Collagen Cross-Linking in Sun-Exposed and Unexposed Sites of Aged Human Skin

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A recently described nonreducible, acid-heat stable compound, histidinohydroxylysinonorleucine (HHL), is a collagen cross-link isolated from mature skin tissue. Its abundance is related to chronologic aging of skin. The present communication describes the quantity of HHL from aged human skin of the same individuals in sun-exposed (wrist) and unexposed (buttock) sites. Punch biopsies were obtained from these sites from nine people of age 60 or older. HHL contents (moles/mole of collagen) at these sites were for wrist 0.13 ± 0.07 and for buttock 0.69 ± 0.17 (mean $\pm$ SD, $p < 0.001$). In addition, it was found that acute irradiation of the cross-linked peptides with UVA (up to 250 J/cm^2) and UVB (up to 1 J/cm^2) had no effect on HHL structure. The same treatment significantly degraded another nonreducible, stable collagen cross-link, pyridinoline. The results suggest that chronic sunlight exposure may be associated with an impediment to normal maturation of human dermal collagen resulting in tenuous amount of HHL. Thus, the process of photoaging in dermal collagen is different from that of chronologic aging in human skin. *J Invest Dermatol 97:938–941, 1991*

Ultraviolet light has profound effects upon dermal connective tissue of human skin [1–6]. Most of these studies have emphasized the dramatic changes that occur in mucopolysaccharides (proteoglycans) and elastic fibers. Light microscopy of photodamaged skin reveals thickened, tangled accretions of degraded elastic fibers [4,6]. The concentration of an elastin cross-link, desmosine, is increased over fourfold in sun-exposed skin compared to unexposed sites in the same person [7].

Less is known about the effect of ultraviolet irradiation upon dermal collagen in human skin. Previous studies have shown that chronic exposure to ultraviolet light could lead to biochemical [1] and ultrastructural changes [4] in dermal collagen. Smith et al [2] also suggested that there was evidence for alteration or "degradation" of insoluble collagen.

Collagen intermolecular cross-links are essential for providing collagen matrices with stability and tensile strength. Because cross-linking is influenced by many factors within tissues (e.g., degree of post-translational modifications, specific molecular packing arrangements, turnover rate, etc.), the cross-linking pattern is tissue specific and may reflect the structural status of the collagen fibrils within a tissue [8,9].

Histidinohydroxylysinonorleucine (HHL) is a newly described stable collagen cross-link found in skin [10] but is absent from other major connective tissues. It is derived from Lys^{ald} (lysine aldehyde, 5-amino-5-carboxypentanal), Hyl, and His of type I collagen (Fig 1) and is a maturational product of an iminium cross-link, dehydro-hydroxylysinonorleucine (deH-HLNL) [10]. Our previous studies have demonstrated that the HHL content in skin tissues increases with chronologic age of the test subject [11]. It has also been shown that HHL is formed in embryonic bovine skin collagen upon in vitro incubation of the tissue [10]. These in vivo and in vitro studies indicate that HHL is an age-related collagen cross-link in skin tissue.

Because alterations in dermal collagen by chronic UV light exposure have been suggested, we examined the content of HHL in human skin that was unexposed (buttock skin) and exposed (wrist skin) to UV irradiation in nine subjects over the age of 60 (five males and four females). In addition, the direct effect of UVA and UVB irradiation on HHL in vitro was studied by using the partially purified cross-linked peptides and compared with that of another nonreducible stable cross-link, pyridinoline.

MATERIALS AND METHODS

Human Skin Samples Normal human adult volunteers were recruited for skin biopsies from sun-exposed and sun-unexposed sites after approval from the University of North Carolina Committee on the Rights of Human Subjects. Nine Caucasian adults (five males and four females) between the ages of 61 and 69 participated in the study. None of the subjects were on medications or had significant medical problems. Identical biopsy sites on the buttocks and the dorsum of wrists were mapped in all patients. The skin sites were cleansed with Hibiclens soap and alcohol and then anesthetized with an intradermal injection of 0.2 ml of 0.5% xylocaine, 1 : 100,000 epinephrine. A 4-mm punch biopsy was obtained from each site, washed briefly in ice cold sterile saline, and immediately frozen in liquid nitrogen. Each sample was coded and subjected to cross-link analysis.

