl-methionyl-leucyl-phenylalanine (fMLP) (Sigma, Saint Louis, MI, USA), 30 minutes incubation time at room temperature.

Pus collection

Pus was collected from patients with ectopic acne during deroofing procedures in the groin area. Freshly obtained pus consisting mainly of neutrophils, was diluted 1:5 in phosphate buffered saline solution (PBS), ready for immediate use.

Immunohistochemistry

Immunohistochemical single staining procedures were carried out using the following monoclonal antibodies: mouse anti-human collagen type I (Sigma; diluted 1:2000); mouse anti-human collagen type III (Sigma; diluted 1:4000); mouse anti-human collagen type IV (Novus Biologicals, Littleton, CO, USA; diluted 1:20); mouse anti-human collagen type VII (Novus Biologicals; diluted 1:10); mouse anti-human fibrillin-1 (Southern Biotechnology Associates Inc, Birmingham, AL, USA; diluted 1:100); and mouse anti-human elastin (Sanbio bv, Uden, the Netherlands; diluted 1:100). Skin sections were fixed in dry acetone containing 0.02% H_2O_2 (7 min) and preincubated with 10% normal human serum (NHuS)/10% normal horse serum/10% bovine serum albumin (BSA) (20 min). Subsequently, the skin sections were incubated with the above-listed primary antibodies diluted in 1% NHuS/1% BSA (60 min), followed by a washing step in PBS containing 0.05% Tween (3 x 3 min). Next, the skin sections were incubated with biotinylated horse anti-mouse immunoglobulin (Vector, Burlingame, CA, USA; diluted 1:300 in 1% NHuS/1% BSA) (30 min), again followed by a rinsing step. The final steps entailed incubation with preformed avidin and biotinylated horseradish peroxidase macromolecular complex (Vector, ABC kit) (45 min), washing in PBS 0.05% Tween, and demonstration of peroxidase activity using aminoethylcarbazole as enzyme substrate (Sigma, AEC chromogen kit) (± 10 min).

For each staining procedure relevant controls including isotype controls and omission of the primary antibodies were performed. Counter-staining was performed with Mayer's hematoxylin or, to prevent obscuring of the primary stain and to facilitate digital imaging analysis, not carried out.

Effect of neutrophil elastase, isolated neutrophils, pus, and hydrogen peroxide on elastin staining

Skin sections from mamma reduction skin were incubated with different concentrations of purified human neutrophil elastase (Bio connect, Huissen, the Netherlands), human

peripheral blood neutrophils, pus, and 30% hydrogen peroxide in water (Sigma) for 2 hours at room temperature. The first three in a humidified chamber, the last (hydrogen peroxide) exposed to daylight. Next, the slides were thoroughly rinsed in PBS (3 x 3 min), left to dry in air and skin sections fixed with dry acetone. Subsequently, immunohistochemical staining of elastin was performed as described above. The experiments were repeated at least three times to confirm the results. Neutrophil elastase was diluted in TRIS buffer (50 mmol TRIS-HCl, 150 mmol $CaCl_2$, 0.2 mmol NaN_3, pH 7.6) to a concentration ranging from 40 μg/ml (diluted 1:5) to 0.2 μg/ml (diluted 1:1000). Individual skin sections were incubated with 10 μl of these dilutions. The average concentration of neutrophil elastase in a neutrophil is 1.59 pg[30]. This means that 10 μl of 40 μg/ml neutrophil elastase is equivalent to the amount of neutrophil elastase in approximately 250 000 neutrophils. 0.1 mM of N-methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone (Molecular Probes, Eugene, OR, USA) was used to inhibit elastase activity. 1,10-phenanthroline, 1.0 mM, was used as a general metalloproteinase inhibitor (Molecular Probes). Multiple control experiments were performed in which different steps in the experimental procedures were omitted. Resulting elastin staining was compared with elastin staining observed in sun-exposed elderly white (i.e., photoaged) skin.

Effect of broad band UVB, narrow band UVB, solar-simulating radiation (SSR) and UVA1 on elastin staining

To investigate the direct effect of different wavelength ranges of UV on elastin staining, skin sections from mamma reduction skin were directly exposed to four different UV sources with doses approximating 1 to 10 MED of persons with SPT III. Irradiance was monitored using a UV detector device (Waldmann, Schwenningen, Germany). The UV sources, their emission spectra and/or peak emission, and the physical doses to which skin sections were exposed were as follows: **(1)** Broadband UVB: TL12 (Philips, Eindhoven, the Netherlands); wavelength range 280-340 nm, 58% in the UVB range, peak emission around 310 nm; skin sections exposed to 50 and 500 mJ/cm^2. **(2)** Narrow band UVB: TL01 (Philips); 88% in the UVB range, peak emission around 311/312 nm; skin sections exposed to 500 and 5000 mJ/cm^2 **(3)** SSR: CLEO natural (Philips); wavelength range 290-420 nm, peak emission around 355 nm; skin sections exposed to 5000 and 50 000 mJ/cm^2. **(4)** UVA1: Sellamed 3000W (Sellamed, Gevelsberg, Germany); wavelength range 340-420 nm, this lamp does not emit UVB; skin sections exposed to 25 000 and 250 000 mJ/cm^2. The spectral distribution measurements were performed with a calibrated standard UV-spectrometer (model 752, Optronic laboratories, Orlando, FL, USA).

It is important to realize that since UV is not filtered by the different layers of the epidermis in these experiments, the biological dose of UV to which the ECM was exposed is, in fact, much higher than when the ECM is exposed to UV by irradiating intact skin.

Following irradiation, staining procedures of elastin was performed as described above.

Microscopic evaluation and statistical analysis

Immunohistochemically stained skin sections were examined using a light microscope at 200x, 400x and 1000x magnification. Staining intensity was determined using AxioVision 4.0 software (Carl Zeiss Vision Imaging System, Thornwood, NY, USA).

Statistical analysis was performed using GraphPad Prism5 software (GraphPad software Inc, CA, USA). To compare staining intensity in the different groups a Kruskal-Wallis test with Dunn's post-hoc multiple comparison test was performed. P values < 0.05 were considered significant.

Results

Elastin, fibrillin-1, collagen types I, III, IV and VII staining

Figures 1A and 1B demonstrate immunohistochemical staining of elastin, fibrillin-1, and collagen types I, III, IV and VII in young and elderly, black and white, sun-protected buttock and sun-exposed forearm skin.

The most striking observation was intense elastin staining in the mid and upper dermis of elderly white forearm skin (graphically demonstrated in Figure 2). Additional biopsies taken from the centre of a wrinkle in the neck of three elderly white volunteers showed a similar albeit even more intense elastin staining pattern as compared to their forearm skin. Staining with Mayer's hematoxylin showed 'basophilic degeneration' in photoaged neck skin (Figure 1C, panel a). Counterstaining elastin-stained skin sections showed that the elastin-positive material is superimposed by Mayer's hematoxylin stain (Figure 1C, panel b).

Elastin staining of elderly black forearm skin showed some increase in elastin staining (Figure 2).

Subepidermal fibrillin-1 staining was dependent on the location of the biopsy taken (Figures 1A and 1B). Subepidermal fibrillin-1 positive fibers of buttock skin were long and thick while those of forearm skin were short and slender. The latter observation was most distinct in elderly white forearm skin, followed by young white forearm skin, elderly black forearm skin and young black forearm skin. Subepidermal fibrillin-1 positive fibers of neck skin were noticeably longer and thicker than subepidermal fibrillin-1 positive fibers of forearm skin within the same volunteer (Figure 3). In the biopsies taken from neck skin, mid and upper dermal (excluding the grenz zone) fibrillin-1 staining appeared to be increased and fibrillin-1 positive fibers showed fragmentation. This increase in fibrillin-1 staining and fragmentation of fibrillin-1 positive fibers was not evident in elderly white forearm skin.

Striking differences with respect to collagen types I and III staining were not found between the groups. However, in the biopsies taken from neck skin collagen types I and III staining in the mid dermis appeared to be reduced in areas where elastin staining was particularly intense (Figure 1C, panel d). In elderly white forearm and neck skin

the grenz zone showed normal collagen I and III staining while this zone was devoid of elastin staining and fibrillin staining appeared reduced.

No differences were observed with respect to collagen types IV and VII staining. Collagen type IV was detected in the subepidermal-, perivascular- and periadnexal-basement membrane zones in all biopsies taken. Collagen type VII staining was consistently limited to the subepidermal zone.

Although not specifically investigated in this study, the observed differences in skin pigmentation are quite illustrative (Figures 1A and 1B). The epidermis of both black buttock and black forearm skin was heavily pigmented. Melanin pigment was concentrated at the level of the basal keratinocytes. In white-skinned volunteers, within the same individual, buttock skin could be differentiated from forearm skin by the increased pigmentation of basal keratinocytes in the latter. Furthermore, buttock skin could be differentiated from forearm skin in both black and white skin by more prominent rete ridges in buttock skin, and by the earlier mentioned subepidermal fibrillin-1 staining pattern.

Elastin staining following incubation of skin sections with neutrophil elastase, isolated neutrophils, pus, and 30% hydrogen peroxide

Skin sections exposed to purified human neutrophil elastase, neutrophils isolated from peripheral blood, diluted pus and 30% H_2O_2 showed a significant increase in elastin staining compared to untreated control skin sections (Figures 4A and 4B). Neutrophil elastase-induced elastin staining was dose dependent (Figure 4B, $p < 0.05$ by linear regression). The effect of neutrophil elastase and isolated neutrophils (both fMLP activated neutrophils and not specifically activated neutrophils) on elastin staining was inhibited by 0.1mM N-methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone (inhibitor of elastase activity). In a control experiment 0.1 mM N-methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone did not inhibit peroxidase-aminoethylcarbazole interaction and staining. The effect of neutrophil elastase and isolated neutrophils (both fMLP activated and not specifically activated) on elastin staining appeared not to be influenced by 1.0 mM 1,10-Phenathroline (a general inhibitor of metalloproteinase activity).

Although, as expected, hydrogen peroxide is capable of directly inducing aminoethylcarbazole staining, control experiments demonstrated that thorough rinsing of skin sections (3 x 3 minutes in PBS) after the 2 hour incubation period with 30% hydrogen peroxide excluded this effect. The same applied to isolated neutrophils (both fMLP activated and not specifically activated) and pus, which also naturally exhibit peroxidase activity. As further expected, neutrophil elastase could not directly induce aminoethylcarbazole staining.

Exposing black skin to purified human neutrophil elastase, neutrophils isolated from peripheral blood, diluted pus and 30% H_2O_2 induced an identical elastin staining pattern as compared to white skin exposed to these products (and a similar pattern as seen in photoaged white skin).

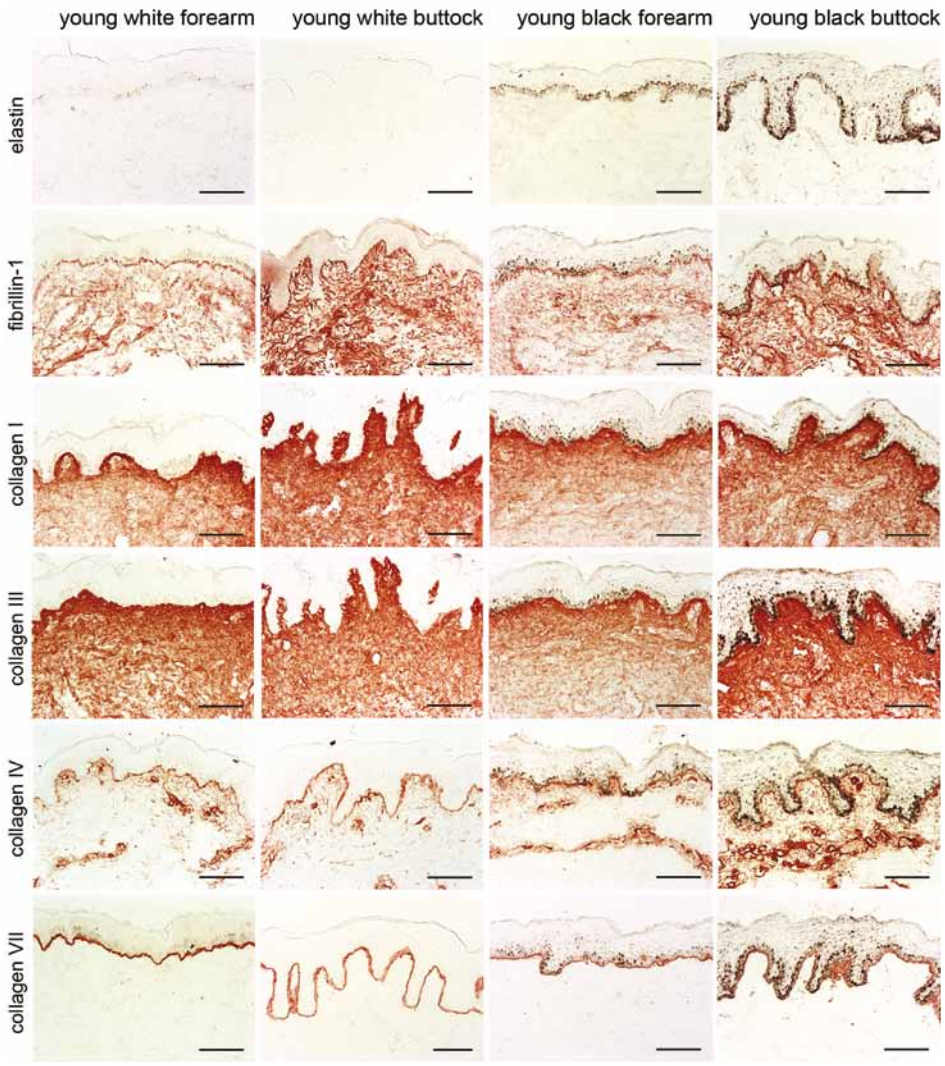
young white forearm
young white buttock
young black forearm
young black buttock
elastin
fibrillin-1
collagen I
collagen III
collagen IV
collagen VII

Figure 1A

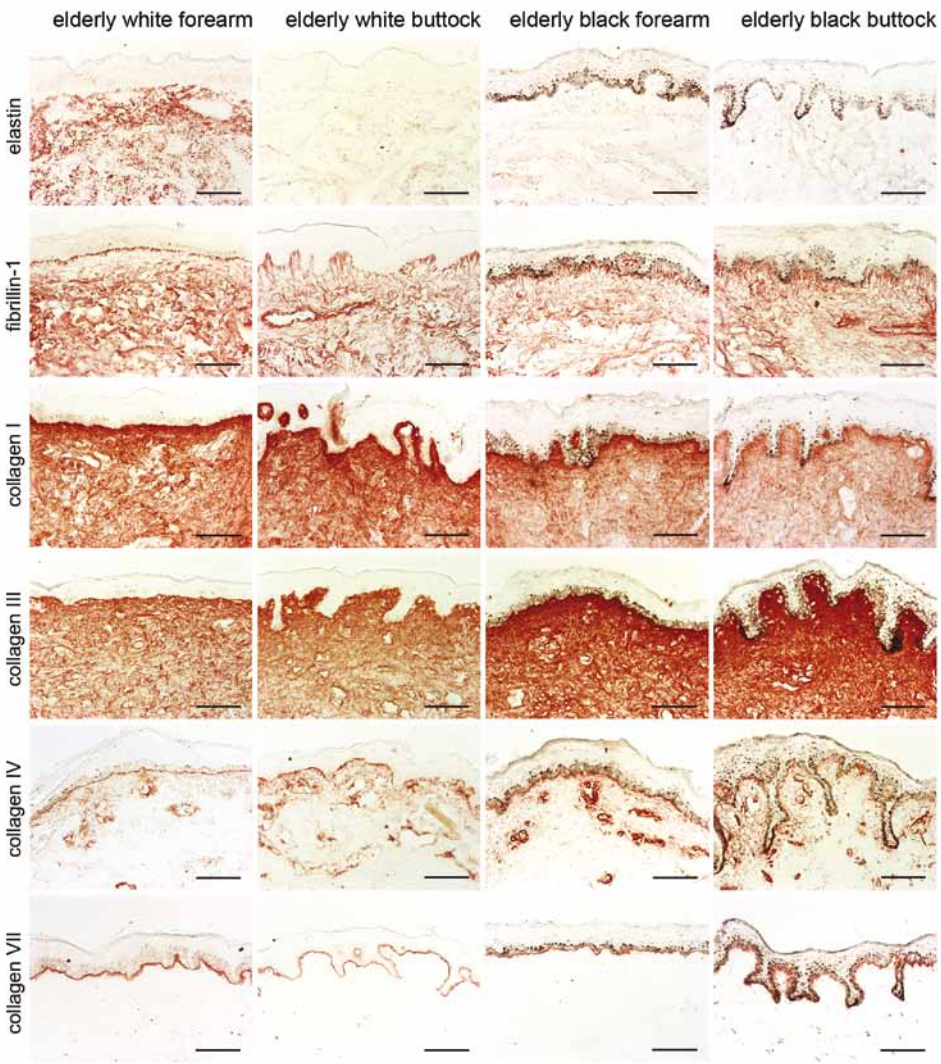
elderly white forearm
elderly white buttock
elderly black forearm
elderly black buttock
elastin
fibrillin-1
collagen I
collagen III
collagen IV
collagen VII

