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The Pathogenesis of Photoaging: The Role of Neutrophils and Neutrophil-Derived Enzymes

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The hallmark of photoaged skin is solar elastosis, which is probably an end product of elastic fiber degradation. Exposure of human skin to a certain threshold of UV, infrared radiation (IR), and heat leads to an influx of neutrophils. These neutrophils are packed with potent proteolytic enzymes capable of degrading collagen and, particularly, elastic fibers. Neutrophil-derived proteolytic enzymes are held responsible for the extracellular matrix (ECM) damage observed in several non-dermatological conditions. Furthermore, neutrophil elastase, a major product of neutrophils, is strongly associated with solar elastosis in mice. Taken together with our data that show *in vivo* proteolytic activity of neutrophil-derived elastase and matrix metalloproteinases (MMPs) in UV-exposed skin, we have hypothesized earlier that neutrophils are major contributors to the photoaging process. Although several groups have shown that MMPs are also induced in skin exposed to relatively low doses of UV, IR, and heat, clinical data indicate that high(er) doses of UV, IR, and heat are necessary to induce photoaging or photoaging-like pathology in the skin. Therefore, we propose that MMPs generated by suberythemogenic doses of UV and low doses of IR/heat are involved in cellular processes other than ECM degradation.

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CLINICAL AND HISTOLOGICAL CHARACTERISTICS AND THE ORIGIN OF ELASTOTIC MATERIAL: A HALLMARK OF PHOTOAGED SKIN

''Photoaging'' is the process by which sunlight or artificial UV gradually induces clinical and histological changes in skin. Typical photoaged skin is characterized by dryness, a rough texture, irregular pigmentation, telangiectasia, yellowish color, plaque-like thickening, deep creases, and fine wrinkles (Gilchrest, 1989). These clinical changes are reflected by histological changes in both the epidermis and 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 irregular pigmentation and roughening of the skin surface (Gilchrest, 1989).

The most conspicuous dermal histological defect, and the hallmark of photoaged skin, is the accumulation of elastotic material in the upper and middle dermis (so-called solar elastosis). By electron microscopy, fully developed photoaged skin shows alternating areas of fibrous, granular, and homogenous elastotic material (Braverman and Fonferko, 1982; Tsuji, 1984; Matsuta *et al.*, 1987). The fibrous areas consist of increased numbers of thickened and tangled elastic fibers. The granular and homogenous areas are believed to be the result of fragmentation of these thickened and tangled fibers. Although there is convincing evidence that the elastotic material is derived from elastic fibers, its origin has been the subject of much debate (Matsuta *et al.*, 1987). Other hypotheses include that the elastotic material could be (1) a degradation product of collagen fibers (Mitchell, 1967), (2) a degradation product of both collagen and elastic fibers (Niebauer and Stockinger, 1965), and (3) an abnormal product of UV-stimulated fibroblasts (Nurnberger *et al.*, 1978). Braverman and Fonferko (1982) 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. On the basis of electron microscopic studies and immunohistochemical staining patterns of multiple markers of the extracellular matrix (ECM), several groups have concluded that the elastotic material in photoaged skin must be derived primarily from degenerated elastic fibers (Chen *et al.*, 1986; Matsuta *et al.*, 1987).

Other observations in the upper and middle dermis of photoaged skin include the presence of deformed collagen fibers, a decrease in the total amount of collagen, increased amounts of ground substance, and dilated blood vessels (Oikarinen, 1990).

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Abbreviations: ECM, extracellular matrix; IR, infrared radiation; MED, minimal erythema dose; MMP, matrix metalloproteinase; SSR, solar simulating radiation

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THE ROLE OF UV-INDUCED REACTIVE OXYGEN SPECIES: DIRECT AND INDIRECT DAMAGE TO THE ECM

In vivo, under normal circumstances, low levels of reactive oxygen species are generated continuously. These reactive oxygen species are involved in signaling pathways, cell activation, cell proliferation, and cell differentiation (Sauer *et al*., 2001). The body defends itself against excessive endogenous or exogenous oxidative stress of this sort with antioxidant enzymes and non-enzymatic antioxidants. During UV exposure, however, this protective system can be overwhelmed, resulting in direct and indirect damage to cellular and extracellular components, including the ECM proteins (Sander *et al*., 2002). Furthermore, depending on the threshold of damage, oxidative stress can result in an influx of inflammatory cells including neutrophils (Scharffetter-Kochanek *et al*., 1997)