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Abbreviations:

deH-HLNL: dehydro-hydroxylysinonorleucine
ELISA: enzyme-linked immunoadsorbant assay
HHL: histidinohydroxylysinonorleucine
His: histidine
Hyl: hydroxylysine
Hyp: hydroxyproline
Lys^{ald}: lysine aldehyde (5-amino-5-carboxypentanal)
MED: minimal erythema dose
TES: N-trismethyl-2-aminoethanesulfonic acid
UVA: ultraviolet A
UVB: ultraviolet B

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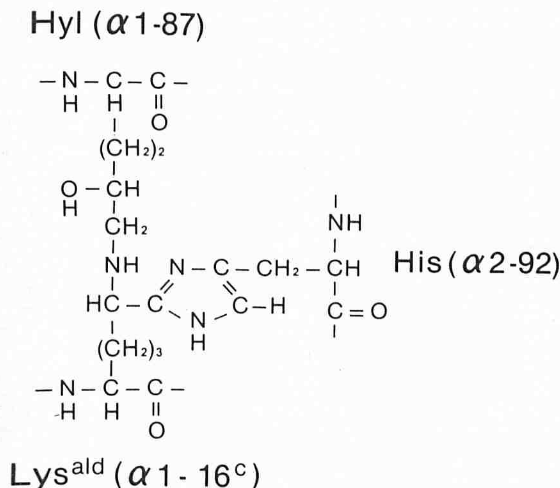

Figure 1. Structure of histidinohydroxylysinonorleucine (HHL). The cross-link is derived from Lys^ald (α1-16^C), Hyl (α1-87), and His (α2-92) of mature skin type I collagen molecules [10,14].

Cross-Link Analysis HHL cross-link was analyzed by quantitative amino acid analysis as previously described [11]. Briefly, skin samples were cut into small pieces by a razor blade while cooled on ice. The pieces were washed with 0.015 M N-trismethyl-2-aminoethanesulfonic acid (TES) buffer, pH 7.4, and then with distilled water by repeated centrifugation at 5000 × *g* at 4°C for 20 min and then lyophilized. The dried samples were hydrolyzed with 6 N HCl in vacuo in an N_2 atmosphere for 24 h at 110°C. The hydrolysates were evaporated by speed vacuum centrifugation (Savant Instruments, Inc.) and the residues were dissolved in 0.5–1.0 ml of distilled water. After the sample solutions were filtered, Hyp and HHL contents of each sample were directly quantified by an amino acid analyzer (Varian 5560 liquid chromatography, AA911 column, Interaction) using a modified gradient system described previously [12]. The ninhydrin reactant was monitored by absorbance at 440 nm to quantify Hyp and at 570 nm for HHL. The wavelength change from 440 to 570 nm was set at 65 min (elution time of Hyp and HHL were at 16.5 min and 76.4 min, respectively). In order to confirm the values for HHL by this system, HHL content of some samples was also measured by the second independent method that has been described previously [11]. Briefly, after Hyp in the hydrolysate was determined, an aliquot was applied to the standardized P-2 column (1.5 × 55 cm, 400 mesh) equilibrated with 0.1 N acetic acid to remove the bulk of amino acids. Fractions that encompassed the elution position of standard HHL [10] were pooled, dried, dissolved in distilled water, and applied to the amino acid analyzer. HHL content in both cases was quantified based on its ninhydrin color factor obtained from the amino acid composition of an apparently pure tryptic peptide cross-linked by HHL [13,14]. HHL content from each sample was quantified as moles per mole of collagen based on the value of 300 residues of Hyp per collagen molecule. Elastin cross-link standards, isodesmosine and desmosine, were obtained from Elastin Products Company, Inc., as well as from the hydrolysate of purified elastin prepared in our laboratory [11]. The means of HHL contents in both sites were compared by using the Student t test and $p < 0.001$ as the significant level.