Figure 1B

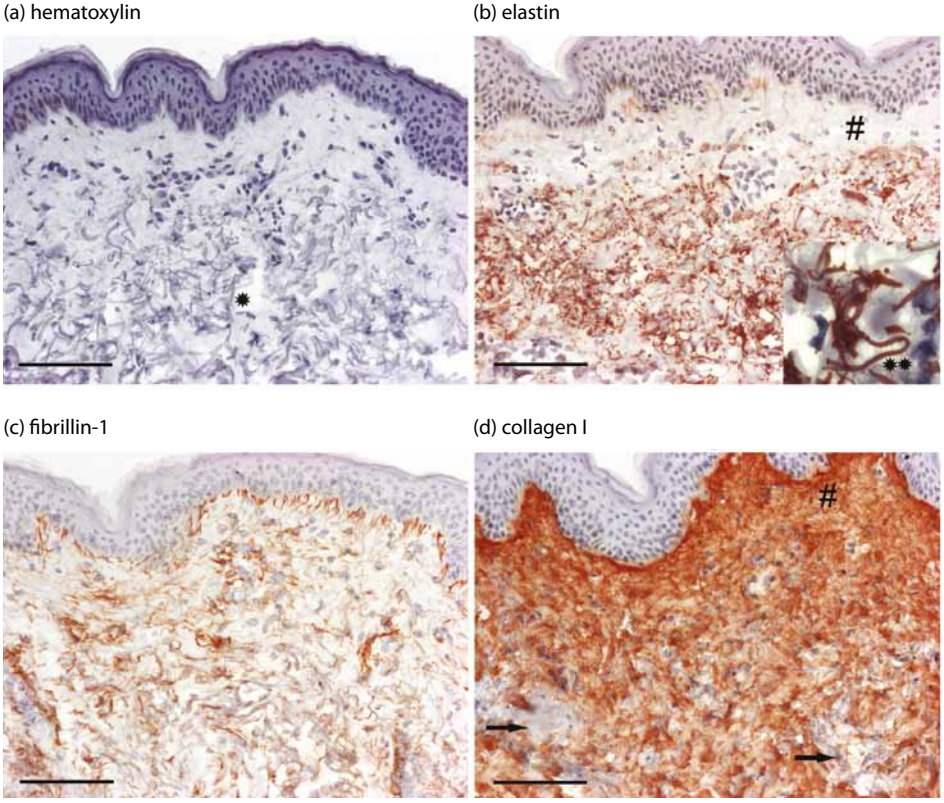

Figure 1C

Figures 1A, 1B and 1C. Immunohistochemical staining of elastin, fibrillin-1, collagen types I, III, IV and VII of biopsies taken from sun-exposed (dorsal forearm) and sun-protected (buttock) skin of young and elderly, black and white volunteers (Figures 1A and 1B). The most striking observation was intense elastin staining of the mid and upper dermis in elderly white forearm skin, reflecting solar elastosis (Figure 1B, top left hand corner). A similar but even more intense elastin staining pattern was observed in biopsies taken from the centre of a wrinkle in the neck of three elderly white volunteers (Figure 1C, panel b). Basophilic material (*) is observed in the mid and upper dermis of neck skin sections stained with Mayer's hematoxylin (Figure 1C, panel a). Basophilic hematoxylin stain superimposes the increased immunohistochemically stained elastin protein (** Figure 1C, panel b, inset: *400x magnification*). Mid and upper dermal fibrillin-1 staining (excluding the grenz zone) also appeared to be increased in photoaged neck skin (Figure 1C, panel c).
Striking differences in collagen staining between the groups were not observed (Figures 1A and 1B). However, in biopsies obtained from the neck region, collagen I (and III, not shown) staining did appear to be reduced, disorganized and fragmented in areas where elastin staining was particularly intense (Figure 1C, panel d, →). Notice that in photoaged skin (elderly white arm and neck skin) the grenz zone shows normal collagen I and III staining while it is relatively devoid of elastin staining and fibrillin-1 staining is reduced (grenz zone marked with # in Figure 1C, panels b and d). *Scale bars: 1A, 1B and 1C: 100 µm.*

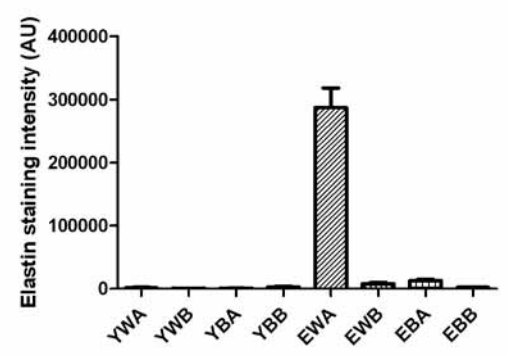

Figure 2. Elastin staining in sun-exposed and sun-protected, young and elderly, black and white skin. Mean elastin staining intensity determined by computer-assisted analysis of digital images captured. Strongly increased elastin staining in elderly white forearm skin was statistically significant, $p<0.05$.
YWA, young white arm; YWB, young white buttock; YBA, young black arm; YBB, young black buttock; EWA, elderly white arm; EWB, elderly white buttock; EBA, elderly black arm; EBB, elderly black buttock; AU, arbitrary units; Error bars, standard error of the mean.

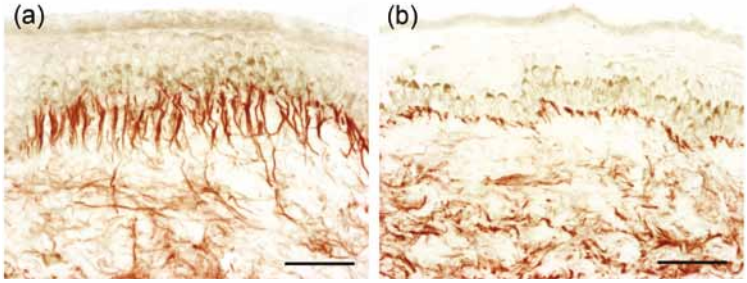

Figure 3. Fibrillin-1 staining of photoaged neck and photoaged dorsal forearm skin within the same individual. Subepidermal fibrillin-1 positive fibers of neck skin (panel a) were noticeably longer and thicker than subepidermal fibrillin-1 positive fibers of forearm skin (panel b). *Scale bars: 50 µm.*

Elastin staining following direct exposure of skin sections to broad band UVB, narrow band UVB, SSR and UVA1

The elastin staining pattern was not affected by irradiation with the different UV sources at any dose.

High magnification of elastin staining: pattern observed in non-photoaged skin and similarity between elastin staining of photoaged skin and neutrophil elastase exposed non-photoaged skin

Photoaged elderly white forearm and neck skin showed intense elastin staining. Elastin staining of young, black or white, sun-protected or sun-exposed skin, and elderly, black or white, sun-protected skin was much more subtle: when examined at high magnification, minimally to non-staining, rod-like structures with stained tips were observed

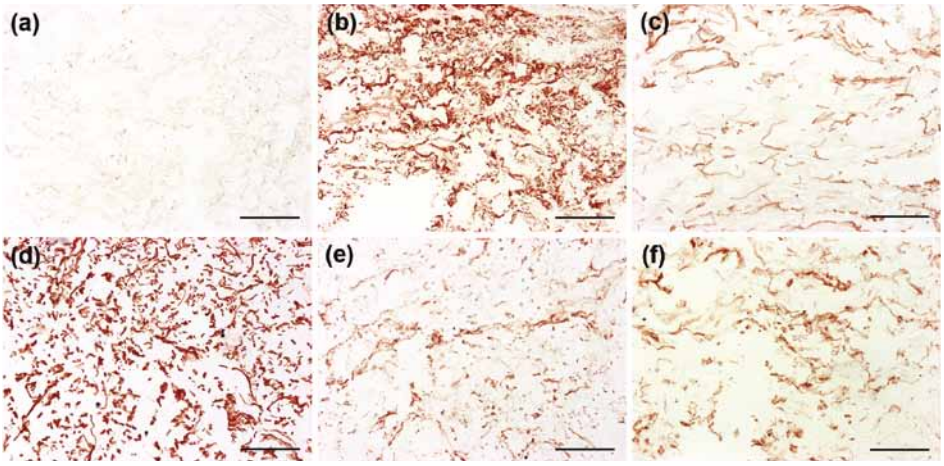

Figure 4A. Elastin staining induced by hydrogen peroxide, neutrophil elastase, isolated neutrophils and pus. Incubating 6 µm skin sections obtained from mamma reduction skin with 30% hydrogen peroxide (panel c), 40 µg/ml neutrophil elastase (panel d), isolated neutrophils (panel e) and diluted pus (panel f) for 2 hours at room temperature induced an elastin staining pattern resembling that observed in photoaged skin. Panel (a): untreated mamma reduction skin. Panel (b): sun-exposed elderly white (i.e., photoaged) skin. *Scale bars: 100 µm.*

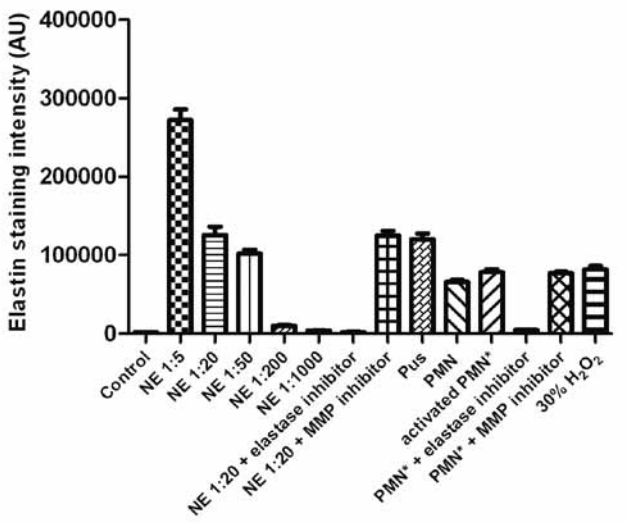

Figure 4B. Intensity of elastin staining following incubation of 6 µm skin sections with hydrogen peroxide, neutrophil elastase, isolated neutrophils, and pus determined by computer-assisted analysis of digital images. Neutrophil elastase-induced elastin staining was dose dependent ($p < 0.05$ by linear regression). The effect of neutrophil elastase (diluted 1:20) and isolated, fMLP-activated neutrophils on elastin staining was inhibited by 0.1 mM N-methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone, an inhibitor of elastase activity. The effect of neutrophil elastase (diluted 1:20) and isolated, fMLP-activated neutrophils on elastin staining was not inhibited by the general metalloproteinase inhibitor 1,10-Phenathroline (1.0 mM).
AU, arbitrary units; Error bars, standard error of the mean.

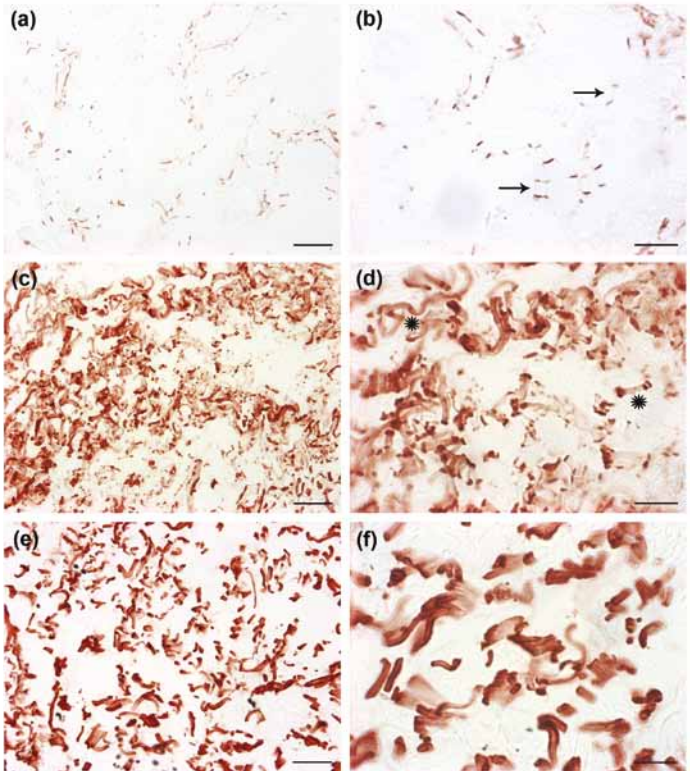

Figure 5. High magnification (*400x and 1000x*) of the elastin staining pattern in sun-protected skin, photoaged skin and skin sections exposed to neutrophil elastase. In sun-protected skin (panels a and b) minimally or non-stained, rod-like structures with stained tips were observed (→). The length of the rod-like structures approached the thickness of the cut skin sections (i.e., 6 µm). In photoaged skin (panels c and d) and skin sections exposed to neutrophil elastase (panels e and f) similar rod-like structures were observed, but here elastin staining was not limited to the tips of the rod like structures: in photoaged and neutrophil elastase exposed skin the entire rod like structure was stained (*). *Scale bars: (a), (c) and (e): 30 µm, (b), (d) and (f): 15µm.*

(Figure 5, panels a and b). The intensity of the dot-like elastin staining pattern observed in non-photodamaged skin varied independently of the 'group' (young, elderly, etc.).

Figure 5, panels c-f demonstrate the similarity in elastin staining pattern between photoaged skin and skin sections exposed to neutrophil elastase.

Discussion

Solar elastosis is the hallmark of photoaged skin. The source of this 'elastotic material' in the mid and upper dermis of photoaged skin has been subject of much debate. Here we provide compelling evidence that it is a degradation product of elastic fibers, and that it

can be induced by neutrophil elastase and ROS. Furthermore, our findings confirm that black skin is less susceptible to solar elastosis/photoaging.

Significantly increased elastin staining in photoaged skin has been described by numerous authors.[2;3;10;31-35] Likewise, we observed intense elastin staining in sun-exposed elderly white skin. Furthermore, we demonstrated that the typical basophilic material observed in photoaged skin (skin sections stained with Mayer's hematoxylin) is the same material we stained with specific anti-elastin antibodies (Figure 1C, panels a and b), i.e., elastin protein appears to be a major component of the elastotic material observed in photoaged skin. Therefore we conclude that increased elastin-staining is a primary marker for photoaged skin.

Although solar elastosis has also been described in black skin[8] and our elderly black sun-exposed skin samples did show some increase in elastin-staining, this increase was insignificant compared to elderly white sun-exposed skin. Thus, in the elderly group, histologically, black skin shows less signs of photoaging.

Elastin staining of young, black or white, sun-protected or sun-exposed skin, and elderly, black or white sun-protected skin, was very subtle compared to sun-exposed elderly white skin: when examining the elastin-stained skin sections more closely, minimally to non-staining, rod-like structures with stained tips were observed (Figure 5, panels a and b). This observation appears to confirm that, broadly speaking, elastic fibers consist of a core of elastin surrounded by a mantle of fibrillin-rich microfibrils.[2;36] When elastic fibers are cut perpendicular to their long axis, the core of the elastic fiber becomes visible and elastin staining shows the characteristic dot-like pattern observed in Figure 5, panels a and b.

We have shown that exposure of skin sections to neutrophil elastase, isolated neutrophils, pus and hydrogen peroxide induces an elastin staining pattern similar to that observed in photoaged skin (Figure 5). Based on these results, we propose the following mechanism: neutrophil elastase and ROS break down the fibrillin-rich microfibrillar mantle of elastic fibers, thereby exposing elastin epitopes (Figure 6). Exposed, cross-linked, insoluble elastin is highly resistant to further proteolysis[37] and gradually accumulates in the skin. Although less resistant to proteolysis than elastin, partially degraded fibrillin-rich microfibrils may also contribute to the accumulation of elastotic material in photoaged skin. Similar to wound healing where repair of the elastic fiber network has been described,[38] a new elastic fiber network may be formed following UV damage. However, this repair process is probably slow and already accumulated elastin may structurally interfere with elastic (and collagen) fiber network regeneration. Newly formed elastic fibers can in turn be degraded and may further contribute to the accumulation of elastin. *In vivo*, the degradation of elastic fibers and accumulation of elastotic material takes place gradually. Our *ex vivo* experiments allowed us to mimic this process in an accelerated fashion. Although, similar to white skin, elastin staining could be induced

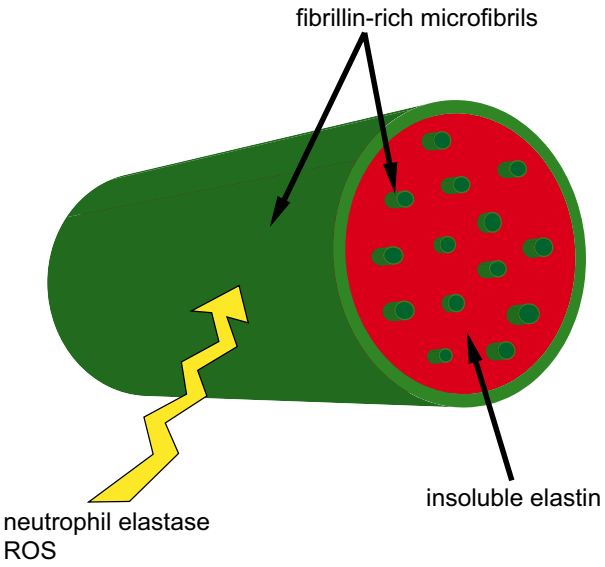

Figure 6. Schematic cross-section of a mature elastic fiber. During the synthesis of elastic fibers, fibrillar glycoproteins (of which fibrillin-1 is a major component) form a scaffold on which elastin is deposited. In a mature elastic fiber a mantle of fibrillin-rich microfibrils surrounds an elastin core. We hypothesize that during photoaging, the mantle of the elastic fiber is degraded thereby exposing elastin. This cross-linked insoluble elastin is highly resistant to further proteolysis and accumulates, resulting in solar elastosis. A process of repeated damage to - and partial repair of - the elastic fiber network may further contribute to excessive deposition of elastotic material. Based on our results, ROS and neutrophil elastase appear capable of inducing solar elastosis.

in black skin *ex vivo*, a heavily pigmented epidermis protects black skin against solar elastosis *in vivo*.

Elastin, fibrillin-1, collagen types I, III, IV and VII staining of skin obtained from the centre of a wrinkle in the neck (a primary site of photoaging) showed a staining pattern similar to photoaged skin obtained from the dorsal forearm of the same individual. Compared to dorsal forearm skin, elastin staining of neck skin was, however, more intense. In areas where elastin staining was particularly intense collagen types I and III appeared to be decreased and/or disorganized. Furthermore, in neck skin, Fibrillin-1 staining appeared to be increased in the mid and upper dermis (excluding the grenz zone). Subepidermal fibrillin-1 staining has been described and used as a marker for photoaging: photoaged skin shows shortening and thinning of subepidermal fibrillin-1 fibres.[32-34] In this respect we made the following interesting observation. Although clinically photoaged neck skin was more wrinkled than photoaged forearm skin, subepidermal fibrillin-1 positive fibers of the neck were noticeably longer and thicker than subepidermal fibrillin-1 positive fibers of the forearm. Furthermore, young white forearm skin, although clinically not photoaged, also showed shortened and thinned subepidermal fibrillin-1 positive fibers.