NEUTROPHIL INFLUX INDUCED BY ERYTHEMOGENIC BUT ALSO SUBERYTHEMOGENIC DOSES OF UV

Neutrophils are known for their destructive capacity and are considered to be responsible for the ECM damage observed in several conditions (Janoff, 1972; Weiss, 1989; Gadek, 1992; Golub *et al*., 1995). Furthermore, neutrophils have been reported to infiltrate the skin following exposure to erythemogenic doses of UVB, solar simulating radiation (SSR), and natural sunlight (Fisher *et al*., 2001; Rijken *et al*., 2004; Cho *et al*., 2008). There are several explanations concerning why neutrophils have received relatively little attention with respect to photoaging. First, 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 small. A neutrophil elastase staining or other markers for neutrophils are often necessary to appreciate the number of infiltrating neutrophils (Rijken *et al*., 2005). Second, the hypothesis by which keratinocyte- and fibroblast-derived MMPs are considered responsible for the ECM damage seen in photoaged skin (Fisher *et al*., 1997) has received wide support and may have diverted the attention from other possible mechanisms. Third, neutrophils and their products can only be detected in skin that has recently been exposed to UV. Finally, a certain threshold of damage is necessary for neutrophils to infiltrate the skin. This threshold, however, should not be overestimated. For example, depending on several factors, 15 minutes (or even less) of sun exposure can be enough for white-skinned persons to develop subtle sunburns (Samanek *et al*., 2006).

It has been established that a single erythemogenic dose of sunlight/UV is accompanied by infiltrating neutrophils (Rijken *et al*., 2004, 2005). Moreover, depending on the dose (which must not be too low) and the interval (which must not be too long), multiple suberythemogenic doses of sunlight can also lead to erythema and an influx of neutrophils. Novakovic *et al*. (2001) showed that exposure of white skin to a 0.75 minimal erythema dose (MED) of SSR for 4 consecutive days would produce the same intensity of erythema as one exposure to 3 MED of SSR. Lee *et al*. exposed white skin to 0.5 MED of SSR for 4 consecutive days, which led to erythema and an influx of neutrophils (manuscript in preparation

Most studies investigating the effect of chronic UV exposure on the skin of laboratory animals have not been focussed on infiltrating neutrophils. In addition, tissue samples in these studies may have been collected at a time when the neutrophilic infiltrate had already resolved. Furthermore, multiple ''suberythemogenic'' doses of UV can also induce a neutrophil influx in animal (mouse) skin (Gomi *et al*., poster presentation IID, 2008). Importantly, mouse skin exposed to ''erythemogenic'' doses of UV tends to show edema rather than erythema (van Weelden *et al*., 1988). In sum, we believe that it is important to use neutrophil-specific markers to determine the presence or absence of neutrophils in irradiated human or animal skin.

KERATINOCYTE- AND FIBROBLAST-DERIVED MATRIX METALLOPROTEINASES *VERSUS* NEUTROPHIL-DERIVED PROTEOLYTIC ENZYMES

A hypothetical model for the pathophysiology of photoaging has been presented by Fisher *et al*. (1996, 1997). 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*. (1996, 1997) postulated that this microscopic damage is caused by specific matrix metalloproteinases (MMPs). They showed that MMP-1, MMP-3, and MMP-9 are induced following exposure of human skin to suberythemogenic doses of UVB. These MMPs are capable of breaking down skin collagens. On the basis of the (1) localization of immunohistochemically stained MMPs, (2) *in situ* hybridization 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, however, not based on co-localization of MMPs and a specific marker for fibroblasts, but 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 located mostly in the epidermis and MMP protein was also induced mainly in this compartment (Fisher *et al*., 1997). They concluded that keratinocytes are the major source of MMPs. However, the ECM damage observed in photoaged skin is located mostly in the upper and middle dermis. To explain this, they proposed that keratinocyte-derived MMPs diffuse from epidermis into the dermis, where most of the MMP enzyme activity had been detected. In a separate study, Fisher *et al*. (2001) 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.

