Acta Dermatovenerol Croat 2007;15(2):65-71 ORIGINAL SCIENTIFIC ARTICLE

Increase in Dermal Collagen Fibril Diameter and Elastogenesis with UVB Exposure: an Optical and Ultrastructural Study in Albino Balb/c Mice

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SUMMARY Cutaneous aging is a complex biological phenomenon, dependent not only on the innate or intrinsic process ("biological clock"), but also on extrinsic elements, primarily chronic sun exposure (photoaging). In order to verify dermal morphological changes in the elastic fiber system and collagen associated with aged skin, we performed a light and electron microscopic study on exposed-shaved albino mice, which were exposed to UVB radiation. The experimental group consisted of 48 exposed animals, randomly distributed in three groups and submitted to different radiation doses (A, 28800 J/m^2; B, 57600 J/m^2; and C, 86400 J/m^2) and studied 0, 30, 60 and 90 days of exposure discontinuation. Nonexposed-shaved and nonexposed-nonshaved animals were included as controls. From the day of exposure discontinuation and subsequently, the elastic system and collagen network were progressively modified. The increase in collagen fibril diameter was prominent in the 60 and 90 day groups ($p<0.05$), as noticed on electron microscopy. Elastic fiber density also increased after irradiation ($p<0.05$). On electron microscopy, elastogenesis was seen in the deep dermis.The comparative study among the groups disclosed clear relationship between doses and "elastotic changes". It also showed that chronological aging of mice skin was apparently intensified after UVB exposure. Skin elastogenesis seems to be a major consequence of UVB exposure, apart from elastolysis, and occurs not only in humans but also in hairless mice submitted to continuous, long-term UVB exposure.

KEY WORDS: skin aging, connective tissue, elastic tissue, ultraviolet ray

INTRODUCTION

Cutaneous aging is a complex biological phenomenon which depends not only on the innate or intrinsic process ("biological clock") but also on extrinsic elements, mainly chronic sun exposure (photoaging). As a result of intrinsic modifications, aged human skin has a characteristic fine wrinkling and appears smooth while photoaged skin shows a variety of clinical manifestations, includ-

ing coarseness, wrinkling, telangiectasia, irregular pigmentation, increasing fragility, impaired wound healing, and neoplasia (1). Alterations in collagenous proteins, the major component of skin, have been suggested as the cause of the clinical changes observed in photoaged and naturally aged skin (2,3).

It has been demonstrated in naturally aged skin that the distribution of type I and III collagens was altered only after the 8th decade, while in photodamaged skin the relative staining density of both decreased from the 5th decade on (4). Deposition of fibrillar collagen in unexposed aged skin has been described (5), as well as an increased expression of procollagen alfa-1 mRNA in sun-exposed skin, as seen in biochemical assays. However, this could be counterbalanced by the high level of metalloproteinase-1, which would result in a diminished protein level (6). Differently, Talwar *et al.* (7) and Varani *et al.* (8) demonstrated a reduction of type I collagen synthesis. Despite it, relatively little attention has been given to the collagen network structure.

Photochemical reactions take place immediately after the absorption of UVB radiation (9), but biological responses are usually observed many years later (10). In fact, systematic investigations in humans have not been done, obliging the use of animal models in order to understand the kinetics of the ongoing lesions in skin aging as well as its morphological consequences. Albino hairless mice have been used in many studies (11-16) and are considered a model for UV-induced changes because of the similarity with human sun-exposed skin (11). Moreover, unlike humans, UV dosimetry can be monitored accurately (9,13).

The present investigation addressed the question of morphological and ultrastructural changes in the elastic system and dermal collagen after exposure to different UVB doses and further modifications of these changes on days 0, 30, 60 and 90 of UVB discontinuation.