Irradiation of Cross-Links with UVA and UVB The peptides cross-linked by HHL were partially purified from a tryptic digest of 9-year-old bovine skin collagen by means of Sephadex G-50 superfine column chromatography [13,15]. The fraction that encompassed the major HHL cross-linked peptides [13,14] was desalted by a P-2 column and lyophilized. The sample that contained approximately 40 to 50 nanomoles of HHL cross-linked peptides was dissolved in 1.0 ml of phosphate-buffered saline, pH 7.4, and filtered. For comparison, the pyridinoline cross-linked peptides were also prepared from a tryptic digest of 2-year-old bovine bone collagen [15] and about the same amount of the cross-linked peptides was subjected to the same treatment. An aliquot was removed from the filtrate as control and an equal amount of cross-linked peptide was dispensed into five wells of an ELISA titer plate. These sample solutions were directly irradiated with a Spectra Mini B ultraviolet light unit (Daavlin Company, Bryan, Ohio) equipped with FS24T12-ERE-OH lamps covered with window glass for UVB irradiation or a Spectra Mini A ultraviolet light unit (Daavlin Company, Bryan, Ohio) equipped with Votarc F24T12-BL-OH lamps and covered with window glass for UVA irradiation. The intensity of the lamps was measured by an International Light 443 radiometer at a distance of 20.2 cm, the distance between the lamps and the ELISA plates. The output of the UVB lamps was 2.2–2.3 mW/cm^2, whereas that of the UVA lamps was 12 mW/cm^2. The ELISA wells were irradiated with 0, 70, 140, 210, 280, and 1000 mJ/cm^2 of UVB or 50, 100, 150, and 250 J/cm^2 of UVA. The sample in each well was collected after each dose of irradiation, lyophilized, and hydrolyzed with 6 N HCl in the same manner described above. The hydrolysates were dried by a Speed Vac Concentrator (Savant), dissolved in distilled water, and assayed for HHL and pyridinoline by the amino acid analyzer (vide supra) [12].

RESULTS

Figure 2 depicts typical chromatograms of direct analysis for both Hyp and HHL. The chromatograms represent the hydrolysates from wrist *(A)* and buttock *(B)* from patient I (see Table I). In this system, HHL elutes prior to isodesmosine and desmosine. The HHL content expressed as moles per mole of collagen for each sample is summarized in the table. Some variation in HHL content was observed among different individuals. However, in each case the content of HHL was very low in wrist skin (sun exposed) and high in skin samples from the buttock (unexposed). The mean values and SD for these sites were 0.13 ± 0.07 and 0.69 ± 0.17 moles of HHL/mole of collagen, respectively ($p < 0.001$).

Figures 3 and 4 depict the changes of HHL and pyridinoline cross-link content after these cross-linked peptides were irradiated by UVA and UVB, respectively. The maximum dose of UVB used for the experiment was approximately twentyfold greater than the minimal erythema dose in human skin. The maximum dose of UVA irradiation was approximately two- to threefold greater than the UVA minimal erythema dose in human skin. Throughout the acute irradiation with both UVA and UVB, the concentration of HHL remained constant, indicating that the acute UV irradiation has no effect on HHL structure. In contrast, pyridinoline content was significantly diminished by irradiation with either UVA or UVB. Approximately 80% of pyridinoline was degraded after 1 J/cm^2 of UVB irradiation. More than 80% of pyridinoline was degraded with 50 J/cm^2 of UVA and essentially no pyridinoline remained after 250 J/cm^2 of UVA irradiation. The photolysis of pyridinoline in the peptide form obviously occurs by UV light irradiation as an isolated pyridinoline (not peptide form) does [16].

DISCUSSION

HHL is a stable cross-link isolated from mature bovine skin collagen. It is formed by a condensation between iminium cross-link, deH-HLNL and the imidazole C-2 carbon atom of His [10] (Fig 1). The molecular locus of HHL within the collagen fibril was identified to be Lys^ald (α-16^C), Hyl (α1-87), and His (α2 -92) of type I collagen [13,14] (Fig 1). The density of HHL at this molecular locus indicates that the three-dimensional structure of collagen fibrils in skin is fundamentally different from that of skeletal tissue [12,14]. This cross-link is virtually absent from other major collagenous tissues like bone, dentin, tendon, and ligament.