Together with the fibrillin-1 staining pattern observed in young and elderly black skin, our findings suggest that next to photodamage,[32-34] skin location also plays a role in the observed fibrillin-1 staining pattern. Our findings suggest that the length and thickness of subepidermal fibrillin-1 fibers do not consistently mirror the clinical picture which appears to be better correlated to elastin staining intensity.

Based on our findings and literature data one can speculate on the mechanism of wrinkle formation. Deep wrinkles are observed at sites exposed to mechanical stress due to underlying muscle movement, such as the neck, forehead and lateral to the eyes (crow's feet). Contrary to young skin which has an intact elastic fiber network, photoaged inelastic skin will have difficulty to regain its original form when put under strain and in time wrinkles are formed. Wrinkle formation may, in fact, accommodate movement of inelastic skin. In other words, continuous bending and stretching of inelastic skin, possibly in combination with ECM remodeling, may be the key ingredients to wrinkle formation.

We chose to investigate elastin, fibrillin-1, collagens I, III, IV and VII because they are important ECM components of the skin and/or have previously been studied in conjunction with photoaging.[2;3;10;11;31-35;39-41] Some research groups found greater differences in collagen staining and smaller differences in elastin staining when comparing photoaged skin with non-photoaged skin. This variation in results may partly be explained by differences in age groups of the volunteers and skin locations studied. Several authors report that fibrillin-rich microfibrils are reduced in moderately to severely photoaged skin.[32-34] These authors focused particularly on subepidermal fibrillin-rich microfibrils and took biopsies from buttock, forearm and facial skin, but not neck skin.

None of the above-mentioned studies investigated differences between black and white skin. By including black and white skin in our study we were able to compare these ECM components in elderly, sun-exposed, significantly photoaged (white) skin and elderly, sun-exposed, relatively non-photoaged (black) skin.

The effect of particular proteases on the ECM has also been studied by Braverman and Fonferko.[2] Their experiments included exposing human skin slices to porcine pancreatic elastase and bovine chymotrypsin, and studying their effect on elastic fibers by means of electron microscopy. These enzymatic studies produced alterations in elastic fibers that resembled abnormalities associated with chronoaged but not photoaged skin.[2] Our experiments performed with human purified neutrophil elastase did produce immunohistochemical alterations resembling those found in photoaged skin.

It has been shown that serine proteases such as neutrophil elastase are potent effectors for the physiological and pathological catabolism of fibrillin-rich microfibrils.[42] It has also been shown that several MMPs (MMP-2, MMP-3, MMP-9, MMP-12, MMP-13 and MMP-14) are capable of cleaving fibrillin-rich microfibrils.[43;44] UV-induced MMPs can be derived from different cell types including infiltrating neutrophils, macrophages, fibro-

blasts and keratinocytes. Thus, as has been suggested by these and other authors, MMPs may also contribute to elastic fiber degradation in the process of photoaging.[33;34;45;46] This effect and the relative contributions of MMPs, serine proteases and ROS to the induction of solar elastosis require further investigation.

Because elastic and collagen fibers can absorb UV directly,[47] we investigated whether exposure of skin sections to different UV sources could simulate the effect of neutrophil elastase, isolated neutrophils, pus and hydrogen peroxide on elastin staining. However, in these experiments, elastin staining of skin sections was not altered following UV exposure. We also investigated the effect of the different UV sources on skin sections with respect to the induction of thymine dimers, an important UV photoproduct (data not shown). Nuclei of cells in all parts of the skin sections (epidermis and dermis) directly exposed to broadband UVB, narrow band UVB and SSR showed intense, dose-dependent, thymine dimer staining. Interestingly, skin sections exposed to high doses of UVA1 showed subtle thymine dimer staining. Thymine dimer staining of non-irradiated skin sections was negative. Thus, *ex vivo* UV-exposed skin sections did show DNA damage, but elastin staining was unaffected.

In summary, our findings indicate that the elastotic material observed in photoaged skin is a degradation product of elastic fibers and can be induced by neutrophil elastase and ROS. We found no evidence that UV directly participates in the formation of 'solar elastosis'.

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Conflict of interest

The authors state no conflict of interest.

After the study was initiated Dr. Piet L.B. Bruijnzeel became employed at Organon now Merck, Sharp & Dohme, Department of Translational Medicine, ECREM, Oss, the Netherlands. Recently he has accepted a position at AstraZeneca as Medical Science Director, Loughborough (Charnwood), United Kingdom. Organon nor Merck, Sharp & Dohme nor AstraZeneca have contributed to this manuscript, either financially or otherwise. Dr. Piet L.B. Bruijnzeel holds an honorary research position at the Department of Dermatology of the University Medical Center Utrecht.

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Chapter 6

Photoaged skin: the role of neutrophils, preventive measures and potential pharmacological targets

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Abstract

Photoaged skin is characterized by epidermal changes and damaged elastic fiber and collagen fiber networks. Sunburned skin, including minimal asymptomatic ultraviolet radiation (UV)-induced erythema, is characterized by infiltrating neutrophils. Neutrophils are potent cells capable of degrading elastic fibers and collagen fibers and are probably important players in the pathophysiology of photoaging. Therefore, prevention of sunburn and/or prevention of neutrophil influx after exposure to artificial sources of UV appear to be key factors in the prevention of photoaging. The wearing of protective clothing and the use of sunscreens are important preventive measures. Drugs that interfere with the cascade of events that eventually lead to neutrophil influx are potential antiphotoaging agents. In contrast, current and/or future therapies that are accompanied by neutrophil influx, particularly when these therapies are administered or performed repetitively, require a critical review. Here we discuss clinical and histopathological features of photoaging, the pathophysiology of photoaging, potential pharmacologic targets with respect to infiltrating neutrophils, and some key points in prevention and therapy of photoaged skin.

Photoaged skin

Photoaging is the process by which natural sunlight and/or artificial sources of ultraviolet radiation (UV) damage the skin. Clinically, severely photoaged skin is characterized by a rough texture, irregular pigmentation, dryness, lack of elasticity, wrinkles, a yellowish color and teleangiectasia.[1] Unlike a 'healthy' tan, these skin changes are generally perceived as unattractive (Figure 1). Histologically, changes can be observed in the epidermis and dermis (Figure 2). Epidermal changes contribute to rough texture and irregular pigmentation and include atypical keratinocytes and irregular distribution of melanocytes and melanin.[1] Dermal changes include the hallmark of photoaged skin, so-called solar elastosis: this accumulated elastotic material in the mid and upper dermis is most likely

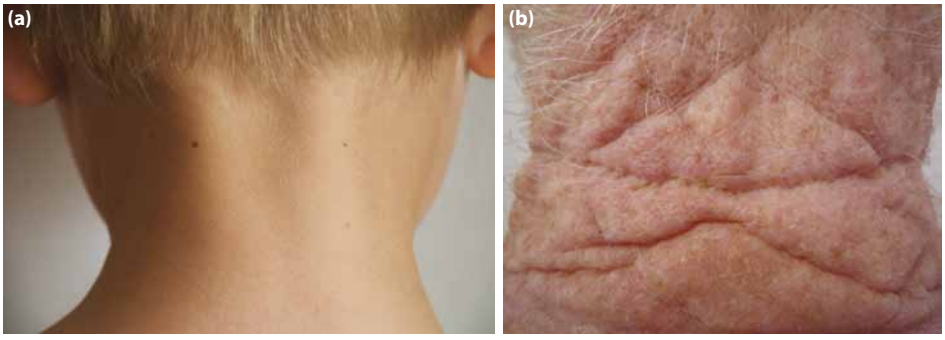


Figure 1. The neck region is readily, and often unknowingly, exposed to sunlight and is a primary site of photoaging. **(a)** Non-photodamaged, youthful skin is smooth and elastic. **(b)** Elderly, photoaged skin is rough, mottled, rigid, and wrinkled.

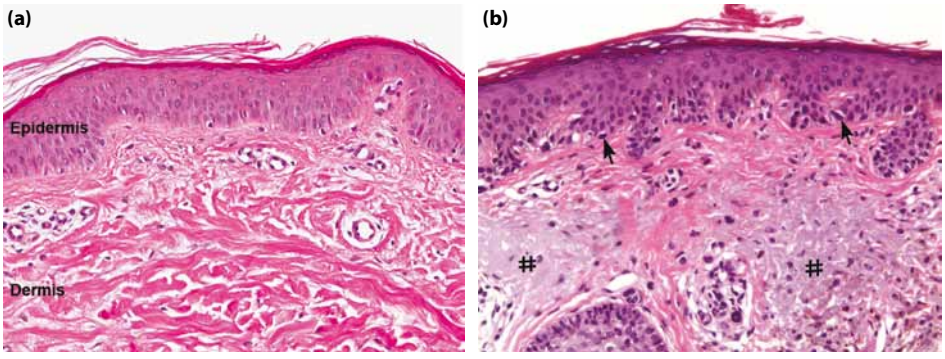


Figure 2. Histo(path)logy of **(a)** normal skin and **(b)** photoaged skin. Photoaged skin is characterized by atypical keratinocytes and irregular distribution of melanocytes (arrows) in the epidermis and conspicuous fibrous and amorphous basophilic material (crosses) in the mid and upper dermis, so-called solar elastosis. Panel 2a courtesy of the Department of Pathology at the University Medical Center Utrecht; panel 2b courtesy of the Department of Pathology at Diakonessenhuis Hospital.

a breakdown product of elastic fibers.[2] Other dermal changes include reduction in collagen fibers, increase in ground substance, and teleangiectasia.[3] Changes in the dermis are responsible for the sallow hue and inelasticity of photoaged skin.

Pathophysiology of photoaging

The changes in the epidermis are due mostly to accumulated damage to keratinocytes and melanocytes. Damage to these cells eventually leads to disturbed proliferation, differentiation, melanogenesis and melanin transfer.[4] The genetic material of keratinocytes and melanocytes is protected by melanin produced by melanocytes. Melanin is concentrated mainly in the supra-nuclear caps of keratinocytes located in the basal cell layer.[5;6] Basal keratinocytes are actively proliferating cells responsible for continuous renewal of the epidermis. Melanocytes are located between the basal keratinocytes. DNA damage after UV exposure can be demonstrated by immunohistochemical staining of cyclobutane pyrimidine dimers.[5;6] If the DNA damage is too extensive, the cells will undergo apoptosis.[7] If DNA damage is limited, it is reparable. However, some DNA damage, although irreparable, may be insufficient to induce apoptosis or may remain unnoticed, and this damage may slowly accumulate.[7] The degree of skin pigmentation plays an important role in how well actively proliferating basal keratinocytes are protected. The importance of pigmentation in protecting against photoaging is demonstrated by the fact that dark skin photoages at a much slower rate than fair skin.[8] Actinic keratosis, an advanced (premalignant) stage of UV-induced epidermal damage, is common in individuals of negroid ethnicity with albinism but rare in the normal dark-skinned population.[9] Actinic keratosis is also common in aged, fair, sun-damaged skin.[10] When normal fair skin and normal dark skin (skin phototype VI) are exposed to the same dosage of UV, the level of induction of UV-induced cyclobutane pyrimidine dimers in suprabasal keratinocytes is similar in both types of skin. However, whereas in fair skin these dimers are induced in basal keratinocytes as well, dark skin is virtually spared this effect.[6] These data support the concept that, with regard to epidermal changes, protection of the basal keratinocytes against UV damage is particularly important.

Besides nucleic acids, other cellular and extracellular components of the epidermis and dermis (e.g., proteins and lipids) are directly or indirectly damaged by UV.[11] Reactive oxygen species (ROS) play a pivotal role in the damaging effect of UV.[11] The depth of penetration into the skin and the type and intensity of damage depend on the wavelength of UV. UV-induced skin damage leads to the induction and release by resident cells of chemokines and cytokines which can trigger an inflammatory infiltrate.[12]

Studies investigating the pathophysiology of photoaging have focused primarily on the changes observed in the dermis in photoaged skin, i.e., solar elastosis. Various

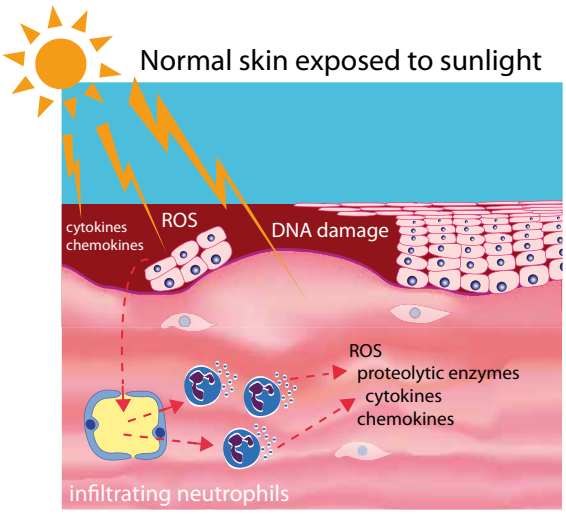
hypotheses have been proposed with respect to the pathophysiology of solar elastosis. A much-cited hypothesis focuses on degradation of collagen fiber by keratinocyte- and fibroblast-derived matrix metalloproteinases.[13] Histopathologically, however, damage to elastic fibers appears to be more important. The elastotic material observed in photoaged skin is strongly positive for elastin, which is a major component of the elastic fiber and is highly resistant to proteolysis.[2;14] It would seem, therefore, that accumulation of elastin is an important factor in the pathophysiology of photoaging; it may interfere structurally with repair of the elastic fiber and collagen fiber networks. The accumulation of elastotic material and damage to the elastic and collagen fibers results in a dysfunctional extracellular matrix and lead to loss of elasticity of the skin, wrinkle formation, and telangiectasia.

Our group has suggested that UV-induced neutrophils play an important role in the pathophysiology of solar elastosis.[6;15] Neutrophils infiltrate the skin after a certain threshold of UV damage has been reached. In susceptible individuals, this threshold may be reached after <10 minutes of exposure to sunlight. Clinically, infiltration of neutrophils is accompanied by erythema of the skin, i.e., sunburn. This may be limited to mild erythema without irritation or pain and thus remain unnoticed by the affected individual. The threshold for neutrophil influx is much higher in dark skin (particularly skin phototype VI) and, under normal circumstances, is unlikely to be reached.[6]

Neutrophils are potent cells capable of causing significant tissue damage.[16;17] They are packed with proteolytic enzymes, including neutrophil elastase and metalloproteinases. Furthermore, activated neutrophils generate and release ROS. Infiltrating neutrophils can thus damage collagen fibers and particularly elastic fibers.

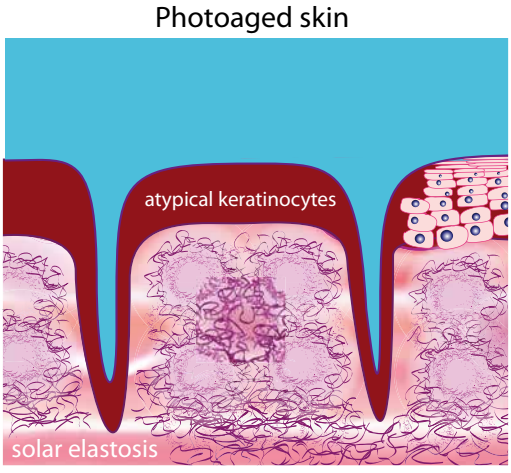
Prevention and pharmacological targets: focus on neutrophils

There are several potential methods and targets for prevention and therapy related to photoaging of the skin (Figure 3). The preventive measures and therapeutics in current use include sunscreens, antioxidants (including vitamins C and E, coenzyme Q10, bioflavonoids, and green-tea extracts), DNA-damage repair enzymes, dietary lipids, osmolytes, hydroxy acids, retinoids, fluorouracil, imiquimod, chemical peels, photodynamic therapy, laser therapy (vascular, pigment, ablative and non-ablative), cryosurgery, dermabrasion, face-lifts, fillers and botulinum toxin.[18;19] Although there is scientific evidence to support the effectiveness of each of these approaches, important questions remain. How effective are they, what is the risk of complications, and what are the long-term results? A discussion of the effectiveness and short- and long-term risks involved in using these products and treatments is beyond the scope of this article. However, their mechanism of action may be relevant in preventing or inducing an influx of neutrophils. Few, if any, studies investigating antiphotoaging products or treatments have specifically focused on infiltrat-



Intervention methods

sunscreens
stimulation of melanogenesis
DNA repair enzymes
antioxidants
anti-inflammatory drugs
antiproteases



pharmacological
laser
surgical
chemical

Figure 3. Summary of the (potential) methods of intervention. The top left schematic portrays the UV-induced events that eventually lead to photoaged skin and shows the important role infiltrating neutrophils play in the process. UV exposure leads to DNA damage and ROS formation, induction of chemokines and cytokines, and, ultimately, infiltration of neutrophils. Neutrophils release potent proteolytic enzymes, ROS and proinflammatory chemokines and cytokines, thereby damaging the ECM. The list of intervention methods shown at the top right of the figure may interfere with these UV-induced events, thereby preventing or slowing down the photoaging process. The bottom left schematic portrays actual photoaged skin. Photoaged skin may be treated with topical drugs, chemicals, lasers and surgical techniques. The underlying mechanisms of these techniques vary: physical or chemical repair and/or removal of the damaged epidermis; surgical removal of areas of full-thickness skin, thereby tightening the remaining skin; removal of underlying muscle tension (using botulinum toxin); laser ablation of telangiectasia; and others. A major remaining challenge is selective removal of the elastotic material and complete repair of the dermal ECM. ECM, extracellular matrix; ROS, reactive oxygen species.
Adapted from ref. 15

ing neutrophils, mainly because the role of neutrophils has been relatively neglected in the context of the pathophysiology of photoaging. Given that neutrophils are probably important contributors to the damage to the dermis in photoaged skin, this research is necessary. For example, fractional laser treatment of the skin may provide skin tightening and a younger appearance in the short run, but a single treatment may be accompanied by an influx of neutrophils and multiple treatments may actually lead to accelerated photoaging. Apart from the necessity of studying the effect of current (and future) treatments on neutrophil influx, a focus on neutrophils also creates new opportunities: (i) UV-induced neutrophil influx can be used as a parameter for comparing sunscreens and other anti-photoaging products; (ii) potential new products may be evaluated with regard to their ability to influence UV-induced influx of neutrophil and/or neutrophil-derived products such as proteolytic enzymes, ROS, cytokines and chemokines; and (iii) products known to influence neutrophil influx may be investigated for possible antiphotoaging properties.