More recently, our group has suggested that neutrophils, rather than keratinocytes and fibroblasts, may be the key players in photoaging (Rijken *et al*., 2006). In a series of

experiments we showed that, following exposure of white skin to erythemogenic doses of SSR, infiltrating neutrophils and not keratinocytes or fibroblasts were the major sources of proteolytic enzymes (particular MMPs and neutrophil elastase, the latter being quantitatively most prominent). Furthermore, prominent *in vivo* protein staining and *in situ* enzyme activity was detected only in skin exposed to erythemogenic doses and not in skin exposed to suberythemogenic doses of SSR (Rijken *et al*., 2004, 2005).

Despite a significant amount of literature and clinical data that directly or indirectly supports our view (Janoff, 1972; Weiss, 1989; Nathan, 2006), neutrophils had only been reported as likely important contributors to photoaging in a murine model (Starcher and Conrad, 1995). Neutrophils are the most abundant white blood cell and, as mentioned above, following a certain threshold of tissue damage, they leave the bloodstream rapidly and migrate toward sites of tissue damage. A major product of activated neutrophils and an important immunohistochemical marker is neutrophil elastase (Shapiro, 2002). Together with cathepsin G and proteinase-3, neutrophil elastase belongs to the group of serine proteases produced by neutrophils. These serine proteases are normally rapidly and irreversibly inhibited by effective plasma antiproteinases (α1-proteinase inhibitor, α2-macroglobulin, and secretory leucoproteinase inhibitor) present in the blood and the interstitium (Weiss, 1989). Neutrophil elastase is a potent proteolytic enzyme capable of degrading both elastic and collagen fibers (Travis, 1988). The biological functions of neutrophil elastase include involvement in migration of neutrophils by means of focal proteolysis, killing of microorganisms, and degradation or activation of various proteins, including ECM proteins, receptors, cytokines, and chemokines (Shapiro, 2002; Chua and Laurent, 2006).

Neutrophils have been shown to express MMP-8, MMP-9, and MMP-12 (Murphy *et al*., 1980; Ilumets *et al*., 2007), and in our studies MMP-1 also co-localized with neutrophil elastase in infiltrating cells following SSR exposure (Rijken *et al*., 2005). Although, similar to other cells including fibroblasts, these proteolytic enzymes are normally stored in vesicles in an inactive form, our studies provide convincing evidence that they are, in fact, enzymatically active in UV-exposed skin (Rijken *et al*., 2004, 2005; Chua and Laurent, 2006). The probable mechanism by which neutrophil-derived proteolytic enzymes are activated (and/or prevented from being inactivated by antiproteinases) and cause ECM damage is reviewed by Weiss (1989) and Chua and Laurent (2006). It involves complex interaction with nicotinamide adenine dinucleotide phosphate oxidase-derived oxygen metabolites, which inactivate antiproteinases and activate MMPs, and it occurs at or just outside the plasma membranes of neutrophils. It is likely that in this setting, neutrophil-derived enzymes, particularly neutrophil elastase, cause ECM damage that may eventually result in solar elastosis.

Although the role of neutrophils has been under-rated in photoaging, neutrophils and their products have been identified as a major cause of tissue destruction in several other conditions, including lung emphysema, rheumatoid arthritis, periodontitis, and wound infection (Gadek, 1992; Golub *et al*., 1995; Edwards and Hallett, 1997; Singer and McClain, 2002). The important role of neutrophil elastase in photoaging of mouse skin has convincingly been shown by Starcher and Conrad (1995), who investigated the effect of chronic UV exposure on neutrophil elastase-deficient mice. Neutrophil elastase and wild-type mice were exposed to a combination of UVA/UVB, three times a week, for a period of 6 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.

In humans, black skin is much less susceptible to sunburn and photoaging than white skin, most likely due to differences in pigmentation (Taylor, 2002). A higher melanin content and a different melanosomal dispersion pattern in black skin provide better protection against the damaging effects of sunlight and increase the physical dose of sunlight necessary to induce a sunburn. As mentioned above, sunburn is accompanied by an influx of neutrophils. Thus, in black skin, the threshold for neutrophils to begin infiltrating the skin is increased and, as a consequence and in accordance with our model, photoaging would be delayed. Taken together, direct and indirect evidence suggests that neutrophils and neutrophil-derived proteolytic enzymes are likely to contribute to the process of photoaging.