MATERIALS AND METHODS

UVB irradiation

Forty eight albino Balb/c mice (six to eight weeks old) were dorsally irradiated three days a week (Monday, Wednesday and Friday) with Hanovea hot quartz lamp with a spectral range of 290-320 nm of UVB and peak output at 302 nm. The minimal erythema dose was 480 J/m^2, and doses up to 6000 J/m^2 were not able to cause more than mild redness. Exposure rate was 16 J/m^2 of UVB-rays at a distance of 50 cm, on a 6-minute exposition - 5760 J/m^2. The radiometer used was an UVB-550 C model (National Biological Corporation, Twensburg, Ohio, USA). Animals were housed in conventional cages (4 *per* cage) on litter and given commercial rodent chow and water *ad libitum*. Each week animals were shaved on the dorsum with a razor blade three times a week (Tuesday, Thursday and Saturday) and submitted to radiation which varied from 12 (group A, total dose of 28,800 J/m^2) through 24 (group B, total dose of 57,600 J/m^2) to 36 days (group C, total dose of 86,400 J/m^2). Another 16 mice (group D) were also shaved but not exposed to UVB, and four animals were neither shaved nor irradiated (group E). All animals were sacrificed under anesthesia and skin samples were collected and processed for histopathologic analysis and ultrastructural studies. Biopsies were obtained on days 0, 30, 60, and 90 of the last UV exposure for each UV dosage group.

Skin samples

Two skin samples were obtained from irradiated area for both light and electron microscopy analysis. One specimen of each animal was fixed in 10% formalin and embedded in paraffin. Sections (5 μm) were stained with hematoxylin-eosin and modified sirius red (17).The other specimen was fixed in 2% buffered glutaraldehyde solution, post-fixed in 1% buffered osmium tetroxide, dehydrated and embedded in epoxy resin (Embed-812, Electron Microscope Sciences, Fort Washington, Pennsylvania, USA). Semi-thin sections were stained with a 1% alkaline toluidine blue solution. Areas of skin containing epidermis and dermis were considered for ultra-thin sectioning.

Histomorphometry (surface density of dermal elastic system)

Elastic fibers of previously oxidated orcein sections were measured at 400x magnification on photomicrographs taken with a Nikon camera attached to a light microscope. Fifteen fields of both superficial and reticular dermis of all samples were measured using computer based Image-Pro Plus software (Cybernetics).

Ultrastructural morphometry

Collagen fibril diameters from groups C, D and E were measured on electron micrographs obtained on a Carl Zeiss EM-9S transmission electron microscope, at 4700x magnification, positive print enlarged 3.9x. They were scanned in a scanner of high quality and images were measured using computer

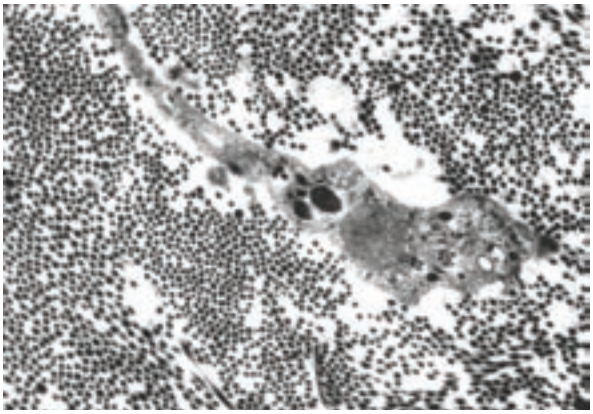


Figure 1. Electron micrograph of non-irradiated dermis of 90 day mice showing enlarged electron-lucent areas which contained flocculent material or had an ill-defined boundary, with a diffuse distribution of fibrils. (x4700, original magnification)

based Image-Pro Plus software. Five measures of the thinner and the thickest transverse diameters of fibrils present in collagen bundles were obtained.

Statistical analysis

Measures obtained from each group were analyzed by Student's t-test when data had normal distribution or by Mann-Whitney rank sum test when the normality test failed. One-way ANOVA test was used to compare more than two groups. One-way Ranks-Kruskal Wallis analysis of variance was used to compare more than two groups when the normality test failed. Differences were considered significant at $p<0.05$.