When UV-induced skin damage crosses a threshold limit, there is an influx of neutrophils. DNA damage, directly induced by UV radiation and UV-induced ROS, is most likely the first step in a cascade of events involving receptor activation, intracellular signal transduction, and cytokine/chemokine production and release, which eventually lead to neutrophil infiltration.[11;12] The extent of damage to the basal keratinocytes, and perhaps to dermal components, appears to hold the key to whether an inflammatory infiltrate is induced.[6] In addition to avoidance of exposure to UV and wearing protective clothing, the application of a sunscreen preparation to the skin is a primary intervention technique that will prevent the aforementioned cascade of events. However, depending on the UVA and UVB absorbing properties of the ingredients of the sunscreen, as well as on its protection factor, some UV will still penetrate the skin. Moreover, the user may fail to apply sunscreen evenly, or it may be washed away by perspiration or swimming. Therefore, further developmental efforts are required for the production of application-friendly (quick and effective), cosmetically pleasing, and waterproof sunscreens. In an era when discussion of the pros and cons of sun exposure and its relationship to cancer induction and prevention is still very much alive, we believe that avoiding sunburn is key to preventing photoaging. Sunburn, as mentioned above, is accompanied by the influx of neutrophils. Recently, Lee *et al.* showed that UVA can also induce a neutrophilic infiltrate in the skin, although UVB was more active in this regard.[20] Consequently, agents that are capable of absorbing both UVA and UVB are required in order to prevent neutrophils from infiltrating the skin.

Systemic or topical use of antioxidants, DNA-damage repair enzymes, and skin pigment-inducing products should, in theory, inhibit UV-induced neutrophil influx. However, these products have not been investigated with respect to their effectiveness for this purpose. Furthermore, there have been no published reports of spectacular clinical

results from the use of these product with regard to the prevention of photoaging or the repair of photoaged skin.

UV-induced inflammatory stimuli that attract and/or activate neutrophils include formyl peptides, interleukin-1β, tumor necrosis factor-α, granulocyte macrophage-colony stimulating factor, interleukin-8 (CXCL8), LTB4, and C5a,[21;22] So-called end-target chemoattractants such as formyl peptides derived from mitochondria of dying cells are dominant over regulatory cell-derived attractants such as interleukin-8 and LTB4. This dominance allows neutrophils to move away from endothelium-derived chemoattractants towards their final target in the skin.[22] Drugs that interfere with the synthesis, release, or function of UV-induced cytokines/chemokines may have a blocking effect on infiltration (and/or activation) of neutrophils. Neutrophils are believed to play an important role in the pathogenesis of rheumatoid arthritis, a condition that can be effectively treated with anti-tumor necrosis factor-α drugs.[23;24] Similarly, anti-tumor necrosis factor-α drugs may offer protection against photoaging. However, rheumatoid arthritis is a debilitating disease, whereas photoaging *per se* is mainly a cosmetic problem. Furthermore, in contrast to rheumatoid arthritis, for which the etiologic agent is unknown, etiologic intervention is possible in photoaging. These essential differences should be taken into account when answering the questions; how effective is the drug, what are its side-effects, risk of complications, potential severity of complications, long-term results, and costs; and what are the alternatives?

Therapeutic measures that ameliorate a sunburn reaction after excessive exposure to sunlight may also reduce neutrophil influx. However, the use and effectiveness of particular drugs in sunburn management are controversial. Topical or systemic corticosteroids may not be effective if administered soon after sun exposure.[25;26] Similarly, topically or systemically administered non-steroidal anti-inflammatory drugs such as acetyl-salicylic acid and indomethacin may not reduce UV-induced inflammation.[27;28] Dapsone is a systemic drug commonly used in skin conditions characterized by infiltrating neutrophils.[29;30] Although its mechanism of action has not been fully elucidated, on the basis of its side-effect profile, dapsone is not a candidate drug for use in systemic antiphotoaging therapy. Nevertheless, clarification of its working mechanism could be relevant to developing new antiphotoaging drugs.

ROS released in the vicinity of neutrophils during oxidative bursts may be neutralized by ROS scavengers. Likewise, antiproteases (e.g., elastase- and metalloproteinase-inhibitors) would be expected to reduce the damage inflicted by the arsenal of proteases released by infiltrating neutrophils.

Formerly thought to be a population of terminally differentiated leucocytes involved exclusively in acute inflammation, neutrophils are now believed to be important contributors to the development of chronic inflammation as well.[21] Therefore, therapies aimed at inhibiting neutrophil-derived cytokines and chemokines may be useful in the treatment

Table 1.

Neutrophils and photoaging: opportunities, challenges, queries, and future investigations
1. Using infiltrating neutrophils as a parameter when comparing antiphotoaging products
2. Do current treatments prevent or actually promote neutrophil infiltration?
3. Using sunscreen preparations, DNA-repair enzymes and pigment-inducing drugs to increase the threshold at which neutrophils infiltrate the skin
4. Applying topical and systemic anti-inflammatory drugs to prevent neutrophils from infiltrating the skin and/or counteract their proinflammatory cytokines and chemokines[a]
5. Deactivating neutrophil-derived potent proteolytic enzymes, including multiple serine proteases and matrix metalloproteinases[a]
6. Applying topical and systemic antioxidants to neutralize reactive oxygen species generated by activated skin-infiltrating neutrophils
7. What is the beneficial role of UV-induced infiltrating neutrophils?

[a]Content of neutrophil granules and secretory vesicles.[33]

of chronic inflammation. However, chronic inflammation does not appear to be a major characteristic of photoaged skin. Importantly, unlike pathologic skin conditions such as so-called neutrophilic dermatoses, an influx of neutrophils after UV exposure is, in fact, a physiologic response, and interfering with a physiologic response may have negative consequences. The queries, opportunities, and challenges faced in acknowledging the important role of neutrophils in photoaging are summarized in Table 1.

Repair of the damage inflicted to the skin is a major area of interest: a distinction must be made between repair of epidermal damage and repair of dermal damage. Effective and relatively safe treatments for the former are unquestionably available (e.g., tretinoic acid, fluorouracil, and imiquimod), although pigment-related changes after a particular treatment may be difficult to control.[31] Treatment of damage to the dermis is much more challenging. As mentioned above, accumulated elastotic material may structurally and biochemically interfere with spontaneous or treatment-targeted repair of the elastic fiber and collagen fiber networks. Aggressive treatments such as CO_2 laser resurfacing, deep chemical peels, and dermabrasion may remove the elastotic material but there is an associated risk of pigment-related changes, secondary infection and scarring.[32] Furthermore, these and other treatment modalities may induce a neutrophilic infiltrate, which can cause additional damage to the extracellular matrix. Such additional extracellular matrix damage may become clinically relevant if these treatments are regularly performed.

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Conflict of interest

The authors declared no conflict of interest.

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Chapter 7

The protective effect of sunscreens, vitamin E 6% cream and betamethasone 0.1% cream on solar simulating radiation-induced erythema and neutrophil influx

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Submitted

Abstract

Background Exposure of white skin (skin phototypes I-III) to erythemogenic doses of solar-simulating radiation (SSR) results in an influx of neutrophils. We have previously shown that neutrophils are a major source of enzymatically-active, photoaging-associated, proteolytic enzymes, and thereby appear to be important contributors to photoaging. Preventing neutrophils from infiltrating the skin following SSR exposure should therefore limit the amount of SSR-induced skin damage.
Objectives To investigate the protective effect of sunscreens, a topical antioxidant and a topical corticosteroid on SSR-induced erythema and infiltrating neutrophils.
Methods Sun-protected buttock skin of six volunteers was pretreated with a broadband sunscreen (Vision SPF 28), vitamin E 6% cream and betamethasone valerate 0.1% cream, and exposed to 2 MED of SSR. Erythema was quantified using a Minolta chromameter and biopsies were taken immediately following and 24 hours after SSR exposure. Eight other volunteers underwent a similar procedure, including measurement of erythema immediately following and 24 hours after SSR-exposure, except that in this group only one control biopsy was taken and, additionally, two other sunscreens (Vision SPF 16 and Penetrase 17) were applied before SSR exposure.
Results Significant prevention of SSR-induced erythema and neutrophil influx was observed only after pretreatment with sunscreens. Skin-application of all other products did not statistically influence SSR-induced erythema or neutrophil influx.
Conclusion Only sunscreens are protective against the photoaging effects of neutrophils.

Introduction

Sunlight, more specifically ultraviolet radiation (UV), can damage human skin. Acute exposure can lead to inflammation of the skin, commonly known as a sunburn. Histopathologically, sunburned skin is characterized by multiple sunburn cells in the epidermis, dilated vessels in the superficial dermis and a mixed inflammatory infiltrate.[1] Chronic exposure of the skin can lead to so-called photoaged skin. Chronic exposure involves multiple exposures to both erythemogenic doses of UV (i.e., sunburn) and suberythemogenic doses of UV. Photoaged skin is characterized by clinical changes such as irregular pigmentation, plaque-like thickening, loss of skin tone, deep furrowing and fine wrinkle formation. Histopathologically the dermis of photoaged skin is characterized by an accumulation of elastotic material, disappearance of elastic fibers and reduced collagen fibers.[2]

Sunburned skin, including mild asymptomatic erythema, shows infiltrating neutrophils. Depending on several factors including skin phototype, UV-spectrum and UV intensity, 10 minutes of UV exposure can be enough to induce a sunburn reaction.[3] Multiple suberythemogenic doses of sunlight/UV (exposures on consecutive days) can also induce erythema and neutrophil influx (Lee *et al*., personal communication). Neutrophils contain and/or generate a variety of potent proteolytic enzymes and reactive oxygen species (ROS) which, when released from the cells, are capable of serious tissue damage.[4] By this capacity, and since they infiltrate the skin following UV-exposure, we have hypothesized that neutrophils play a major role in the pathophysiology of photoaging.[5] Preventing neutrophils from infiltrating the skin could thus slow down the photoaging process. Topically applied sunscreens, antioxidants and corticosteroids are potential candidates to prevent such neutrophil influx.

Sunscreens have been shown to prevent or reduce UV-induced erythema.[6] Inorganic sunscreens contain compounds that reflect and scatter UV, organic sunscreens contain compounds or chemicals that absorb UV.[7] The absorption spectrum of the latter is chemical dependent: i.e., some chemicals absorb mainly UVB wavelengths, some chemicals absorb mainly UVA wavelengths and some absorb both spectra. UVB wavelengths are 600-1000 times more effective in inducing erythema or sunburn than UVA wavelengths.[8] The action spectrum of photoaging in humans (i.e., the wavelengths responsible for photoaging) is difficult to determine and therefore still not entirely clear.[7]

UV-induced ROS play a major role in the cascade of events that eventually leads to skin inflammation.[9] Vitamin E is an essential nutrient and a major lipophilic antioxidant present in plasma and tissues. Several studies have demonstrated that topical vitamin E applied onto the skin prior to UV exposure is capable of reducing acute skin responses such as erythema and edema.[10;11]

Topical corticosteroids are widely used for different inflammatory skin conditions including those where infiltration of neutrophils are involved.[12] Several studies have shown that UV-induced erythema can be suppressed by topical corticosteroids.[13;14]

Although there are numerous studies that have investigated the effect of sunscreens, vitamin E and corticosteroids on UV-induced erythema, the influence of these products on UV-induced neutrophil influx are rare or absent.

The objectives of this study are to determine and compare the effect of sunscreens, a topical antioxidant and a powerful topical corticosteroid on solar-simulating radiation (SSR)-induced erythema and neutrophil influx.

Materials and methods

Volunteers

Fourteen healthy fair-skinned [skin phototypes (SPT) I-IV] volunteers were recruited. Volunteer characteristics are summarized in Table 1. Informed consent was obtained from all participants. The study was approved by the medical ethical committee of the University Medical Center Utrecht and the study was monitored by the Julius Institute, Utrecht, the Netherlands.

Table 1. Volunteer characteristics

No.	SPT	Gender	Age (years)	MED (mJ per cm^2)
1	III	male	22	9200
2	II	female	18	8600
3	IV	male	24	9600
4	IV	female	46	9600
5	III	male	22	9200
6	III	male	22	8900
7	II	female	23	8700
8	I	female	23	6400
9	III	female	22	9000
10	II	female	23	8500
11	III	female	23	8900
12	II	male	22	6600
13	III	female	22	8900
14	III	female	24	9000

SPT, skin phototype ; MED, minimal erythema dose.

SSR source, minimal erythema dose (MED) determination and measurement of erythema

CLEO Natural lamps (Philips, Eindhoven, the Netherlands) were used as a source of SSR. CLEO Natural lamps emit mostly UVA and 3% UVB. This UVA/UVB ratio is comparable to midday-sunlight during summer in Amsterdam (52° N), the Netherlands. The spectral energy distribution of CLEO Natural lamps ranges from 290 nm to 420 nm, with a maximum around 355 nm. Throughout the experiments the output of the CLEO Natural lamps was measured with a Waldmann UV-detector device (Waldmann, Schwenningen, Germany).

A specifically designed test device with nine 3 by 10mm windows that open consecutively exposing the underlying skin, was used to determine the MED.

A chromameter (Chromameter; Minolta Camera Co ltd, Osaka, Japan) was used to measure erythema of the skin. This instrument detects brown, blue and red pigments in the skin, allowing SSR-induced erythema to be quantified.

Topical products investigated

The following topical products were investigated:

(1) Vision SPF 28 and Vision SPF 16 (Nycomed Nederland B.V., Breda, the Netherlands): broadband organic sunscreens containing both UVA and UVB filters.
(2) Penetrase SPF 17 (Nycomed Nederland B.V., Breda, the Netherlands): a narrowband organic sunscreen containing UVB filters.
(3) Tocopherol-acetate 6% cream (Fagron, Nieuwerkerk a/d Ijssel, the Netherlands): a thermostable ester of vitamin E.
(4) Betamethasone-17-valerate 0.1% cream: a potent corticosteroid (and its vehicle).
(5) Cetomacrogol cream (Fagron, Nieuwerkerk a/d Ijsel, the Netherlands), the vehicle of tocopherol-acetate 6% cream.

Spectral transmission measurements

Spectral transmission through all products under investigation was performed with a calibrated standard UV-visible spectrometer (model 752, Optronic Laboratories, Orlando, FL, USA) and 150W halogen lamp (Osram, Augsburg, Germany). Products were diluted in ethanol/ether (50/50) to a concentration of 1.5 mg/ml and transferred into a 5mm thick fused quartz cuvette, after which the measurements were performed. The chosen concentration of 1.5mg/ml is based on the normal amount of sunscreen applied onto the skin (1.5mg/cm^2).

Volunteer procedures

Six of the fourteen volunteers (no. 1-6 in Table 1) underwent the following procedure: (i) MED determination; (ii) application, for 2 hours under occlusion, of Vision SPF 28,

vitamin E 6% cream, and betamethasone 0.1% cream on three separate 2 by 2 cm areas of buttock skin; (iii) exposure to 2 MED of SSR; (iv) immediately following SSR exposure: taking of 4mm skin-biopsies from the three pretreated areas, from an untreated SSR-exposed area and from an untreated non-irradiated area; (v) 24 hours after SSR exposure: taking of 4mm skin-biopsies from the three pretreated areas and from the untreated SSR-exposed area.

The eight other volunteers (no. 7-14 in Table 1) underwent a different procedure: (i) MED was determined; (ii) Vision SPF 28, Vision SPF 16, Penetrase SPF 16, vitamin E 6% cream, betamethasone 0.1% cream and cetomacrogol cream were each applied twice (i.e., six fields on the left buttock and six fields on the right buttock) on six separate, 1 by 1 cm areas of buttock skin; (iii) one buttock was exposed to 18 000 mJ/cm² of SSR* (i.e., exposure of six pretreated areas and a seventh control area); (iv) 24 hours after SSR exposure erythema of all pretreated- and untreated-areas, SSR-exposed and non-exposed (i.e., both buttocks) was measured; (v) a single 4 mm control biopsy was taken per volunteer from a particularly pre-treated, irradiated site to confirm the presence or absence of neutrophils in visibly erythematous or non-erythematous skin.

Biopsy processing

All obtained skin biopsies were snap-frozen in liquid nitrogen, embedded in Tissuetek (Sakura,Torrance, CA, USA) and cut into 6µm thick skin sections using a freezing microtome. Skin sections were mounted on Superfrost® Plus coated slides (Thermo scientific, Waltham, MA, USA), left to dry on Silicagel (MERCK, Darmstadt, Germany), and stored at -80°C.

Immunohistochemistry

Immunohistochemistry was performed to investigate the presence of neutrophils in the biopsies collected. Neutrophil elastase was used as a marker for neutrophils[15;16]. The immunohistochemical staining procedure was as follows: skin sections were fixed in dry acetone containing 200µl hydrogen peroxide and pre-incubated with 10% normal human serum (NhuS)/normal horse serum (NhoS)/bovine serum albumin (BSA). Next, the sections were incubated with mouse anti-human neutrophil elastase immunoglobulin (DAKO A/S, Glostrup, Denmark; diluted 1:100 in 1% NhuS/BSA). Following a washing step in phosphate-buffered saline (PBS)/Tween 0.05%, sections were incubated with biotinylated horse anti-mouse immunoglobulin (Vector, Burlingame, CA, USA; diluted 1:300 in 1% NhuS/BSA). Following another washing step in PBS/Tween 0.05%, sections were incubated with horseradish peroxidase-conjugated avidin-biotin complex (Vectastain ABC-HRP, Vector, diluted 1:1000 in 1% NhuS/BSA). A final washing step in PBS/

* 18 000 mJ/cm² of SSR is equivalent to 2-3 MED in SPT I-III.

Tween 0.05% was followed by staining with 3-amino-ethyl-carbazole chromogen (Scytec, Logan, Utah, USA). Staining was halted with distilled water.

