3D 피부세포 배양계를 이용한 피부광노화 연구

강상진

LG 화학 화장품연구소

Skin photoaging in reconstituted skin culture models

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Abstract

Skin is continuously exposed to external stimuli including ultraviolet radiation, which is a major cause of skin photoaging. According to recent discoveries, UVA with a lower energy but deep-penetrating properties, compared to UVB, is likely to play a major part in causing skin photoaging. The clinical and histochemical changes of photoaging are well characterized, but the biochemical mechanisms are poorly understood partly due to the lack of suitable experimental systems.

In this work, three-dimensional, reconstituted skin culture models were prepared. After certain period of maturation, the equivalent models were shown to be similar in structure and biochemical characteristics to normal skin. Mature dermal and skin equivalent models were exposed to sub-lethal doses of UVA, and the effects of UVA relevant to dermal photoaging were monitored, including the production of elastin, collagen, collagenase(MMP-1), and tissue inhibitor of metalloproteinases-1 (TIMP-1).

Interestingly, dermal and skin equivalents reacted differently to acute and chronic exposure to UVA. Elastin production was increased as soon as one week after commencing UVA irradiation by chronic exposure, although a single exposure failed to do so. This early response could be an important advantage of equivalent models in studying elastosis in photoaged skin. Collagenase activity was increased by acute UVA irradiation, but returned to control levels after repeated exposure. On the other hand, collagen biosynthesis, which was increased by a single exposure, decreased slightly during 5 weeks of prolonged UVA exposure. Collagenase has been thought to be responsible for collagen degeneration in dermal photoaging. However, according to the results obtained in this study,

elevated collagenase activity is not likely to be responsible for the degeneration of collagen in dermal photoaging, while reduced production of collagen may be the main reason.

It can be concluded that reconstituted skin culture models can serve as useful experimental tools for the study of skin photoaging. These culture models are relatively simple to construct, easy to handle, and are reproducible. Moreover, the changes of dermal photoaging can be observed within 1 ~ 4 weeks of exposure to ultraviolet light compared to 4 months to 2 years for human or animal studies. These models will be useful for biochemical and mechanistic studies in a large number of fields including dermatology, toxicology, and pharmacology.

The aging of human skin is a complex process in which many factors contribute to the age-related changes. One of these factors relates to UV-radiation, but the exact mechanistic details are not well established. Skin is directly exposed to environmental stresses, particularly ultraviolet(UV) radiation from the sun, which induces various sorts of photodamage. The short-term exposure to UV results in inflammation, skin darkening, and decreased immune function. Beside the acute effects, repeated exposure to the sun for long periods induces deep wrinkles, loss of elasticity, and premature aging, usually referred to as "photoaging"[1-3]. Accumulating evidence suggests that alterations in the dermal extracellular matrix are primarily responsible for the clinical appearance of photodamaged skin[1, 4]. There is an accumulation of amorphous mass of elastotic material in the papillary dermis[5, 6] with degeneration of collagen[7] and changes in the proteoglycan contents[8].

Within sunlight, UVB is primarily responsible for most biological effects including inflammation, changes in pigmentation, immunosupression and carcinogenesis. However, UVA radiation, once believed harmless to skin, is now recognized as being capable of producing profound damage to most epidermal and dermal components[3, 9]. It is not only the larger amount of UVA reaching to the skin that makes UVA an important cause of photoaging, but also UVA can penetrate deep into the dermis[10]. Solar UVB radiation is known to induce various effects in many cases as a result of the direct absorption of photons by DNA and proteins in mammalian skin[11, 12]. In contrast, the skin-damaging effects of UVA appear to result from oxygen-mediated photodynamic reactions in which UVA in the presence of certain photosensitizing chromophores(e.g. riboflavin, porphyrins, NADPH etc.) leads to the formation of reactive oxygen species[13]. Because of these differences, UVA and UVB show different biological effects on the skin. The important point with respect to the present work is that the effects of UVA are closely related to the changes occurring on a result of aging process such as free radical formation[13], impairment of cutaneous antioxidant defense system[13], protein crosslinking[14, 15], and age-associated changes of dermal protein metabolism, such as increased elastin and collagenase production[16, 17]. Thus UVA probably plays a more important role in dermal photoaging

than UVB.

Almost all the studies published to date about photoaging were performed *in vitro* using monolayer cell culture[18-21] or *in vivo* on animal and human skin[22-26]. Both of these systems have some shortcomings. Cells in monolayer culture, in a situation far removed from that *in vivo*, show different behavior and respond differently to external stimuli including UV radiation. These cells show rapid proliferation and are actively producing extracellular matrix components and remodeling enzymes. Moreover, skin fibroblasts and keratinocytes can not be maintained long enough to reveal signs of aging without passage, which change may overwhelm or negative the effects of UV exposure. On the other hand, *in vivo* models are good for endpoint studies of the effects of long-term UV exposure. However, it is difficult to perform controlled experiments on the study of the mechanism and control of photoaging, because these systems are too complicated and it takes long time to see the initial signs of photoaging[22, 23]. Since reconstituted three-dimensional cultures of skin were developed[27, 28], many improvements have been made for equivalent models to produce structure and function similar to those in *in vivo* state[29, 30].