RESULTS

Chronological aging

Non irradiated and irradiated skin presented progressive diminution of thickness with time, an aspect depicted on HE stained slides. All specimens showed almost complete lack of inflammatory cells or vascular congestion. Moreover, sirius red staining showed progressive compactness of collagen bundles in all groups. Comparing these shaved animals to age-matched normal ones, it was possible to recognize and characterize the focal, rare, scar zones induced by shaving. These scars characteristically exhibited a zone of accumulated collagen fibers oriented in parallel to the epidermis major axis and lacking elastic fibers. In fact, all observations related to photodamaged skin were done in zones distant from scars.

Ultrastructural study of non-irradiated groups revealed loose organization of collagen bundles in

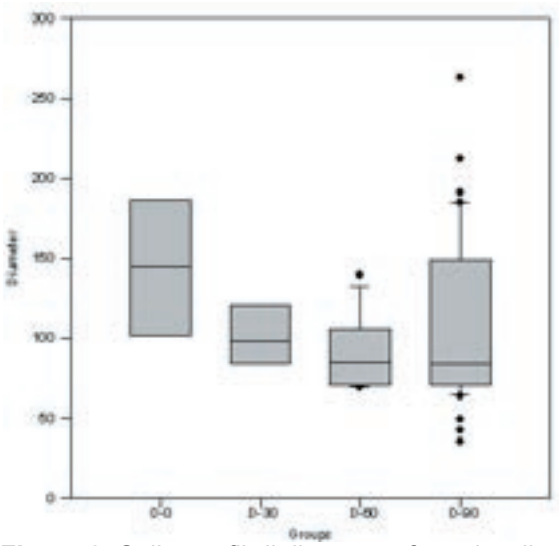


Figure 2. Collagen fibril diameter of non-irradiated group ($p>0.05$).

papillary dermis, in contrasting to densely packed bundles present in reticular dermis. Despite the apparent dense organization of collagen fibers, these were either separated by enlarged electron-lucent areas which contained aggregated 10 nm microfibrils and flocculent material or had an ill-defined boundary, with a diffuse distribution of fibrils (Fig. 1). UVB irradiated groups showed a slight progressive disorganization of extracellular matrix in reticular dermis. Collagen bundles, in this localization, had varied diameters, and were separated by electron-lucent zones, sometimes lacking the bundle arrangement similar to that seen in aged skin.

Transverse sections of group D-0 (non-irradiated animals) collagen bundles showed homogeneous rounded structures, with apparent similar diameters which ranged from 97 to 191.4 nm. With time, collagen bundles showed some heterogeneity in collagen fibril diameter, ranging from 35 to 263 nm, the differences being not statistically significant ($p>0.05$) when compared with group D-0 mice, with major modifications occurring in 90 day-mice (Figs. 2 and 3). Moreover, the most prominent change was the irregularity in collagen fibril shape; angulated irregular structures were seen interspersed between the round ones (Fig. 4).

Elastic fibers were only present in reticular dermis and consisted of irregular outlined electron-dense structures, intermingled among collagen fiber bundles. In the upper dermis, elaunin fibers were observed, and consisted of smaller structures, with less electron-dense core surrounded by 10-12 nm microfibrils. On the other hand, oxytalan

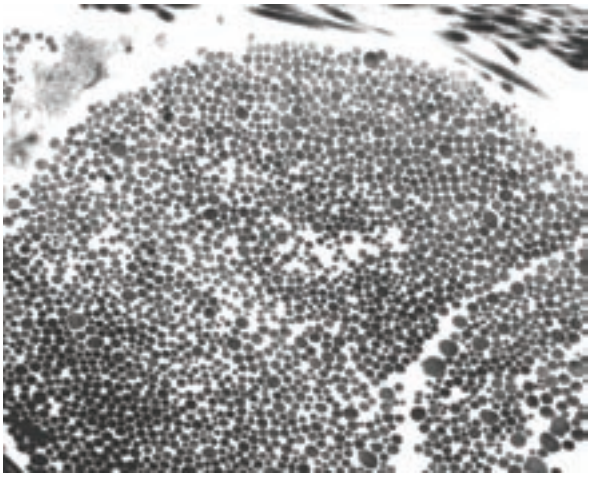


Figure 3. Electron micrograph of irradiated dermis of 90 day mice showing irregularity in collagen fibril shape, and heterogeneity of collagen fibril diameter. (x4700, original magnification)

fibers, constituted only of microfibrils, were present in papillary dermis near to the dermoepidermal junction. Elastolysis images and modifications of any component of the elastic fiber system were never seen in this group.