Light microscopy

Immunohistochemically stained skin sections were examined by light microscopy at 200x and 400x magnification. For quantification of cell numbers, stained cells in an area below and parallel to the dermal-epidermal junction (band-form; 0.445mm broad) were counted. Stained cells in 8 fields at 400x magnification were counted. To prevent bias from occurring during quantification of stained cells, slides were blinded and received a code. The code was broken at the end of the counting procedures. Results are expressed as mean number of positive cells per mm^2.

Statistical analysis

Data (erythema intensity and cell numbers) are shown as mean ± standard error of the mean. Statistical analysis was performed using GraphPad Prism 5 software (GraphPad software Inc, CA, USA). To compare the parameters under investigation a Kruskal-Wallis test with Dunn's post-hoc multiple comparison test was performed. P values < 0.05 were considered significant. Correlation analysis was performed using a Spearman's Rank test.

Results

Intensity of SSR-induced erythema correlates with neutrophil influx

SSR-induced neutrophil influx (Figure 1) was quantified by counting the number of neutrophil elastase positive cells (Table 2). Correlation analysis indicated that the intensity of erythema and the number of infiltrating neutrophils are positively correlated (Spearman Rank correlation coefficient: R neutrophil erythema/elastase =0.59 ($P<0.0006$): i.e., an increase in erythema was associated with an increase of infiltrating neutrophils (Figure 2a).

Effect of sunscreens on SSR-induced erythema and neutrophil influx

Application of sunscreens suppresses the induction of erythema and prevents neutrophils from infiltrating the skin (Table 2, Figures 2a and 2b). Skin pretreated with Vision SPF 28, Vision SPF 16 and Penetrase SPF 17, and exposed to18 000 mJ/cm2 of SSR, showed no erythema of the skin 24 hours after irradiation (observed with the naked eye). Erythema measured with the chromameter appeared to show some increase in the Vision SPF 16- and Penetrase SPF 17-pretreated skin compared to Vision SPF 28 pretreated and non-irradiated skin, but the difference was not statistically significant.

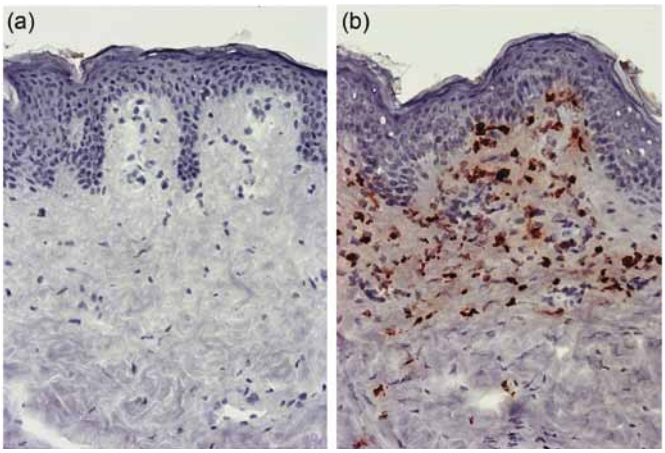

Figure 1. Example of the neutrophil elastase staining pattern in skin biopsies taken from sun-protected buttock skin before (a) and 24 hours after exposure to 2 MED of solar-simulating radiation (b). Neutrophil elastase staining reflects the infiltration of neutrophils into the skin.
MED, minimal erythema dose.

Table 2. Number of neutrophil elastase positive cells (per mm²) in collected skin samples

Volunteer no.	Non-irradiated control	Vision SPF 28	Vitamin E 6%	Betamethasone 0.1%	Radiated control
1	0	0	116	18	97
2	0	0	241	130	223
3	0	0	64	6	50
4	0	0	101	5	62
5	0	0	291	40	129
6	0	0	165	179	299

Neutrophils were not detected in the irradiated skin pretreated with Vision SPF 28 (exposed to 2 MED of SSR). Nor were neutrophils detected in the two irradiated skin samples collected from Vision SPF 16 and Penetrase SPF 17 pretreated skin (exposed to 18 000 mJ/cm2 of SSR).

Effect of vitamin E and betamethasone on SSR-induced erythema and neutrophil influx

Application of vitamin E 6% cream or its vehicle alone and betamethasone 0.1% cream did not prevent SSR-induced erythema (Figures 2a and 2b).

Although the chromameter showed a trend towards slightly decreased erythema in SSR-exposed skin pretreated with the products above (compared to the erythema induced in the irradiated untreated skin sample), the difference was not statistically significant.

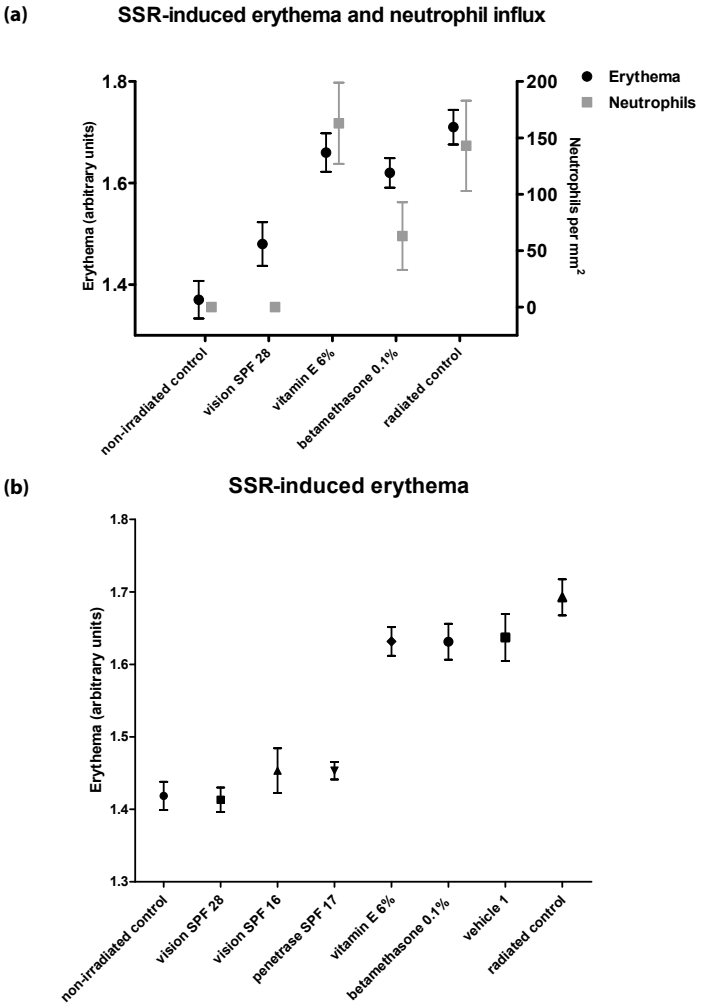

Figure 2. The effect of sunscreens, vitamin E 6% cream and betamethasone 0.1% cream on solar-simulating radiation (SSR)-induced erythema and neutrophil influx. (a) Skin of healthy volunteers (n=6, SPT I-IV) was pretreated with Vision SPF-28, vitamin E 6% cream, and betamethasone 0.1% cream, and exposed to 2 MED of SSR. 24 hours after SSR exposure erythema was quantified using a chromameter and biopsies were taken from the pretreated sites, from a non-irradiated control site and from an irradiated control site. Neutrophil influx was quantified by counting neutrophil elastase positive cells. The degree of erythema correlated significantly with the degree of neutrophil influx (Spearman rank correlation coefficient R=0.6, $p<0.05$). Vision SPF 28 significantly inhibited SSR-induced erythema and neutrophil influx. The other pretreatments showed no significant protective effect with regard to erythema and neutrophil influx (Kruskal-Wallis test: $p< 0.05$).
(b) Skin of healthy volunteers (n=8, SPT I-III) was pretreated with Vision SPF-28, Vision SPF 16, Penetrase SPF 17, vitamin E 6% cream, betamethasone 0.1% cream and vehicle 1 (vehicle of vitamin E), and exposed to 18 000 mJ/cm^2 of SSR. 24 hours after SSR-exposure erythema was quantified using a chromameter. All tested sunscreens significantly inhibited SSR-induced erythema. The other pretreatments did not show a significant protective effect with regard to erythema.

Application of vitamin E 6% cream and betamethasone 0.1% cream did not prevent neutrophils from infiltrating the skin (Figure 2a)

Pretreatment of the skin with vitamin E 6% cream had no effect on the number of infiltrating neutrophils following exposure to SSR. Pretreatment of the skin with betamethasone 0.1% did not prevent neutrophils from infiltrating the skin, but did appear to reduce their numbers (although statistically not significant with Kruskal-Wallis test with Dunn's post-hoc multiple comparison test).

Spectral transmission through the products applied

To check whether the tested products had the capacity to absorb UV, the spectral transmission of all products was measured (Figures 3a and 3b). Vision SPF 28 and Vision SPF 16 both absorbed radiation in the UVB spectrum (280nm-320nm) and UVA spectrum (320nm-400nm). Vision SPF 28, however, absorbed SSR more strongly than Vision SPF 16 in the UVB and UVA2 spectra (320nm-340nm). Penetrase SPF 17 mostly absorbed radiation in the UVB spectrum. Vitamin E 6%, betamethasone 0.1% and their vehicles showed very limited absorption of SSR in the UVB, UVA and visible light spectra.

Discussion

Exposure of human skin to SSR can result in erythema and an influx of neutrophils. Here we show that application of commercially available sunscreens effectively prevents SSR-induced eythema and neutrophil influx. Topical application of commercially available vitamin E does not significantly influence SSR-induced erythema or neutrophil influx. Topical application of betamethasone possibly reduces the number of skin-infiltrating neutrophils following SSR exposure, but may have less influence on the intensity of SSR-induced erythema. Considering that skin-infiltrating neutrophils are likely important contributors to the photoaging process, sunscreen application appears to be the most effective preventive treatment against its progression.

The relatively small number of volunteers and small number of investigated skin samples in the present study may have impacted the outcomes and statistics, particularly with respect to pretreatment of the skin with betamethasone. However, the findings relating to sunscreens are highly convincing and require no greater volunteer- or skin sample- numbers. Other possible confounding factors that should be considered are; (1) the curvature of the buttock, leading to an increased dose of SSR in the elevated fields and a reduced dose in the lower fields, and (2) the errors that can occur when determining the MED, including the fact that the MED was determined using the naked eye.

When using SSR as a UV source, the intensity of erythema induced by and large parallels the number of skin-infiltrating neutrophils induced. Taking skin biopsies is a

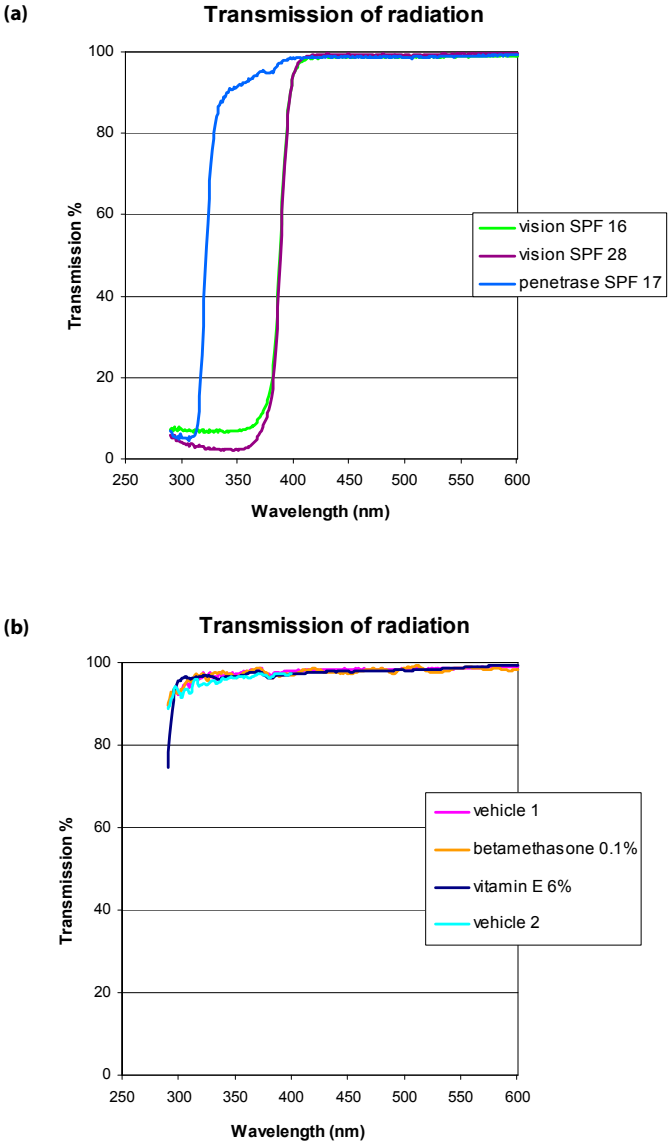

Figure 3. Spectral transmission of the products tested.
(a) Spectral transmission of Vision SPF 16 (green line); Vision SPF 28 (purple line); Penetrase SPF 17 (blue line). All products show clear absorption of wavelengths in the UV range and no absorption in the visible light range. Vision SPF 16 and 28 absorb both UVA and UVB. Penetrase SPF 17 almost exclusively absorbs in the UVB range.
(b) Spectral transmission of vehicle 1, the vehicle of vitamin E (pink line); betamethasone 0.1% cream (orange line); vitamin E 6% cream (dark blue line); and vehicle 2, the vehicle of betamethasone (light blue line). Only very limited absorption of SSR in the UVB, UVA and visible light spectra was observed. The SPF of these products is negligible.

minimally invasive procedure while determining skin-erythema using a chromameter is a non-invasive procedure. Thus, the latter parameter and procedure appears to be the preferred method to compare the effectiveness of antiphotoaging products with respect to their influence on skin-infiltrating neutrophils. However, the possibility that the product under investigation may have a greater impact on neutrophil influx than on erythema (e.g., topical betamethasone in the present study) must also be taken into account. With respect to investigating antiphotoaging products, exposing skin to SSR is, in itself, not entirely harmless or without side effects. The chromameter is much more sensitive in detecting SSR-induced erythema than the naked eye and objectively quantifies the erythema induced. Using a chromameter thus allows investigators to apply lower doses of SSR in their experiments and allows for an accurate and unbiased quantitative analysis of erythema. Taken together, although the welfare of the volunteers is of primary concern, we advocate the use of both (chromameter determined) erythema and neutrophil influx as readouts when investigating antiphotoaging products.

When evaluating the protective effect of sunscreens we found that Vision SPF 16 and Penetrase SPF 17 equally prevented SSR-induced erythema. No neutrophils were detected in the two skin samples taken from Vision SPF 16 and Penetrase SPF 17 pretreated skin. Considering that Penetrase SPF 17 virtually only absorbs UVB (Figure 3a), these data suggest that UVB photoabsorbers are particularly important in preventing neutrophil influx. These data are in accordance with that of Lee *et al.*[16] The UV-action spectrum of neutrophil influx appears to parallel that of the action spectrum of UV-induced erythema which is known to parallel that of UV-induced thymine dimers.[17]

The spectral transmission experiments are a valuable tool to exclude the possibility that the effect of a particular product on UV-induced erythema or neutrophil influx is due to the UV-absorbing properties of that product. Vitamin E 6% cream, betamethasone 0.1% cream and their vehicles showed very limited absorption of SSR. 95% transmission of SSR through these products means that they have a sun protection factor of 1.05, which is negligible. Their mode of action must therefore be locally in the skin by counteracting UV-generated ROS and inflammatory mediators.

Tocopherol-acetate, the thermostable ester form of vitamin E is marketed as an anti-(photo)aging agent. Together with directly UV-induced DNA damage, UV-induced ROS are believed to play an important role in UV-induced inflammation. Our data suggest that pretreatment of the skin with tocopherol-acetate 6% cream has little or no effect on UV-induced inflammation (i.e., erythema and neutrophil influx). Thus the ROS scavenging capacity of this form of treatment is inadequate.

As mentioned above, betamethasone 0.1% cream only had fractional UV-absorbing properties and its effect is locally in the skin. Topical corticosteroids can influence downstream factors involved in UV-induced neutrophil influx.[18-20] Topical betamethasone valerate has been shown to influence UVB-induced erythema.[20] UVB-induced neutrophil

influx has been shown to be inhibited by pretreatment of the skin with clobetasol.[21] Our experiments performed with a slightly less potent corticosteroid than clobetasol failed to show significant inhibition of SSR-induced neutrophil influx. As discussed above, this result may be influenced by the small number of samples investigated. Nevertheless, the effect of topical corticosteroids on neutrophil influx is obviously inferior to that of sunscreens.

In the present study we have not investigated the effect of prolonged or repetitive application. Taking into account that both tocopherol-acetate and betamethasone may need more time/applications to accumulate in the skin, and that tocopherol-acetate has to be hydrolyzed in the skin to its active form, further study is required.

In summary, application of sunscreens is the most effective treatment to prevent UV-induced erythema and neutrophil influx.

Conflict of interest

The authors state no conflict of interest.

After the study was initiated Dr. Piet L.B. Bruijnzeel became employed at Organon now Merck, Sharp & Dohme, Department of Translational Medicine, ECREM, Oss, the Netherlands. Recently he has accepted a position at AstraZeneca as Medical Science Director, Loughborough (Charnwood), United Kingdom. Organon nor Merck, Sharp & Dohme nor AstraZeneca have contributed to this manuscript, either financially or otherwise. Dr. Piet L.B. Bruijnzeel holds an honorary research position at the Department of Dermatology of the University Medical Center Utrecht.

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Chapter 8

Summary of Results and General Discussion

I. Introduction

Prior to commencing this thesis we searched for literature on 'the pathophysiology of photoaging' and learned that the dermal defects observed in photoaged skin were probably due to ultraviolet radiation (UV)-induced matrix metalloproteinases (MMPs) derived from fibroblasts and particularly keratinocytes[1] (the hypothesis on the pathophysiology of photoaging further referred to as the 'current hypothesis' in this discussion). Photoaging is often used synonymously with solar elastosis which is the hallmark of photoaged skin and describes the dermal changes observed in photoaged skin.[2] Oddly enough, the current hypothesis on the pathophysiology of photoaging focuses almost exclusively on collagen degradation, while literature on the histopathology of photoaged skin/solar elastosis focuses more on degraded elastic fibers.