In this study, dermal and skin equivalent models were prepared and subjected to repeated exposure to sublethal doses of UVA radiation, to see if these models are suitable for the investigation of dermal photoaging.

Dermal equivalents, consisted of normal human skin fibroblasts and type I collagen isolated from rat tail, showed extensive contraction, which ceased at about two weeks after fabrication. During this period, fibroblasts underwent striking morphological changes ; the cells which were stellate in the early stages lost their dendrites and assumed a bipolar morphology(Fig. 1a, b). Parallel with the contraction, which is believed to be similar to what happens in the wound healing process, the production of elastin, one of the extracellular matrix components, was enhanced and returned to its normal level followed by a transient increase in the level of remodeling enzyme(collagenase) activity (Fig. 2). These results indicate that an initial maturation period was required during which fibroblasts remodel the extracellular matrix and regain their normal physiological state. The rapid proliferation of fibroblasts which occurring during the initial stages also decreased and, after this maturation period, the number of fibroblasts remained approximately constant.

To prepare skin equivalent, normal human keratinocytes were seeded on top of the dermal equivalent. About 48 hours after seeding onto dermal equivalents the keratinocytes were confluent, and the cultures were then raised to the air/liquid interface. Stratum corneum and suprabasal layers were apparent as early as 3 days after air/liquid exposure, which became mature, with 10-15 suprabasal layers and thick stratum corneum, within 3 weeks. The deposition of basement membrane components, type IV collagen and laminin, were visualized by immunohistochemistry. The patches of type IV collagen were recognized one week after air/liquid exposure, and continuous deposition of

type IV collagen and laminin were observed at three weeks.

In this study, fibroblasts in dermal and skin equivalents, after certain period of maturation, showed relatively normal metabolic features of the dermal extracellular components and have produced a good basement membrane. Therefore, the effects of extrinsic stimuli on 'normal' cells may be studied in these models, which are simple and in which it is easy to control the experimental conditions compared to the more complex *in vivo* model systems.

To evaluate the effects of single exposure to UVA radiation, monolayer culture of fibroblasts and mature dermal and skin equivalents were exposed to varying doses of UVA(0 to 40 J/㎠). At least 95% of fibroblasts in dermal equivalents were viable following exposure to 5 J/㎠ UVA. Interestingly, as seen in normal human skin, sunburn cells could be observed in skin equivalents, 24 hours after irradiation with 40 J/㎠ UVA.

Elastin biosynthesis was measured in the conditioned media of cultures irradiated with varying doses of single UVA irradiation (0 to 30 J/㎠). There was no difference in elastin levels/unit cell number in the conditioned media from fibroblasts both in monolayer cultures and in dermal equivalents. Acute UVA seems to have little or no effect on elastin biosynthesis in fibroblasts in monolayer culture and in equivalent models.

[^{3}H]-proline incorporation into collagenous protein by fibroblasts in dermal equivalents was increased 3-fold by as little as 5 J/㎠ UVA irradiation, and no further increase was observed by higher UVA dosages(Fig. 3■). On the other hand, collagen biosynthesis in sham-irradiated monolayer cultures was similar to that of irradiated dermal equivalents and the level was decreased dose-dependently by UVA irradiation(Fig. 3◆).

Collagenolytic activities in conditioned media from dermal and skin equivalent cultures were assayed after UVA irradiation. Little activity was detected in the medium without APMA treatment, indicating that most of the MMP-1 is present in latent form. Although the total activities were not significantly changed by UVA irradiation, the activity per unit cell number showed dose-dependent increase(Fig. 4). The mRNA levels of MMP-1 were dramatically elevated both in dermal and skin equivalents with higher levels in skin equivalents, while TIMP-1 gene expression showed slight increase(Table 1).

To evaluate the effects of chronic UV exposure, Dermal and skin equivalents were exposed to sublethal doses of UVA(5 J/㎠) five days a week for up to 5 weeks. Strikingly, dermal and skin equivalent models responded differently to acute and chronic exposure to UVA light. In contrast to the acute effect, elastin concentrations released into the conditioned media from both dermal and skin equivalents were increased by repeated exposure to UVA irradiation within a week and increased further until 3 weeks(Fig. 5a). Similar elevation patterns were observed in both dermal and skin

equivalents.