UVB irradiated skin

Slight progressive skin atrophy was also observed in this group of animals. Neither oxytalan nor elaunin fiber plexus were modified, whereas thick, shorter orcein reactive fibers, present in deep reticular dermis, were increased in number, an aspect more evident in group C animals ($p<0.05$) (Figs. 5a and 5b). Collagen fibril diameters, in UVB irradiated group, ranged from 70 to 269 nm; these extreme values were recorded in 90 day animals (C-90). Significant changes in collagen fibril diameter were encountered in 60 and 90 day of irradiated mice ($p<0.05$), when compared to 60 and 90 day controls (Figs. 6 and 7). Ultrastructural study showed slight progressive disorganization of extracellular matrix in reticular

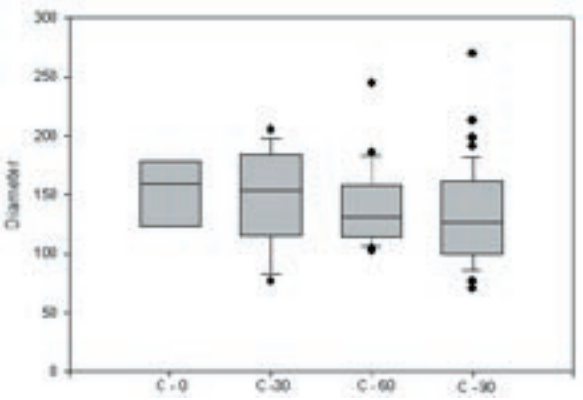
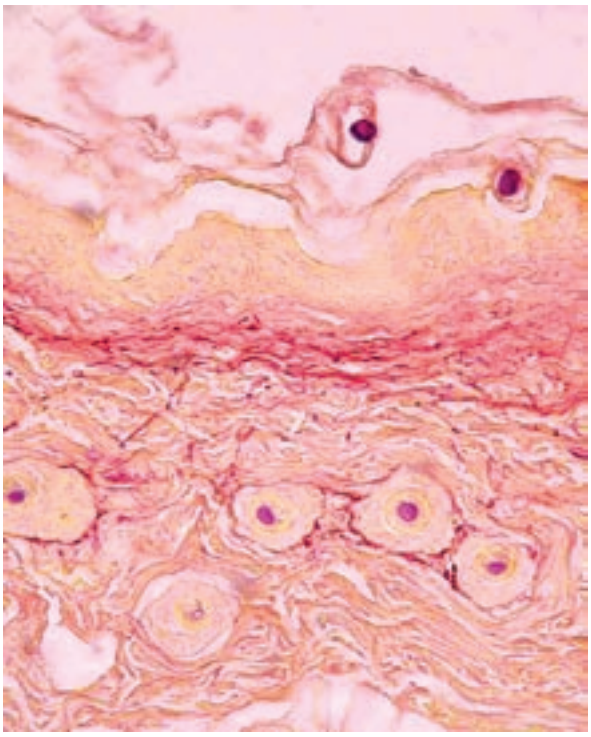


Figure 4. Collagen fibril diameter of group C after UVB irradiation, total dose 86,400 J/m^2 ($p>0.05$).