It has been reported that the content of HHL from human thigh skin and bovine skin increases with chronologic aging [11]. In this report, we demonstrated that HHL content in human skin depended on the body site from which the skin was obtained. It was low in wrist that was exposed to sunlight and high in buttock that was unexposed and the difference is statistically significant (p <

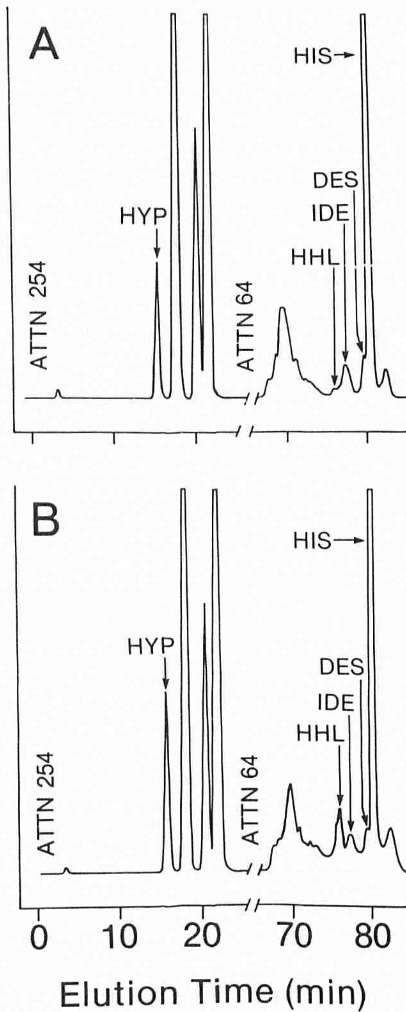

Figure 2. Typical chromatographic profiles of direct HHL cross-link analyses of *(A)* wrist skin and *(B)* buttock skin hydrolysates. See *Materials and Methods* for details. (ATTN, attenuation; HYP, hydroxyproline; HHL, histidinohydroxylysinonorleucine; IDE, isodesmosine; DES, desmosine; HIS, histidine.)

Table I. HHL Content in Different Sites of Aged Human Skin (>60Y)

Patient	Moles/Mole of Collagen: Wrist (sun-exposed)	Moles/Mole of Collagen: Buttock (unexposed)
I	0.14	1.08
II	0.14	0.80
III	0.10	0.76
IV	0.05	0.58
V	0.08	0.66
VI	0.11	0.47
VII	0.27	0.55
VIII	0.07	0.71
IX	0.23	0.66
Mean ± SD	0.13 ± 0.07	0.69 ± 0.17

0.001). The mean value of the former site represented only less than 20% of the latter. The values for buttock shown are similar to those obtained from other sun-unexposed sites, thigh (0.5–0.7 m/m collagen [11]) and underarm (0.4–0.5 m/m collagen, Yamauchi and Woodley, unpublished data) from human beings in a similar age group. The results clearly indicate that collagen cross-linking is different between these different sites of the body that are exposed to different amounts of sunlight. Because the HHL is quantified as moles per mole of collagen, the values obtained are independent of the thickness of dermis or collagen fibrils in these different sites.

The reasons for the low concentration of HHL in the sun-exposed site of skin could be due to either photolysis of HHL or impediment in HHL formation because of UV irradiation. It has been shown that pyridinoline, another stable collagen cross-link, is susceptible to UV light, especially in neutral and alkaline solution [16]. The results shown in this paper also demonstrate the occurrence of photolysis of pyridinoline in the peptide form by UV irradiation as was shown by Wu and Eyre [17]. In contrast, no structural changes of HHL occurred by irradiation with either UVA or UVB light. The minimal erythema dose (MED) for UVB irradiation (280–320 nm) in normal human beings is in the range of 30–45 mJ/cm^2. UVB irradiation penetrates human skin to the depth of the epidermal-dermal junction and has minimal penetration into the dermis [18]. UVA irradiation (320–400 nm), in contrast to UVB, only causes erythema at very high doses. However, natural sunlight emits over 10 times more UVA than UVB light, and UVA light penetrates into the dermis of human skin. Human skin has a number of components that filter or absorb UV light and serve as a protective barrier to irradiation, such as melanin pigment and urocanic acid, two barriers that absorb large amounts of UV light. However, in the experiments described in this paper, the HHL cross-link, the major collagen cross-link in aged human skin, was directly irradiated with UVB and UVA light in a clear fluid. Therefore, in this assay, there were no protective components present as one would find in human skin. We used doses of UVB and UVA irradiation that would induce erythema and marked phototoxicity in human skin. For example, the 70 mJ/cm^2 dose of UVB light is well above the usual MED for white human skin. The intensity of UV light (250 J/cm^2 of UVA and 1 J/cm^2 of UVB) in an in vitro assay without any protective elements means that the UV irradiation received by HHL cross-linked peptides in the wells of ELISA titer dishes greatly exceeded that which occurs in human skin under normal sunlight exposure or during UV irradiation for the treatment of skin diseases.