UV-INDUCED MMPs DERIVED FROM DIFFERENT CELL TYPES: ECM DEGRADATION OR OTHER (EXTRA)CELLULAR FUNCTIONS?

In our studies, significant amounts of MMPs were induced only in skin exposed to erythemogenic doses of SSR, and these MMPs were linked to infiltrating neutrophils (Rijken *et al*., 2004, 2005). Lahmann *et al*. (2001) also found that MMP-1 mRNA was induced only by erythemogenic doses of SSR. However, it has been shown that specific MMPs are induced by suberythemogenic doses of UVB and by combinations of infrared radiation (IR)/visible light, and even by solar heat alone (Fisher *et al*., 1996; Cho *et al*., 2008). These MMPs are probably keratinocyte and/or fibroblast derived, as there are very few or no infiltrating neutrophils at this stage.

It is generally assumed that these keratinocyte- and/or fibroblast-derived MMPs are major contributors to photoaging. We here suggest that sunlight-induced keratinocyte-derived MMPs may actually be involved in cellular processes other than ECM damage.

Recently, Cho *et al*. (2008) showed that significant amounts of MMP-1 and MMP-9 mRNA and protein (up to 90% of the expression in non-protected sunlight-exposed skin) are induced in sun-exposed, cloth-covered skin. This indicates that solar heat induces MMP-1 and MMP-9. However, in their experiments, solar heat did not induce an erythema reaction, and consequently neutrophils were not detected in solar heat-exposed skin. 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. Therefore, solar heat-induced MMPs most likely serve another function.

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. 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 (Figure 1) (Lookingbill *et al.*, 1995). 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 signs of photoaging. On the basis of the experiments of Cho *et al.* (2008), one would expect to find MMP induction in solar heat-exposed, cloth-covered skin of albino patients, but apparently these MMPs do not contribute to photoaging.

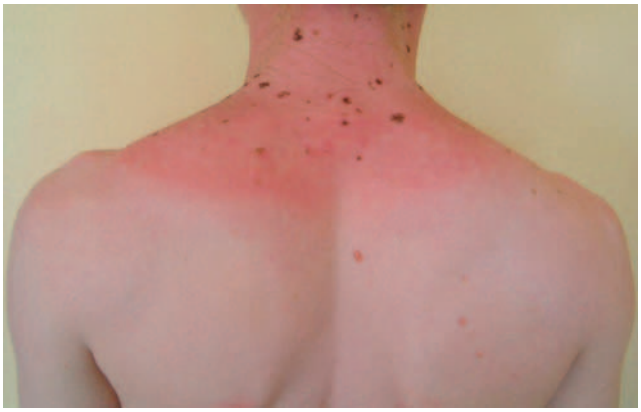

Figure 1. **An albino patient showing sunburn and photoaging in the neck region.** Note that solar heat-exposed but UV-protected, cloth-covered skin shows no signs of acute or chronic photodamage.

Incidentally, papers propagating the role of (solar) heat or IR in photoaging often refer to the 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 (Finlayson *et al.*, 1966; Chan and Chiu, 2007). However, contrary to the doses involved in sun-exposed, cloth-covered skin, 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 (Finlayson *et al.*, 1966). Therefore, also in this condition, neutrophil-derived proteolytic enzymes may be responsible for the histopathological changes that are present.

Besides their function in ECM remodeling, MMPs are known to be involved in signal transduction and, particularly, chemokine and cytokine (in)activation (Parks *et al.*, 2004; Pardo and Selman, 2005). For example, MMP-1, -2, -3, -7, -9,