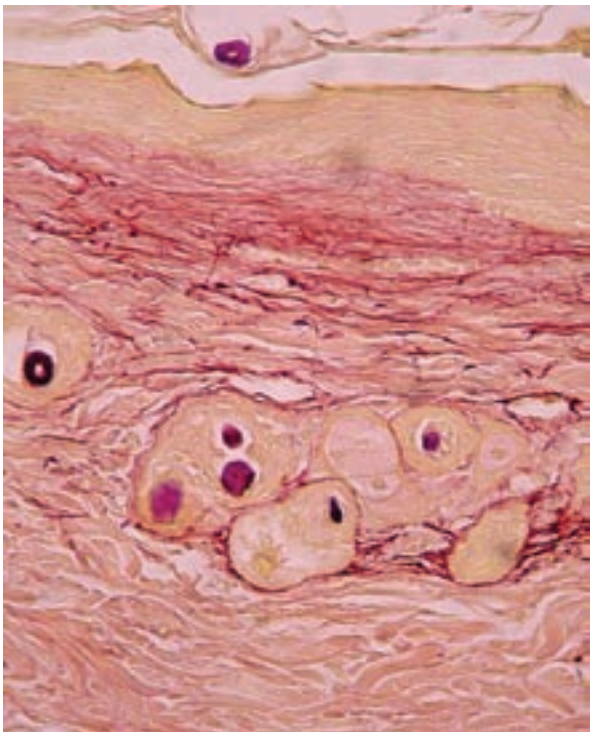


Figure 5a and b. Photomicrograph of non-irradiated (left) and irradiated (right) of 90 day mice: skin showing an increased number of elastic fibers in the irradiated group. (orcein, x400)

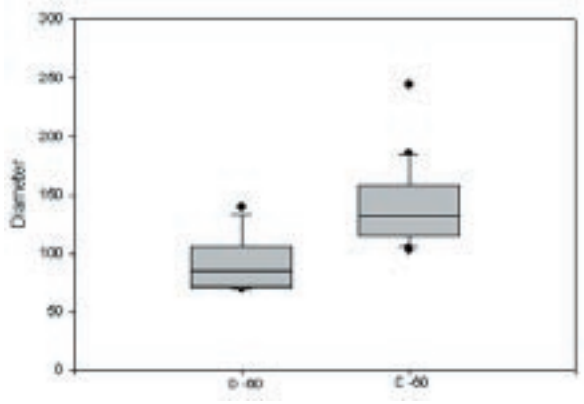


Figure 6. Collagen fibril diameter after 60 days of UVB discontinuation in irradiated group (C-60) and non-irradiated group (D-60) ($p<0.05$).

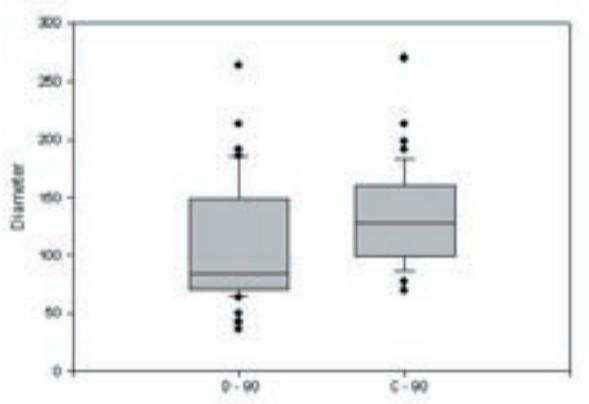


Figure 7. Collagen fibril diameter after 90 days of UVB discontinuation in irradiated group (C-90) and non-irradiated group (D-90) ($p<0.05$).

dermis. Electron-lucent zones present between collagen bundles were also depicted in irradiated animals. The increase in the amount of microfibrillar material inside the electron-lucent areas present between collagen bundles was one of the most prominent modifications in this group. Neither oxytalan nor elaunin fibers were modified in structure or number in this group. The prominent finding was seen in deep reticular dermis where an increased elastogenesis was evident (Fig. 8).