Our goal at the time, was to investigate the possible contributing role of activated macrophages to the pathophysiology of photoaging. A study protocol wherein healthy volunteers were exposed to solar-simulating radiation (SSR) was initiated. The study protocol included a dose-response study and a time-course study. Multiple markers of tissue damage and tissue damaging mediators were investigated including MMPs and neutrophil elastase. By chance, a volunteer with skin phototype (SPT) VI was included in the study and this led to a switch in the cell of interest (i.e., a switch to the neutrophilic granulocyte) and, we dare say, this booklet.

II. Aims of this thesis

As stated in the general introduction the primary objectives of this thesis were:

(i) To determine the role of neutrophils in the pathophysiology of photoaging.
(ii) To critically examine the current hypothetical model for the pathophysiology of photoaging.

Secondary objectives were:

(i) To determine the expression, activity and origin of photoaging-associated proteolytic enzymes in 'young' and 'elderly' skin, different SPTs ('black' and 'white' skin), sun-exposed and sun-protected skin, and SSR-exposed skin.
(ii) To show that in chronically sun-exposed skin elastic fiber breakdown is more important than collagen breakdown.
(iii) To provide further evidence for the role of reactive oxygen species (ROS) in the pathophysiology of photoaging.
(iv) To determine the protective effect of skin pigmentation, sunscreens, topical vitamin E and betamethasone against SSR-induced erythema and neutrophil influx.

III. Key results summarized

In this thesis we were able to show that:

(i) Neutrophils infiltrate the mid and upper dermis of sun-protected buttock skin exposed to erythemogenic doses [≥1 minimal erythemal dose (MED)] of SSR. Skin-infiltrating neutrophils express neutrophil elastase, MMP-8 and MMP-9. *In situ* zymography showed prominent elastase and gelatinase activity in the mid and upper dermis, indicating that neutrophil elastase and MMP-9 may actively contribute to tissue damage in the dermis. Accordingly, neutrophils can contribute to the dermal changes observed in photoaged skin (chapters 2 and 3).

(ii) MMP-1 (interstitial collagenase) which is considered to be a key player in the process of photoaging according to the current hypothesis was mainly detected in the epidermis in all SPTs. Collagenase enzyme activity was also mainly detected in the epidermis (again, all SPTs). The staining intensity of MMP-1 and collagenase activity remained virtually unchanged when skin was exposed to erythemogenic doses of SSR. Our data question a major role of MMP-1 for photoaging (chapter 4).

(iii) Elastin staining was strongly increased in the mid and upper dermis of chronically sun-exposed elderly white (SPT I-III) forearm skin. Staining of collagen types I, III, IV and VII was not clearly affected in these skin samples. Subepidermal fibrillin staining appeared to be influenced by (chronic) sun exposure but also appeared to be dependent on skin location. These data indicate that elastin staining is a key marker for photoaged skin (chapter 5).

(iv) Skin biopsies taken from the center of a wrinkle in photoaged nucheal skin showed a similar staining pattern as described above. Because the neck region is a primary site of photoaging, elastin staining was even more intense and collagens I and III staining appeared reduced in areas of intense elastin staining. These findings show that the histopathology of a wrinkle does not substantially differ from that adjacent to the wrinkle and suggest that wrinkle formation is simply due to mechanical stress and inelasticity of the skin (chapter 5).

(v) Black skin (SPT VI) is protected against the damaging effects of UV. Melanin, which absorbs UV photons and scavenges UV-induced ROS, is particularly concentrated in basal keratinocytes. SSR-induced DNA damage (shown by staining of thymine dimers) was limited to the suprabasal keratinocytes demonstrating that UV does not penetrate into and past this layer in black skin. 18 000 mJ/cm^2 (equivalent to 2-3 MED of white skin) failed to induce a neutrophilic infiltrate in black skin. This demonstrates that black skin is better protected against sunburn and the potential damaging effects of skin-infiltrating neutrophils. Compared to chronically sun-exposed white skin, chronically sun-exposed black skin only showed minimally

increased elastin staining in the mid and upper dermis. This indicates that black skin is better protected against photoaging (chapters 2, 4 and 5).

(vi) Incubation of 'undamaged' (i.e., young and sun-protected) skin sections with pus, isolated neutrophils, neutrophil elastase and hydrogen peroxide induced an elastin staining pattern similar to that observed in chronically sun-exposed elderly white skin (i.e., photoaged skin). These data provide additional evidence that neutrophils may contribute to the pathophysiology of photoaging. Furthermore they indicate that UV-induced ROS and/or neutrophil-derived ROS may also directly contribute to the dermal changes observed in photoaged skin (chapter 5).

(vii) Compared to topical betamethasone and commercially available vitamin E, sunscreens are superior in preventing SSR-induced erythema and neutrophil influx. Based on the spectral absorbance of the sunscreens applied, it appeared that UVB is mostly responsible for the induction of erythema and neutrophil influx (chapter 7).

Based on the findings above and literature data, we have proposed a new model for the pathophysiology of photoaging: a model in which neutrophils, neutrophil-derived proteolytic enzymes and ROS play a central role (Figure 1).

IV. Our hypothesis on the pathophysiology of photoaging

Summary of our view on the photoaged skin: key characteristics, pathophysiology, and natural protection

Photoaging of human skin involves epidermal and dermal changes. The hallmark of photoaged skin is an accumulation of elastotic material in the mid and upper dermis, so-called solar elastosis. This elastotic material is mostly derived from degraded elastic fibers and consists mainly of insoluble elastin. In areas of severe solar elastosis collagen fibers are reduced and fragmented. Photoaged skin is inelastic and severe wrinkling occurs at sites of mechanical stress as the skin is continuously stretched and bent (e.g., neck and face). Neutrophil-derived mediators and proteolytic enzymes, particularly neutrophil elastase, play a key role in the development of solar elastosis (Figure 1). Neutrophil-derived MMP-9 contributes to collagen degradation. The role of keratinocyte-derived MMP-1 is insignificant and the role of MMP-1 derived from dermal cells is limited. Together with directly UV-induced DNA damage, UV-induced ROS play a major role in the cascade of events that precede UV-induced inflammation and neutrophil influx. UV-induced ROS and neutrophil-derived ROS can also contribute directly to the ECM damage observed in photoaged skin. Prevention of sunburn which is accompanied by skin-infiltrating neutrophils is key in the prevention of photoaging. Black skin is much

less prone to photoaging because it is much less likely to develop a sunburn with accompanying neutrophil influx, and because ROS are less likely to be generated in the dermis.

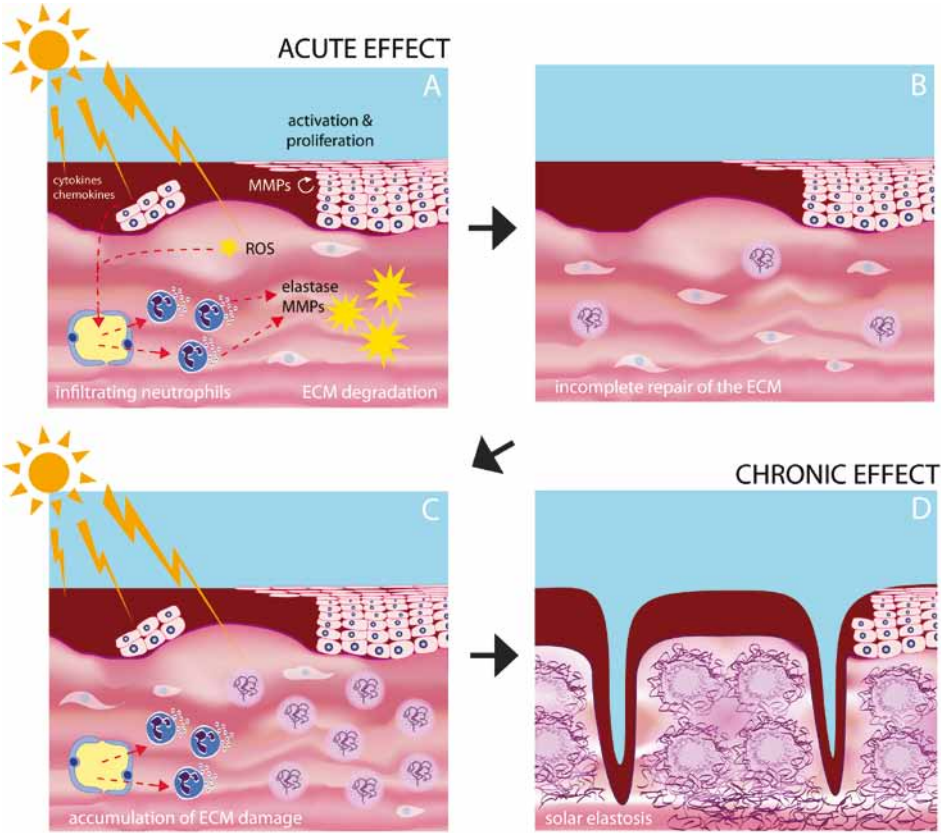

Figure 1. Our hypothesis on the pathophysiology of photoaging: we propose that, besides directly induced ECM damage by UV-induced ROS, neutrophil-derived proteolytic enzymes are the most important contributors to the ECM damage seen in photoaged skin. We further propose that keratinocyte-derived MMPs play more important roles in cellular processes other than ECM damage. UV-induced DNA damage and ROS initiate a cascade of events that can lead to an influx of neutrophils. UV-induced ROS can cause direct damage to the ECM. Through the release of proteolytic enzymes (including neutrophil elastase and MMP-9) and generation of ROS, skin-infiltrating neutrophils can damage the ECM (a). A single exposure to UV results in microscopic ECM damage (b). Multiple exposures lead to an accumulation of microscopic damage (c) and eventually to the clinical and histological signs of photoaged skin (d).

Arguments supporting our view of the pathophysiology of photoaging and arguments against the current hypothesis by Fisher *et al.*[1]

Until now, our hypothesis presented internationally in papers and oral presentations has not received much support. Here we would like to elaborate on our view of the pathophysiology of photoaging and compare it to the above-cited view.

(i) **The hallmark of photoaged skin is an accumulation of elastotic material, most likely derived from degraded elastic fibers, in the mid and upper dermis.** Previous literature data support the concept that photoaged skin is characterized by an accumulation of degraded elastic fibers in the mid and upper dermis.[3;4] As discussed in chapter 1, however, the current hypothesis focuses mainly on collagen degradation: i.e., keratinocyte- and fibroblast-derived UV-induced MMPs (MMP-1, 3 and -9) degrade collagen.[1] Our hypothesis focuses more on elastic fiber degradation. In chapter 5 we compared young and elderly skin, black and white skin, sun-protected and sun-exposed skin and found compelling evidence that 'solar elastosis' is indeed a degradation product of elastic fibers. Photoaged skin showed intense elastin staining as compared to non-photoaged skin, but did not differ significantly from non-photoaged skin with respect to collagen type I, -III, -IV, and -VII staining. Furthermore, we were able to induce similar elastin staining as observed in photoaged skin by incubating skin sections with neutrophil elastase, whole neutrophils and high concentrations of hydrogen peroxide.

(ii) **Repetitive UV exposures lead to photoaged skin.** Like Fisher *et al.*,[1] we believe repetitive exposures to UV eventually lead to the clinical and histopathological signs of photoaging. Unlike Fisher *et al.*[1] we believe erythemogenic doses play a major role in this process (although multiple sub-erythemogenic doses can also induce erythema of the skin, see further on), for the reason that erythemogenic doses are required for neutrophils to infiltrate the skin. Consequently, prevention of sunburn, including prevention of minimal asymptomatic erythema, is key to slowing down the photoaging process.

(iii) **It is unlikely that UV-induced, keratinocyte-derived MMPs diffuse across the dermal-epidermal junction.** The authors of the current hypothesis state that keratinocytes are the major source of UV-induced MMPs.[5] They hypothesize that these keratinocyte-derived MMPs diffuse across the dermal-epidermal junction to the dermis where they degrade collagen fibers.[1] However, when examining their original article closely, their *in vivo* studies show that at 24 hours after UV-exposure, MMP mRNA (e.g., MMP-1) was mostly located in the epidermis and MMP protein was also mostly observed in this compartment, while MMP enzyme activity (*in situ* zymography) at exactly the same time point was located mostly in the dermis (Fisher *et al.*[1], N. Engl. J. Med., 1997, 337, p1422, Figures 1C and D). Surely one would expect to find MMP protein and MMP enzyme activity to be located in the same compartment when these are visualized at the same time point!? Additionally, their immunohistochemical staining of (for example) MMP-1 protein shows a sharp demarcation between the epidermis and the dermis further arguing against diffusion of this protein to the dermis. Furthermore, in severely photoaged skin

the subepidermal grenz zone (i.e., the zone keratinocyte-derived MMPs would first pass through) appears spared of collagen damage.[6] We believe it is unlikely that keratinocyte-derived MMPs are responsible for the dermal changes observed in photoaged skin. After an initial misinterpretation of some of our own results (results concerning MMP-1 expression in chapters 2 and 3, misinterpretation discussed in chapter 4), we have shown that MMP-1 protein is mostly located in the epidermis and that collagenase activity coincides with the location of MMP-1 protein (chapter 4). Staining of MMP-1 protein with different anti-MMP-1 clones all show the above-mentioned sharp demarcation between the intensely stained epidermis and the much less intensely stained dermis (chapter 4).

(iv) **Photoaging-associated MMP-1, -2 -3 and -12 were not clearly induced by SSR. Staining patterns of MMP-1 and -3 are similar in black and white skin.** Numerous authors, including Fisher *et al.*,[1] have shown an induction of various MMPs by UV[5;7-15] (non-exhaustive list of references cited). We investigated the presence of MMP-1, MMP-2, MMP-3, MMP-8, MMP-9 and MMP-12 in elderly and young skin, black and white skin, sun-protected and sun-exposed skin, and SSR-exposed skin (chapters 2-4). By immunohistochemistry, MMP-12 and MMP-2 were not detected in any of the skin samples. MMP-1 and MMP-3 were detected in all skin samples, particularly in the epidermis. Skin pigmentation or SSR-exposure did not clearly influence immunohistochemical staining of MMP-1 or MMP-3 (apart from some increased cellularity in the dermis but this was insignificant compared to the total amount of staining which was concentrated in the epidermis). Although immunohistochemical staining is a less sensitive technique than, for example, western blot, one would at least expect to observe a difference in MMP-1 staining between control and SSR-exposed skin if MMP-1 plays a major role in photoaging. Furthermore, one would not expect to find significant MMP-1 staining and collagenase activity in black control skin (or white control skin, for that matter) as we did. In our studies only MMP-8 and MMP-9 staining was clearly dynamic. MMP-8 and MMP-9 were induced in the dermis by erythemogenic doses of SSR. These MMPs proved to be neutrophil-derived. MMP-9 was shown to be enzymatically active. We cannot explain why other authors have found MMP-1, -3, -2 and -12 to be induced by UV, but our data plead against a major role for MMP-1, -2, -3 and -12 in photoaging. Despite, or perhaps thanks to, our initial inaccurate results with respect to MMP-1 staining, we are confident of the accurateness of our results thereafter. Since immunohistochemistry and *in situ* zymography are prone to various artefacts, measures were taken to rule out our results and conclusions being based on these artefacts: all immunohistochemical staining and *in situ* zymography procedures were repeated a number of times with the same skin samples; skin sections from different biopsies were placed on the same glass slide; positive and negative controls were performed;

different anti-MMP-1 clones were used; counting and scoring was performed in a blinded fashion.

(v) **MMP-1 and MMP-9 are induced by infrared radiation (IR), but solar heat exposed skin shows no clinical signs of photoaging.** In our opinion, the following study further questions a major role for MMPs in photoaging. Cho *et al.*[8] showed that significant amounts of MMP-1 and MMP-9 mRNA and protein are induced in cloth-covered, sun-exposed skin (up to 90 percent of the expression of MMP-1 and MMP-9 in non-covered, sun-exposed skin)[8] which indicates that solar heat induces MMP-1 and MMP-9. In their experiments, however, solar heat did not induce an erythema reaction and consequently neutrophils were not detected in solar heat-exposed skin. In accordance with the current hypothesis on the pathophysiology of photoaging, the authors concluded that as solar heat induces MMPs, it contributes to photoaging. We maintain that if solar heat-induced MMPs really play an important role in photoaging, one would expect to find at least some clinical signs of photoaging in skin surfaces that are regularly exposed to solar heat, such as the swimming trunk- and hair-covered skin. These signs are, however, absent. An especially illustrative example of the lack of signs of photoaging in solar heat-exposed, cloth-covered skin is provided by albino patients living in the tropics (Figure 2). One of the first signs of chronic photodamage in these patients is solar elastosis in the neck region, an area of the skin that is easily and often unintentionally exposed to the sun.[16] In fact, many of these patients almost continuously suffer from sunburn in the neck region. On the other hand, their cloth-protected skin, which is also exposed to high doses of solar heat, does not sunburn and does not show any signs

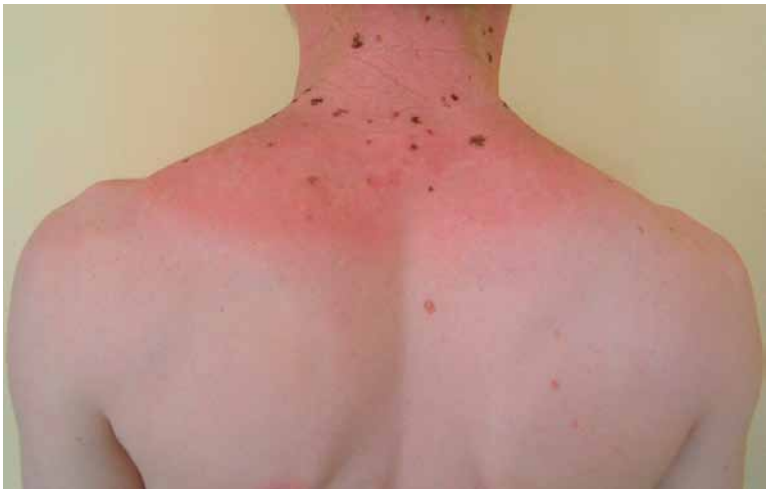

Figure 2. An albino patient showing sunburn and photoaging in the neck region. Note that solar heat-exposed but UV-protected, cloth-covered skin shows no signs of acute or chronic photodamage.

of photoaging. On the basis of the experiments of Cho *et al.*,[8] one would expect to find MMP induction in the solar heat-exposed cloth-covered skin of albino patients, but apparently these MMPs do not contribute to photoaging.