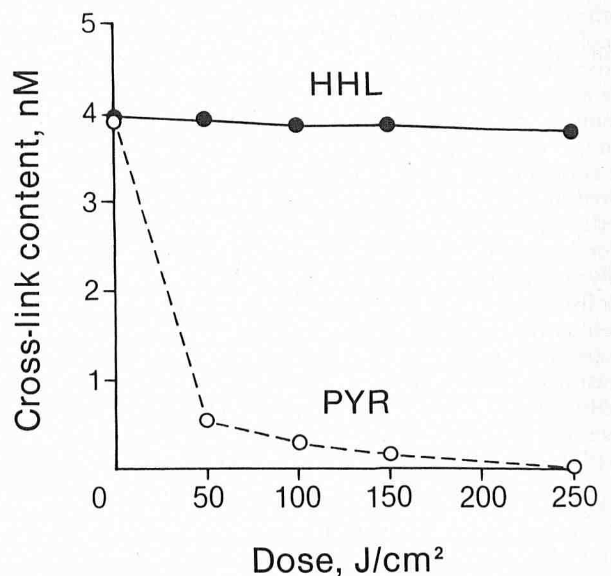

Figure 3. Effect of UVA irradiation on the cross-links histidinohydroxylysinonorleucine (HHL) and pyridinoline (PYR). Approximately 4 nmol of cross-linked peptides were subjected to various doses of UVA irradiation. The cross-link at each point was measured by amino acid analyzer. See details in the *Materials and Methods* section in the text.

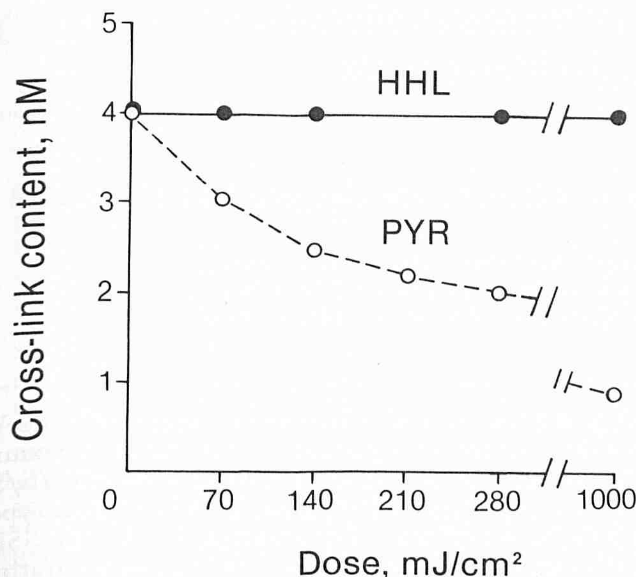

Figure 4. Effect of UVB irradiation on the cross-links histidinohydroxylysinonorleucine (HHL) and pyridinoline (PYR). The experiment was conducted in the same manner as UVA irradiation. See details in the *Materials and Methods* section in the text and the legend of Fig 3.

In earlier studies, Sams and Smith [1] demonstrated that there was an increase in soluble collagen and a concordant decrease in insoluble collagen in sun-damaged human dermis compared to that of unexposed skin [1]. It also has been reported that chronic sun exposure results in structural damage (degradation) of collagen fibrils [4]. Recently, Kligman et al [19] suggested that collagen may be enzymatically hydrolyzed by cells of the inflammatory infiltrate provoked by ultraviolet radiation. The low concentration of HHL in sun-exposed sites could be explained by these findings, although the possibility that HHL structure is affected by toxic products such as reactive oxygen species generated by UV irradiation cannot be ruled out. If collagen fibrils do not properly mature in these skin sites due to UV-induced structural changes and/or degradation, HHL formation may be impeded. In order for the tissue to form HHL cross-links, the stereospecificity of the collagen molecules in the fibrils is critical [14] and a certain period of time for condensation reaction is required [10].