DISCUSSION

The present study investigated histological and ultrastructural alterations in dermal collagen of albino mice after controlled doses of UVB, as well as the ongoing alterations after UVB discontinuation, allowing us to observe aspects of both chronological and photo aging. We found the collagen metabolism to be profoundly altered after UVB irradiation and that it was progressive even after UVB discontinuation. Our results demonstrated that shaved Balb/c mice submitted to UVB irradiation exhibited altered structure and organization of the deep dermis in part similar to the modifications seen in hairless skin dermis, the animal model of photodamage, and in human skin. Our results demonstrated that shaved Balb/c mice submitted to UVB irradiation had an altered structure and organization of the deep dermis and fragmentation of elastic fiber network. In photodamaged skin, we could observe not only the aging induced changes but also those related to UV irradiation. Elastogenesis comprised the appearance of new slender fibers constituted by a dense core of elastin surrounded by microfibrils immersed among collagen bundles and elastic fibers. Morphometric study showed a significant increase of elastic fibers in UV irradiated skin, as also demonstrated elsewhere (2,18). It is possible that the increase in the production of tropoelastin, its deposition and degradation in the upper dermis probably contribute to the accumulation of elastotic materials in photodamaged skin. Skin elastogenesis seems to be a major consequence of UVB exposure, apart from elastolysis, and occurs not only in humans but also in hairless mice submitted to continuous, long-term UVB exposure (18-20).

Previous studies describe an increase in the number and function of fibroblasts as a result of UV radiation, with enhanced collagen synthesis and thickening of the dermis (21,22), a finding that may be indicative of variable amounts of collagen degradation, alterations in collagen production, or both. Studies concerning changes in collagen production in mice as a result of UV exposure have shown variable findings. Increases (23) and decreases (24) in collagen in response to UV exposure have been reported in mouse models. Klig-

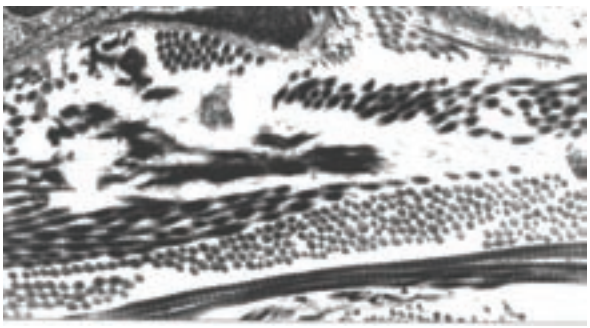


Figure 8. Electron micrograph of irradiated dermis of 90 day mice: smaller, irregular, electron-dense fibers surrounded by microfibrils similar to elaunin fibers seen amongst collagen fiber bundles. (x4700, original magnification)

man *et al.* (25) found that collagen production in mice increased initially (4-16 weeks) after chronic UV exposure, and returned to control levels at week 20. This could be a result of enzymatic digestion by collagenase secreted by cells of the inflammatory infiltrate, and the degradation of collagen could finally exceed the capacity of the UV-irradiated fibroblasts to synthesize new collagen. In our experiment, when comparing the UVB exposed groups with non-exposed ones, there was a significant increase in the collagen fibril diameter after 12 weeks (90 days) in the irradiated group. This is in accordance with the early increase in collagen production observed by Kligman *et al.* (25).

In our study, individual measures performed on ultrastructural assessment were progressively heterogeneous in both non-irradiated and irradiated groups. The mean diameter of collagen fibrils decreased with time in groups C and D. Despite this diminution, the collagen bundles seemed more compact on light examination. Alterations in extracellular matrix components such as a decrease in the levels of glycosaminoglycans, could explain this apparently paradoxical finding. The balance between collagen synthesis and degradation, after chronic UVB exposition, could have disturbed the relatively inert metabolism of collagen bundles in the deep dermis, favoring the extracellular matrix modifications observed.

The heterogeneity of collagen fibril diameter, an aspect more prominent after UV discontinuation, could be due not only to the metabolic disarrangement of collagen turnover but also to impairment of collagen fibrillogenesis. The role of proteoglycans in the regulation of collagen fibrillogenesis and the ability of decorin to influence collagen fibrillogenesis and fibril diameter are well known. Age related changes in the proteoglycans of human skin could be therefore responsible for the altered collagen fibril diameter (26).

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