(vi) **Solar elastosis-like pathology observed in erythema ab igne may be caused by skin-infiltrating neutrophils.** Papers propagating the role of (solar)heat or IR in photoaging often refer to a clinical phenomenon called 'erythema ab igne' or related conditions. Erythema ab igne is a condition caused by repeated exposure to heat and/or IR, and it exhibits histological similarities to solar elastosis.[17;18] However, contrary to the doses involved in sun-exposed cloth-covered skin described above, the induction of erythema ab igne involves much higher doses of heat or IR. Furthermore, erythema ab igne, similar to sunburn, is characterized by an influx of neutrophils in its acute phase.[18] Therefore, also in this condition, neutrophil-derived proteolytic enzymes may be responsible for the histopathological changes that are present.

(vii) **Animal studies and clinical syndromes that directly or indirectly support our view of the pathophysiology of photoaging.** In chapters 2, 3 and 4 we showed that, following exposure of the skin to erythemogenic doses of SSR, infiltrating neutrophils and not keratinocytes or fibroblasts were the major source of proteolytic enzymes detected in the dermis: i.e., prominent *in vivo* MMP-9 and neutrophil elastase protein staining accompanied by prominent *in situ* gelatinase and elastase enzyme activity was demonstrated. Despite a significant amount of literature and clinical data that directly or indirectly support our view, neutrophils had only been reported as likely important contributors to photoaging in a murine model. Starcher *et al.*[19] investigated the effect of chronic UV exposure on neutrophil elastase-deficient mice. Neutrophil elastase-deficient and wild-type mice were exposed to a combination of UVA/UVB, three times a week, for a period of six months. At the end of this period the neutrophil elastase-deficient mice had not developed any significant solar elastosis in contrast to the wild-type mice. Neutrophils are potent cells capable of damaging the ECM:[20-22] next to neutrophil elastase and MMP-9, neutrophils are packed with other proteolytic enzymes[23] and activated neutrophils generate and release ROS.[22] Neutrophils and their products have already been identified as a major cause of tissue destruction in a number of conditions including lung emphysema,[24] rheumatoid arthritis,[25] periodontitis,[26] and wound infection.[27] Elastic and collagen fiber abnormalities have been reported in sun-exposed skin of a 19 year old patient with α_1-antitrypsin deficiency.[28] Massive infiltration of neutrophils leading to acquired cutis laxa has been described in an infant with α_1-antitrypsin deficiency.[29] Mid-dermal elastolysis, a condition characterized by focally wrinkled skin and loss of elastic tissue, has been associated with skin infiltrating neutrophils.[30] In summary, neutrophils are likely contributors to the dermal changes observed in photoaged skin.

V. Miscellaneous

Several issues relevant to this thesis have not yet been discussed or have only been touched on in previous chapters.

(i) Sunburn reaction and solar elastosis in persons with SPT VI

The tendency of a person to develop a sunburn reaction is strongly dependent on the degree of skin pigmentation. According to Table 3 in chapter 1, persons with SPT VI never sunburn and show slow and minimal photoaging. If their skin is exposed to a UV-dose that exceeds their minimal 'erythema' dose, they will develop a sunburn reaction with accompanying neutrophil influx. Importantly, erythema or rubor, one of the cardinal signs of inflammation is difficult, if not impossible, to detect in persons with SPT VI. Instead their sunburned skin appears darker compared to non-sunburned skin.

Solar elastosis has previously been described in SPT VI.[31] More recently, Young *et al.*[32] published a paper entitled: 'are dark-skinned people really protected from UV?'. In their study, facial skin biopsies were taken from 140 light-skinned (I-III) and 147 dark-skinned (SPT IV-VI) persons, and examined histopathologically (hematoxylin & eosin stains) for solar elastosis. Volunteers' age ranged from 30-75 years. Volunteers with SPT VI belonged to the following age groups: 40-49 yrs (n=1), 50-59 yrs (n=12) and 60-69 (n=2). Solar elastosis was graded on a five point scale ranging from no solar elastosis (grade 0) to nodular elastosis (grade 5). Results showed high-grade (=moderate to severe: grade 3-4) solar elastosis in 80% of light skin-skinned volunteers and in 34% of dark-skinned volunteers. In the dark-skinned group (SPT IV-VI), high grade solar elastosis was seen in 47.5% of Hispanic subjects and 10.2% of African American subjects. The prevalence of solar elastosis in the subjects with SPT VI was not specifically mentioned. The authors concluded that photoaging occurs in dark skin as it does in light skin, although to a much lesser extent. Furthermore, solar elastosis appeared to correlate with the degree of skin pigmentation and thus, the degree of melanin photoprotection. Evidently, photoprotection by skin pigmentation is not absolute and susceptibility to photoaging forms a spectrum just as the degree of skin pigmentation forms a spectrum. We have two remarks with respect to the study of Young *et al.*:(1) the diameter of skin biopsies taken was only 2mm, and (2) the photograph of a 66 year-old African American woman, with grade 3 solar elastosis, actually shows quite a light complexion. It is important to realize that even within the SPT VI group of persons, there is a wide range in the degree of skin pigmentation. For example, Africans from Southern Africa with SPT VI generally have a much lighter complexion than Africans from West African countries (Figure 3). Accordingly, the former are less well protected against sunburn and photoaging.

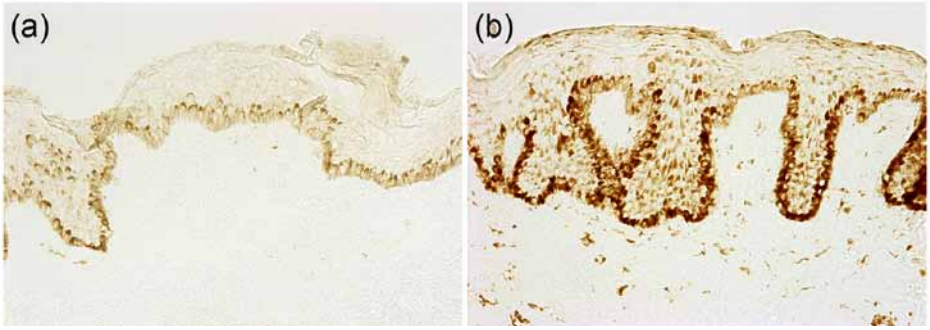

Figure 3. In general Africans from South Africa have a lighter complexion than Africans from West Africa, although both have a SPT VI. Histology shows less intense pigmentation of the epidermis in the former (a).

(ii) UV-hardening of the skin

The MED of a person's skin can be raised by natural tanning (sun exposure) and artificial tanning (using different UV sources). Cripps[33] investigated the effect of natural and artificial tanning on the MED of persons with SPT I-VI: three and a half months of natural sunlight exposure increased the MED by a factor 2.33 (SD 0.048); two weeks of PUVA (mean total dose 29000 mJ/cm^2) increased the MED by a factor 2.70 (SD 0.515), and; four weeks of UVB (mean total dose 3 490 mJ/cm^2) increased the MED by a factor 8.01 (SD 1.86). Although a factor 2.33 is not an impressive increase for a person who develops a sunburn within 20 minutes (he/she would now develop a sunburn after approximately 45 minutes), it is a significant and functional increase for a person who normally develops a sunburn after 3 hours: that person will now be able to spend 6½ hours outdoors before getting sunburned. The mechanisms by which the MED is raised following UV-exposure have not been fully elucidated. As demonstrated by one of the vitiligo patients (patient 2) described in chapter 2b, other factors in addition to increased pigmentation of the skin must also play a role. In this patient, despite the complete absence of pigmentation on his hands, neutrophils did not infiltrate his lesional skin following exposure to 18 000 mJ/cm^2 of SSR. The lesional skin of this patient showed particularly strong hyperkeratosis, supporting the hypothesis that increased thickness of the stratum corneum and epidermis contributes to UV-hardening of the skin. Other mechanisms underlying UV-hardening of the skin could be: attenuated signaling pathway sensitivity, increased ROS scavenging, and changes in (proinflammatory) cytokine expression. In any case, both increased pigmentation and/or strong hyperkeratosis appear to be able to increase the UV-damage threshold at which neutrophils infiltrate the skin.

(iii) The effect of different UV-sources on neutrophil influx

Lee *et al.*[34] investigated the effect of different UV sources on neutrophil influx. With respect to their potency in inducing a neutrophil influx the authors found that: SSR >

narrowband UVB >>> broadband UVB ≈ UVA1. Based on the wavelength spectra emitted by the different UV sources UVB seemed to be more effective than UVA in inducing a neutrophil influx. Furthermore, longer wavelengths within the UVB range appeared mostly responsible for the infiltration of neutrophils in the UV-exposed skin.
These findings support our results in chapter 7 that show that a UVB absorbing sunscreen (Penetrase SPF 17) was able to prevent SSR-induced erythema and neutrophil influx. Thus, with respect to prevention of UV-induced neutrophil influx it is particularly important to apply UVB absorbing sunscreens.

(iv) Neutrophil influx induced by erythemogenic but also suberythemogenic doses of UV

It has been established that a single erythemogenic dose of sunlight/UV is accompanied by infiltrating neutrophils.[35;36] Furthermore, Novakovic *et al.*[37] showed that exposure of white skin to 0.75 MED of SSR on four consecutive days produced the same intensity of erythema as a single exposure to 3 MED of SSR. Lee *et al.* (personal communication) exposed white skin to 0.5 MED of SSR on 4 consecutive days and this led to erythema and an influx of neutrophils, although in this case erythema was less intense and numbers of neutrophils were significantly smaller compared to a single exposure to 2 MED of SSR.

Multiple suberythemogenic doses of UV can also induce a neutrophil influx in mouse skin[38]. Most studies investigating the effect of chronic UV exposure on the animal skin, however, have not studied or focused on infiltrating neutrophils. Additionally, tissue samples in these studies may have been collected at a time point when the neutrophilic infiltrate has already resolved. Furthermore, mouse skin exposed to 'erythemogenic' doses of UV tends to show edema rather than erythema.[39]

In summary, depending on the dose (which must not be too low) and the interval (which must not be too long), multiple suberythemogenic doses of UV can also lead to erythema and skin-infiltrating neutrophils.

(v) Why have neutrophils been neglected with regard to the pathophysiology of photoaging?

Neutrophils are long known to infiltrate the skin following UV-exposure, they are known for their destructive capacity, and they are considered to be important contributors to the ECM damage observed in other conditions.[20;22;24-26] Despite all this, neutrophils have received relatively little attention when it comes to photoaging of human skin.
There are several possible explanations for this neglect:

(1) Neutrophils cannot be recognized morphologically in frozen skin sections and may even be difficult to recognize in paraffin-embedded skin sections when their numbers are limited. Immunohistochemical staining with neutrophil elastase or another

marker for neutrophils (e.g., CD66b) is often necessary to appreciate the number of infiltrating neutrophils.[34;36]

(2) Neutrophils and their products can only be detected in skin that has recently been exposed to UV.

(3) A certain threshold of damage is necessary for neutrophils to infiltrate the skin. This threshold, however, should not be overestimated. Depending on several factors, 15 minutes (or even less) of sun exposure can be sufficient for white-skinned persons to develop a subtle sunburn.[40]

(4) The current hypothesis whereby keratinocyte- and fibroblast-derived MMPs are considered responsible for the ECM damage seen in photoaged skin[1] has received wide support and has probably diverted the attention from other possible mechanisms.

Regardless of the reasons, UV-induced skin infiltrating neutrophils obviously deserve the attention we have given them.

(vi) The role of UV-induced ROS: indirect and direct damage to the extracellular matrix

For our study in chapter 7, we attempted to visualize oxidation reaction products in SSR-exposed, treated and untreated skin. Monoclonal antibodies targeting 4-Hydroxy-2-nonenal (4-HNE) (R&D, Minneapolis, MN, USA), Malondialdehyde (MDA) (Bio connect, Huissen, the Netherlands), 8-Hydroxy-2'-deoxyguanosine (8-OHdG) (Bio connect), and Dibromo-Tyrosine (DiBrY) (Bio connect) were purchased. 4-HNE and MDA are markers of lipid peroxidation, 8-OHdG is a DNA oxidation product, and DiBrY a marker of protein oxidation caused by neutrophils. Immunohistochemical staining procedures described in the product data sheets and in literature data were carried out, but we failed to attain any convincing results. A possible explanation is that most studies examining these oxidation reaction products used paraffin embedded biopsies and we only had frozen biopsies available. Although these experiments failed, as can be deduced from Table 1 in chapter 1, ROS clearly play an important role in UV-induced inflammation and the cascade of events that precede UV-induced neutrophil influx. Next to ROS induced directly by UV, neutrophils themselves are important generators of ROS.[22] In chapter 5 we demonstrated that hydrogen peroxide, a ROS itself, is capable of directly inducing solar elastosis-like pathology *ex vivo*.

Thus, ROS play an important role in UV-induced inflammation and neutrophil influx, but also appear to be able to directly contribute to the ECM damage observed in photoaged skin.

(vii) UV-induced keratinocyte-derived MMPs: extracellular matrix degradation or other (extra)cellular functions?

In our studies, MMPs were significantly induced only in skin exposed to erythemogenic doses of SSR and these MMPs were linked to infiltrating neutrophils.[35;36] However, immunohistochemistry and *in situ* zymography are less sensitive techniques than other detection methods such as northern blot and western blot. As mentioned earlier and in previous chapters, other authors found that MMPs are induced by sub-erythemogenic doses of UV, by a combination of IR/visible light and by solar heat alone.[8;14] Clinical data do not support a major contribution of low doses of UV (e.g., sun-exposed black skin and ventral forearm skin, which are regularly exposed to suberythemogenic doses of UV, show little or no solar elastosis), IR and solar heat to the photoaging process. Here we suggest that UV- and IR-induced keratinocyte-derived MMPs may actually be involved in other cellular processes rather than ECM damage. Besides their function in ECM remodelling, MMPs are known to be involved in signal transduction and particularly chemokine and cytokine (in)activation.[41;42] For example, MMP-1,-2, -3, -7, -9, -12 and -17 can activate TNF[42] in vitro, MMP-1 is associated with cell proliferation,[43] and MMP-9 cleaves desmoglein on apoptotic keratinocytes.[44] Parks *et al.*[42] stated that 'matrix degradation is neither the sole nor the main function of MMPs', and that 'a specific MMP secreted by one cell type would probably carry out a different function than the same MMP produced by another cell type'.

In summary, we believe that keratinocyte-derived MMPs are involved in other cellular processes, such as cell-maturation, -activation, -migration and -proliferation, rather than in ECM degradation (Figure 1).

(viii) Prevention and therapy of photoaged skin

With regard to therapy of photoaged skin, retinoids are the primary drugs named in literature. The therapeutic effect of retinoids is based on: (1) clinical data, (2) the effect retinoids may have on collagen synthesis and UV-induced MMPs, and (3) the hypothesis that MMPs produced by keratinocytes and fibroblasts are key factors in the pathophysiology of photoaging.[1;45;46] The Cochrane review discussed in chapter 1 concluded that topical tretinoin cream (a retinoid) improves skin changes (fine and coarse wrinkles, roughness, freckles and pigmentation) associated with prolonged sun exposure.[45] There is no question that retinoids have a positive, albeit modest, effect on photoaged skin. However, in our opinion, the primary site of action in the skin remains a topic of discussion. For instance, as mentioned in chapter 1, Kossard *et al.*[47] found that 6 months of tretinoin treatment particularly affected the epidermis and had no effect on the degree of solar elastosis.

Prior to our review in chapter 6, papers on preventive measures and therapeutics of photoaging did not focus on UV-induced neutrophil influx. As discussed in chapter

6 we believe prevention of neutrophil influx is key to prevention of photoaging or, at least, slowing it down. Sun- or UV-avoidance, wearing of protective clothing and use of sunscreens are important and effective preventive measures in achieving the above. Data in chapter 7 demonstrate the superior effect of sunscreens compared to betamethasone cream and commercially available vitamin E cream with respect to prevention of UV-induced neutrophil influx. Topical or systemic drugs that interfere with the cascade of events that eventually lead to neutrophil influx are potential antiphotoaging agents. However, since photoaging per se is mainly a cosmetic problem, these drugs need to be safe and cause few or no side-effects. Currently propagated and future therapies that may be or are accompanied by neutrophil influx must be viewed critically, particularly when administered or performed repetitively.

VI. Concluding remarks

The role of neutrophils in photoaging of human skin has been underrated. We believe neutrophils play an important role in the pathophysiology of photoaging. When this concept becomes more widely accepted it will undoubtedly lead to new interventions to prevent their influx and counteract their mediators. However, sun avoidance, wearing of protective clothing and application of sunscreens will remain key methods to prevent photoaging.

Acknowledgements

Parts of the general discussion including Figures 1 and 2 are derived from our article entitled 'the pathogenesis of photoaging: the role of neutrophils and neutrophil-derived enzymes'[48]

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Nederlandse Samenvatting

Introductie

Voordat wij begonnen aan het onderzoek dat opgetekend is in dit proefschrift werd de literatuur over huidveroudering onder invloed van zonlicht (zogenaamde 'photoaging') bestudeerd. Hierin wordt beschreven dat de histologische afwijkingen aangetroffen in de huid in dit kader waarschijnlijk worden veroorzaakt door matrix metalloproteinases (MMPs) afkomstig van fibroblasten en met name keratinocyten. Deze breed gedragen hypothese, geïntroduceerd door de groep van Fisher en Voorhees, richt zich met name op de afbraak van collageenvezels. Echter, de histologie van de door zonlicht beschadigde huid wordt vooral gekenmerkt door een opstapeling van 'elastotisch' materiaal dat vermoedelijk afkomstig is van beschadigde elastische vezels. De opstapeling van elastotisch materiaal in het dermale compartiment van de huid wordt 'solaire elastose' genoemd. In de literatuur worden de begrippen 'solaire elastose' en 'photoaging' vaak als synoniemen van elkaar gebruikt. In feite is 'photoaging' een breder begrip daar ook de door zonlicht geïnduceerde epidermale veranderingen hieronder vallen. 'Photoaged' huid wordt klinisch gekenmerkt door onregelmatige pigmentatie, teleangiëctasiëen, plaque-achtige verdikking, verruwing van de huid en rimpelvorming.