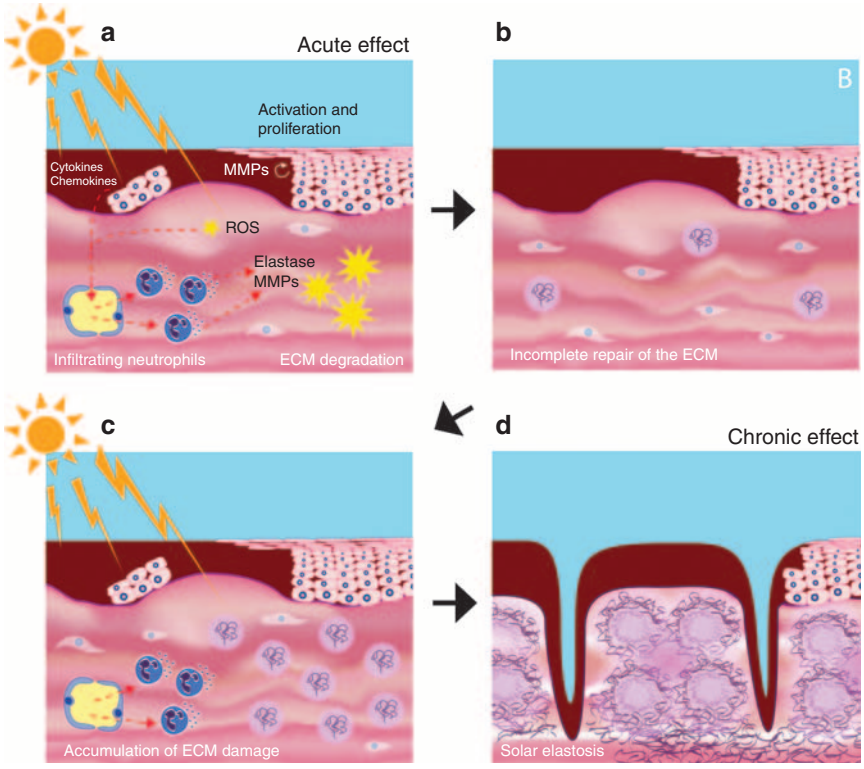

Figure 2. **The pathogenesis of photoaging: we propose that, besides directly induced ECM damage by UV-induced reactive oxygen species, neutrophil-derived proteolytic enzymes are the most important contributors to the ECM damage seen in photoaged skin.** (**a**–**d**) We further propose that UV-, infrared (IR)-, and solar heat-induced keratinocyte-derived matrix metalloproteinases (MMPs) play more important roles in cellular processes other than extracellular matrix (ECM) damage (**a**).

-12, and -17 can activate tumor necrosis factor (Parks *et al*., 2004) *in vitro,* and MMP-9 cleaves desmoglein on apoptotic keratinocytes (Cirillo *et al*., 2007). Parks *et al*. (2004) state 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.''

Taking all of this information together, we believe that keratinocyte-derived MMPs may be involved in other cellular processes (such as cell maturation, activation, migration, and proliferation) rather than in ECM degradation, whereas neutrophil-derived MMPs are the most likely candidates for the latter activity.

SUMMARY

Many studies investigating the pathophysiology of photoaging focus on the induction of MMPs and collagen degradation. However, the most conspicuous histopathological change observed in photoaged skin is related to the elastic and not the collagen fiber network. MMPs have been shown to be induced by suberythemogenic doses of UV, IR, and solar heat. However, clinical data do not support a major contribution of low doses of UV (for example, sun-exposed black skin and ventral forearm skin, which are regularly exposed to suberythemogenic doses of UV but show little or no solar elastosis), IR, and solar heat to the photoaging process. In fact, clinical data indicate that high(er) doses of UV are necessary to induce photoaging. Furthermore, high doses of IR/heat can lead to solar elastosis-like pathology. Exposure of skin to higher doses of UV (for example, subtle sunburn) and IR/heat (for example, erythema ab igne) leads to an influx of neutrophils that are packed with collagen and elastic fiber-degrading proteolytic enzymes. Neutrophil elastase, a major product of neutrophils, is an especially potent proteolytic enzyme, and it is associated with solar elastosis in mice. In humans, neutrophil-derived proteolytic enzymes are held responsible for the ECM damage observed in lung emphysema, rheumatoid arthritis, wound infection, and other conditions. Here we propose that, besides directly induced ECM damage by UV-induced reactive oxygen species, neutrophil-derived proteolytic enzymes are the most important contributors to the ECM damage seen in photoaged skin (Figure 2). We further propose that UV-, IR-, and solar heat-induced keratinocyte-derived MMPs may play more important roles in cellular processes other than ECM damage.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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