In the sun-unexposed portion of skin, on the contrary to the exposed portion, collagen fibrils do mature with time and apparently become more insoluble and resistant to enzymatic degradation. This is likely due to an increase in the stable cross-links such as HHL as our previous study demonstrated [11]. Consequently, collagen bundles become larger rope-like structures [6]. HHL is abundant in such aged insoluble skin tissue [11]. As was demonstrated in the in vitro irradiation experiment, it is highly unlikely that chronic sun-exposure directly causes degradation of preformed HHL.

The photoaging of skin may be a rather destructive process that actually hampers normal maturation and aging and, therefore, is fundamentally different from chronologic aging.

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REFERENCES

1. Sams WM Jr, Smith JG: The histochemistry of chronically sun-damaged skin. J Invest Dermatol 37:447–452, 1961
2. Smith JG, Davidson EA, Sams WM Jr, Clark RD: Alteration in human dermal connective tissue with age and chronic sun damage. J Invest Dermatol 39:347–350, 1962
3. Kligman AM: Early destructive effects of sunlight on human skin. JAMA 210:2377–2380, 1969
4. Lavker RM: Structural alterations in exposed and unexposed aged skin. J Invest Dermatol 73:59–66, 1979
5. Sams WM Jr: Sun-induced aging: clinical and laboratory observations in man. Dermatol Clin 4:509–516, 1986
6. Kligman LH: Photoaging: manifestations, prevention, and treatment. Dermatol Clin 4:517–528, 1986
7. Uitto J, Matsuoka LY, Kornberg RL: Elastic fibers in cutaneous elastoses. In: Rudolf R (ed.). Problems in Aesthetic Surgery: Biological Causes and Clinical Solutions. CV Mosby Co., St. Louis, 1986, pp 307–338
8. Eyre DR, Paz MA, Gallop PM: Cross-linking in collagen and elastin. Annu Rev Biochem 53:717–748, 1984
9. Yamauchi M, Mechanic GL: Cross-linking of collagen. In: Nimni ME (ed.). Collagen, Vol. 1. CRC Press, Boca Raton, Florida, 1988, pp 157–172
10. Yamauchi M, London RE, Guenat C, Hashimoto F, Mechanic GL: Structure and formation of a stable histidine-based trifunctional cross-link in skin collagen. J Biol Chem 262:11428–11434, 1987
11. Yamauchi M, Woodley DT, Mechanic GL: Aging and cross-linking of skin collagen. Biochem Biophys Res Commun 152:898–903, 1988
12. Yamauchi M, Katz EP, Mechanic GL: Intermolecular cross-linking and stereospecific molecular packing in type I collagen fibrils of the periodontal ligament. Biochemistry 25:4907–4913, 1986
13. Yamauchi M, Noyes C, Kuboki Y, Mechanic GL: Collagen structural microheterogeneity and a possible role for glycosylated hydroxylysine in type I collagen. Proc Nat Acad Sci USA 79:7684–7688, 1982
14. Mechanic GL, Katz EP, Henmi M, Noyes C, Yamauchi M: Locus of a histidine-based, stable trifunctional, helix to helix collagen cross-link: stereospecific collagen structure of type I skin fibrils. Biochemistry 26:3500–3509, 1987
15. Yamauchi M, Katz EP, Otsubo K, Teraoka K, Mechanic GL: Cross-linking and stereospecific structure of collagen in mineralized and nonmineralized skeletal tissues. Connect Tissue Res 21:159–169, 1989
16. Sakura S, Fujimoto D, Sakamoto K, Mizuno A, Motegi K: Photolysis of pyridinoline, a cross-linking amino acid of collagen by ultraviolet light. Can J Biochem 60:525–529, 1982
17. Wu JJ and Eyre DR: Identification of hydroxypyridinium cross-linking sites in type II collagen of bovine articular cartilage. Biochem 23:1850–1857, 1984
18. Harbor LC, Bickers DR: Photoprotection. In: Harbor LC, Bickers DR (eds.). Photosensitivity Diseases: Principles of Diagnosis and Treatment. WB Saunders Company, Philadelphia, 1981, pp 85–103
19. Kligman LH, Gebre M, Alper R, Kefalides NA: Collagen metabolism in UV irradiated hairless mouse skin and its correlation to histochemical observations. J Invest Dermatol 93:210–214, 1989