Toen wij begonnen met het onderzoek was ons doel de mogelijke rol van geactiveerde macrofagen in de pathofysiologie van 'photoaging' te bepalen. Een studie waarin gezonde vrijwilligers werden blootgesteld aan ultraviolet straling (UV) werd geïnitieerd. De studie omvatte een dosisrespons studie en een studie waarin vrijwilligers werden blootgesteld aan een vaste dosis UV waarna op verschillende tijdstippen huidbiopten werden afgenomen. Een reeks van markers voor specifieke celtypes, weefselbeschadiging en weefselbeschadigende stoffen, inclusief MMPs en neutrofiel elastase, werd onderzocht. Neutrofiel elastase is een krachtig enzym dat meerdere componenten van de extracellulaire matrix (o.a., elastische vezels en collageenvezels) kan beschadigen en het is een belangrijke marker voor neutrofiele granulocyten. Het feit dat dit proefschrift hier ligt is mede te danken aan het destijds breed inzetten van markers en het per toeval includeren van een vrijwilliger met een zeer donkere huid. De observaties die wij toen deden leidden er toe dat onze aandacht verschoof van de macrofaag naar de neutrofiele granulocyt. Neutrofiele granulocyten zijn potente cellen die tal van weefselbeschadigende producten kunnen vrijmaken. Zij worden reeds in verband gebracht met het ontstaan van weefselschade in aandoeningen zoals longemfyseem en reumatoïde artritis.

Doelstellingen

De primaire doelstellingen van dit proefschrift zijn:

(i) De rol van neutrofiele granulocyten in de pathofysiologie van 'photoaging' te bepalen.
(ii) De huidige, bovengenoemde hypothese over de pathofysiologie van 'photoaging' kritisch onder de loep te nemen.

Secundaire doelstellingen zijn:

(i) De expressie, activiteit en bron van de met 'photoaging' geassocieerde proteolytische enzymen te onderzoeken in 'jonge' en 'oude' huid, 'blanke' en 'zwarte' huid (huid fototype volgens Fitzpatrick I-III en huid fototype VI), zonblootgestelde (buitenzijde onderarm) en zonbeschermde (bil) huid en UV* blootgestelde huid.
(ii) Aantonen dat de afbraak van elastische vezels belangrijker is dan afbraak van collageen in 'photoaging'.
(iii) De rol van reactieve zuurstofdeeltjes in de pathofysiologie van 'photoaging' verder te onderbouwen.
(iv) Het beschermende effect van huidpigment, anti-zonnebrandmiddelen, vitamine E (een antioxidant) en betamethason (een hormoon preparaat en krachtige ontstekingsremmer) te bepalen.

Resultaten

In dit proefschrift tonen wij het volgende aan:

(i) Neutrofiele granulocyten infiltreren de huid wanneer deze wordt blootgesteld aan een dosis UV die tot erytheem leidt [een dosis ≥1 minimale erytheem dosis (MED)]. Neutrofiele granulocyten brengen neutrofiel elastase, MMP-8 en MMP-9 tot expressie. *In situ* zymografie laat opvallende elastase[1] en gelatinase[2] enzymactiviteit zien in eenzelfde patroon als de immuunhistochemische aankleuring van huidinfiltrerende neutrofiele granulocyten. Deze data suggereren dat neutrofiel elastase en MMP-9 afkomstig van neutrofiele granulocyten een actieve bijdrage kunnen leveren aan UV geïnduceerde dermale schade en pleiten voor een rol van neutrofiele granulocyten in de pathofysiologie van 'photoaging' (zie hoofdstukken 2 en 3).
(ii) MMP-1, de matrix metalloproteinase die in de hypothese van Fisher en Voorhees een centrale rol speelt, wordt vooral gedetecteerd in de epidermis, zowel in blanke als in zwarte huid. Collagenase[3] enzymactiviteit wordt ook voornamelijk gedetecteerd in de epidermis (wederom eenzelfde patroon in blanke en zwarte huid). Blootstelling aan UV heeft vrijwel geen invloed op de immuunhistochemische aankleuring van

* Als UV bron maakten wij gebruik van een zogenaamde 'solar simulator': een UV bron die zonlicht benadert qua verhoudingen UVB en UVA golflengtes.
[1,2,3] Weerspiegeling van respectievelijk neutrofiel elastase, MMP-9 en MMP-1 enzym activiteit.

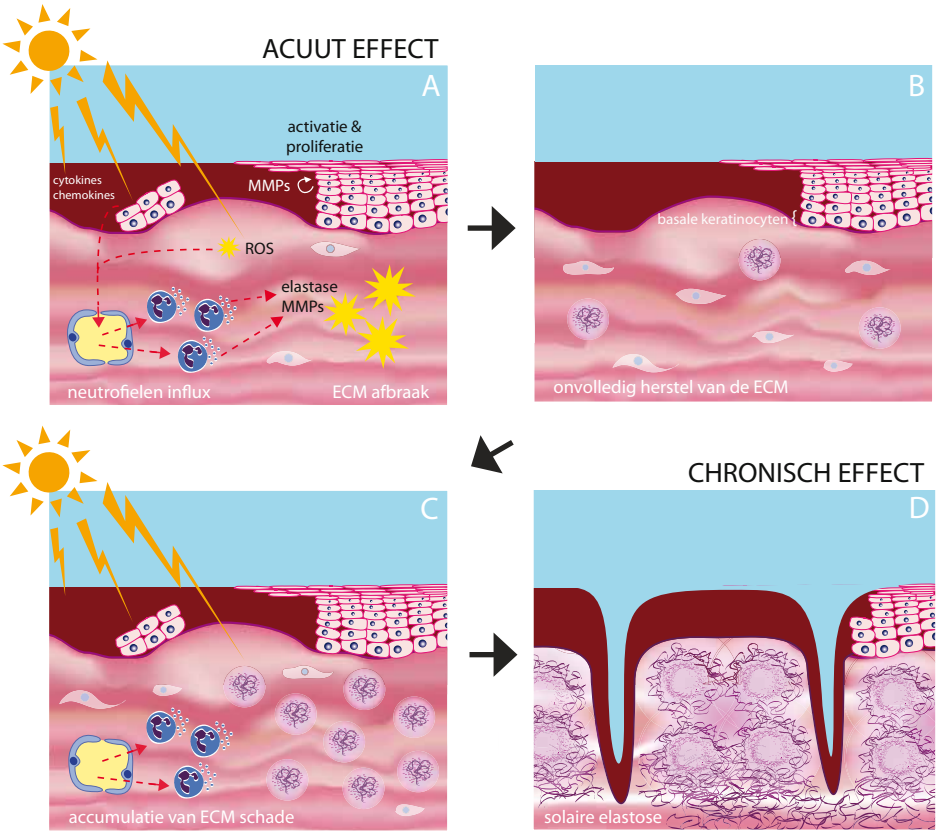
MMP-1 noch op collagenase enzymactiviteit. Deze data plaatsen vraagtekens bij de essentiële rol van MMP-1 in de pathofysiologie van 'photoaging' (zie hoofdstuk 4).

(iii) Elastische vezels bestaan grofweg uit een kern van elastine omgeven door een huls van fibrillines. In hoofdstuk 5 tonen wij aan dat immuunhistochemische aankleuring van elastine in de dermis sterk is toegenomen in de door zonlicht beschadigde, oude, blanke huid. Immuunhistochemische aankleuring van verschillende types collageen (I, III, IV en VII) laat geen opvallende verschillen zien tussen de verzamelde huidbiopten (behalve verminderde collageen I en III aankleuring in gebieden met zeer sterke toename van elastine). Subepidermale fibrilline aankleuring lijkt te worden beïnvloed door (chronische) zonblootstelling maar lijkt ook afhankelijk te zijn van de locatie van het afgenomen biopt. Deze data pleiten voor het gebruik van elastine als marker voor 'photoaged' huid.

(iv) Huidbiopten afgenomen uit het midden van een rimpel in de nek (oude, blanke, zonblootgestelde huid) laten een vergelijkbaar aankleuringspatroon van elastine, fibrilline en verschillende types collageenvezels zien als hierboven beschreven. Echter, de nek is een primaire locatie voor zonbeschadiging (bij mannen vrijwel altijd blootgesteld aan de zon) met als gevolg dat de elastine aankleuring hier nog meer uitgesproken is. Zoals reeds gemeld gaan gebieden met zeer sterke elastine aankleuring gepaard met verminderde aankleuring van collageen types I en III. Deze bevindingen bevestigen het nut van elastine als marker voor 'photoaging' en tonen aan dat de histologie van de huid in het midden van een rimpel niet wezenlijk verschilt van die naast de rimpel. Waarschijnlijk ontstaan rimpels eenvoudigweg als gevolg van verlies van elasticiteit van de huid en herhaaldelijke mechanische stress (zie hoofdstuk 5).

(v) Zwarte huid (huid fototype VI) is beter beschermd tegen de schadelijke effecten van UV. Melanine pigment, dat UV fotonen absorbeert en antioxiderende eigenschappen heeft, is vooral geconcentreerd ter hoogte van de basale keratinocyten (boven de celkernen). UV geïnduceerde DNA schade in zwarte huid (aangetoond door aankleuring van thymine dimeren) wordt vrijwel uitsluitend aangetroffen in de kernen van suprabasale keratinocyten wat aantoont dat UV in zwarte huid tot aan en niet voorbij de basale keratinocyten komt. 18 000 mJ/cm^2 (gelijk aan 2-3 MED in blanke huid) is ontoereikend om een neutrofiel ontstekingsinfiltraat te induceren. Deze data tonen aan dat de zwarte huid beter beschermd is tegen UV/zonverbranding en tegen infiltratie en de potentieel schadelijke effecten van neutrofiele granulocyten. Vergeleken met oude, chronisch zonblootgestelde, blanke huid laat oude, chronisch zonblootgestelde, zwarte huid slechts een geringe toename van elastine in de dermis zien. Dit wijst er op dat zwarte huid beter beschermd is tegen 'photoaging' dan blanke huid (zie hoofdstukken 2, 4 en 5).

(vi) Incubatie van onbeschadigde (jonge, zonbeschermde) huid met (1) pus, (2) geïsoleerde neutrofiele granulocyten, (3) neutrofiel elastase en (4) waterstofperoxide induceert een elastine aankleuringspatroon vergelijkbaar met of zelfs identiek aan dat geobserveerd in oude, blanke, chronisch zonblootgestelde huid (of te wel 'photoaged' huid). Deze bevindingen bieden additioneel bewijs dat neutrofiele gra-



Figuur 1. Onze hypothese ten aanzien van de pathofysiologie van 'photoaging'.
Naast directe schade door UV geïnduceerde reactieve zuurstofdeeltjes spelen neutrofiele granulocyten en hun producten (o.a., neutrofiel elastase en MMP-9) een centrale rol in het ontstaan van de histologische afwijkingen (solaire elastose) die worden gezien in de door zonlicht beschadigde huid. Waarschijnlijk spelen MMPs afkomstig van keratinocyten een rol in andere (lokale) processen dan in beschadiging van elastische en collageen vezels in de dermis.
UV geïnduceerde DNA schade en reactieve zuurstofdeeltjes initiëren een cascade van gebeurtenissen die leidt tot een influx van neutrofiele granulocyten (a). Een eenmalige blootstelling aan UV veroorzaakt microscopische schade die niet volledig hersteld wordt (b). Accumulatie van restschade na herhaalde blootstellingen (c) leidt uiteindelijk tot de klinische en histologische kenmerken van 'photoaged' huid (d). Zwarte huid is minder vatbaar voor 'photoaging' dan blanke huid omdat er voor zwarte huid een significant hogere dosis UV nodig is om een neutrofiel ontstekingsinfiltraat te induceren. Daarnaast is het minder waarschijnlijk dat reactieve zuurstofdeeltjes worden gegenereerd in de dermis van zwarte huid.
ROS, reactieve zuurstofdeeltjes; ECM, extracellulaire matrix.

nulocyten kunnen bijdragen aan de pathofysiologie van 'photoaging'. Voorts tonen deze gegevens aan dat UV geïnduceerde reactieve zuurstofdeeltjes en/of reactieve zuurstofdeeltjes gegenereerd door neutrofiele granuloctyten mogelijk rechtstreeks (door oxidatie van elastische vezels) kunnen bijdragen aan de dermale veranderingen die gezien worden in de door zonlicht beschadigde huid (zie hoofdstuk 5).

(vii) Vergeleken met een commercieel verkrijgbaar vitamine E preparaat en topicaal betamethason zijn anti-zonnebrandmiddelen superieur wat betreft preventie van UV geïnduceerd erytheem en neutrofielen influx. Op basis van de spectrale absorptie van de bestudeerde anti-zonnebrandmiddelen lijkt het er op dat UVB hoofdzakelijk verantwoordelijk is voor inductie van erytheem en neutrofielen influx (hoofdstuk 7).

Op basis van de bovenstaande bevindingen en literatuurgegevens hebben wij een nieuw model voor de pathofysiologie van 'photoaging' ontwikkeld. In dit model spelen neutrofiele granulocyten, proteolytische enzymen afkomstig van neutrofiele granulocyten en reactieve zuurstofdeeltjes een centrale rol (Figuur 1).

Discussie

Wat pleit voor onze hypothese en wat maakt de hypothese van Fisher en Voorhees minder waarschijnlijk? Waarom hebben neutrofiele granulocyten zo weinig aandacht gekregen in het kader van 'photoaging' terwijl het uitermate potente cellen zijn en ze de huid binnentrekken na UV blootstelling? Waarom is het onwaarschijnlijk dat MMP-1 vanuit de epidermis door de basale membraan naar de dermis diffundeert? Wat is de mogelijke functie van MMP-1 in plaats van het berokkenen van weefselschade in de dermis? Deze vragen en meer worden besproken in hoofdstuk 8.

Conclusies

De rol van neutrofiele granulocyten in het kader van veroudering van de huid onder invloed van zonlicht is ondergewaardeerd. Wij bepleiten een belangrijke rol voor neutrofiele granulocyten in de pathofysiologie van 'photoaging'. Wanneer dit concept geaccepteerd wordt zal dat ongetwijfeld leiden tot nieuwe interventies om influx van neutrofielen in de huid te voorkomen en hun producten te neutraliseren. Deze nieuwe kansen worden besproken in hoofdstuk 6. Niettemin zullen ook dan het vermijden van zonblootstelling, dragen van beschermende kleding en gebruik van anti-zonnebrandmiddelen de belangrijkste maatregelen zijn om 'photoaging' tegen te gaan.

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Curriculum Vitae

De in Amsterdam geboren en getogen ouders van Feiko Rijken vertrokken na hun studie geneeskunde naar Suriname. Daar werd Feiko geboren op 5 september 1972 te Brownsweg, distrikt Brokopondo. Toen hij een jaar was reisde het gezin terug naar Nederland waar zijn vader zich specialiseerde tot gynaecoloog. De tropen bleef lonken en in 1979 verhuisden zijn ouders met hem en zijn 3 zussen naar Tanzania.

Na bijna acht jaar Tanzania en een tussenstop van een jaar in Nederland haalde Feiko in Botswana zijn O-levels op Maru a Pula School te Gaborone. Eindexamen (International Baccalaureate) legde hij af op Waterford Kamhlaba United World College in Mbabane, Swaziland. In de voetsporen van zijn ouders besloot hij geneeskunde te gaan studeren. Aanvankelijk in België aan de Katholieke Universiteit Leuven waar hij in 1994 zijn kandidaatsexamen haalde en later aan de Universiteit Utrecht. Hier studeerde hij af en begon hij in 2001 aan het onderzoek naar 'photoaging' op de afdeling dermatologie van het Universitair Medisch Centrum Utrecht onder leiding van dr. P.L.B. Bruijnzeel. Na ruim een jaar werd het onderzoek onderbroken toen hij startte met zijn specialisatie tot dermatoloog met prof.dr. C.A.F.M. Bruijnzeel-Koomen als opleider. Zes maanden van zijn opleiding bracht hij door op het Regional Dermatology Training Center in Moshi, Tanzania, onder leiding van prof.dr. H. Grossmann. Eind 2007 rondde hij zijn opleiding af en had hij reeds een doorstart gemaakt met het onderzoek naar 'photoaging'. Sinds half 2009 werkt hij als dermatoloog in het Diakonessenhuis Utrecht/Zeist/Doorn.

Feiko is getrouwd met Sandra. Samen hebben ze een zoontje Pim en een dochtertje op komst.

Abbreviations

Akt - non-specific serine/threonine protein kinase
AP-1 - activator protein-1
AP- alkaline phosphatase
DNA - deoxyribonucleic acid
ECM - extracellular matrix
EGF - epidermal growth factor
FasR - death receptor on the surface of cells
Gro-α - growth related oncogene-α
ICAM - intercellular adhesion molecule
IL - interleukin
IR- infrared radiation
JNK - c-Jun N-terminal kinase
K - cytokeratin
LT - leukotriene
MED - minimal erythema dose
MMP(s) - matrix metalloproteinase(s)
NADP - nicotinamide adenine dinucleotide phosphate
NF-κβ - nuclear factor-κβ
NO - nitric oxide
PAF - platelet activating factor
PBS - phosphate-buffered saline
PG - prostaglandin
PTEN - phosphatase and tensin homolog
Ref - references
ROS - reactive oxygen species
SMAD - intracellular proteins involved in TGF-β signaling
SPF - skin protection factor
SPT - skin phototype
SSR - solar-simulating radiation
TGF-β - transforming growth factor-β
TIMP - tissue inhibitor of metalloproteinase
TNF-α - tumor necrosis factor-α
UV - ultraviolet radiation
VIP - vasoactive intestinal protein
X mJ per cm^2-CIE eff - calculated wavelength weighted UV doses (X) using the CIE erythema action spectrum
X mJ per cm^2-Waldmann - UV dose (X) computed using direct readings from a Waldmann UV detector device