The initial elevated levels of collagen synthesis in UVA-irradiated dermal and skin equivalents returned to those of non-irradiated controls within one week, and a slow reduction in collagen biosynthesis was observed showing about 20% reduction after 5 weeks of repeated exposure. This effect was similar in both dermal and skin equivalents except that a higher collagen biosynthesis was observed in skin equivalents(Fig. 5b). Collagenase activities in both dermal and skin equivalent models were increased during the early period of UVA exposure. However, after prolonged exposure, these elevated collagenase activities returned to normal. In dermal equivalents, collagenase activity showed a peak at 5 days after the start of irradiation and returned to the level of non-irradiated controls after 2 weeks(Fig. 6■). The similar but slower response was seen in skin equivalents, showing collagenase activities peaking after 1 ~ 2 weeks and returning to normal after 4 weeks of irradiation (Fig. 6▲).

Higher collagenase activities in non-irradiated control were noticed in skin equivalents than dermal equivalents. Also, the return of elevated collagenase activities to normal levels took a longer time in skin equivalents than dermal equivalents.

Elevated collagenolytic activity following UV exposure in fibroblasts in monolayer culture as well as in human or animal skin have repeatedly been reported by several groups[19, 31, 32], suggesting that the reduced collagen content in photoaged skin is a result of increased or accelerated degradation. Most of these data were obtained by single or several exposure to relatively large amount of UV radiation. However, because aging is generally considered as a result of accumulation of minor damage over a long period, it is worth confirming whether the same result is obtained following long-term exposure to low levels UV radiation or whether the damage is produced by different or additional mechanisms. In the study reported here, reconstituted skin cultures were repeatedly irradiated with sublethal doses of UVA and the changes in collagen biosynthesis and collagenolytic activity were monitored.

Acute and chronic UVA exposure brought about different results in dermal metabolism in equivalent models. Elastin production was enhanced only after repeated exposure to UVA. Increased collagen biosynthesis was observed after single exposure, but the opposite was seen following repeated exposure. Also, collagenase activity, which was enhanced by a single UVA irradiation, returned to normal after several weeks of chronic UVA exposure. The mechanisms leading to these different responses are not known. Possible explanations could include feedback control by the extracellular concentration and activity of the proteins and enzymes or their degradation products, or a different set of signals triggered by repeated UV exposure leading to different results. Overall one can conclude that the biochemical modifications that follow repeated exposure to UVA are not the result of simple accumulation of the effects brought about by single exposure and therefore

it is not appropriate to explain the changes in photoaged skin on the basis of data obtained in experiments in which there is single or short-term UV exposure. For example, the enhanced collagenolytic activities were thought to be responsible for the decreased collagen levels in photoaged skin based on results obtained from acute exposure to ultraviolet in *in vivo* and *in vitro* experiments[17, 32, 33]. In contrast, according to the results from the present study, however, decreased collagen biosynthesis might be a more important reason for this change and may help to resolve the confusion originating from different results obtained under varying experimental conditions and in different models.

The early signs of skin photoaging, such as increased elastin production and reduced collagen synthesis, could be recognized in equivalent models within a relatively short period compared to *in vivo* models. These changes could explain the mechanism of solar elastosis and degeneration of collagen in photoaged skin, and agreed well with other results, such as reduced type I and III procollagen levels in photoaged human skin compared with the light-protected skin of the same patients[34], elevated elastin and fibrillin gene expression in photoaged skin[24], and enhanced elastin promotor activities in transgenic mice[35]. The different basal levels of collagen and collagenase, and the different responsiveness to chronic UVA in collagenase activities, between dermal and skin equivalents might reflect the role of keratinocytes and epidermal cytokines in producing dermal changes after UVA exposure. The results in this study suggest that reconstituted skin culture model is a useful tool for the study of photoaging. There are some limitations, however, with present techniques skin equivalent models can only be maintained for relatively short periods, say a maximum of 7 to 8 weeks after exposure at the air/liquid interface, and thereafter lose their structure and viability for the reasons that are not clear. More importantly, these models do not contain other types of cells which are known to play significant roles in UV-induced skin responses, including skin-residing Langerhans' cells, melanocytes, macrophages, and neutrophils[25, 36]. Especially cells from blood vessels secret cytokines as well as various matrix-degrading enzymes, such as collagenase, gelatinase, and elastase, in response to the inflammatory signals including UV radiation[37, 38]. Neutrophil-derived lysozyme and elastase may also participate in producing elastosis[39, 40].

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Table 1.

Dose-dependent elevation of mRNA levels of MMP-1 and TIMP-1 resulting from UVA irradiation of dermal and skin equivalent models. Total RNA was isolated from equivalent models after 24 hours incubation following irradiation with varying doses of UVA. mRNA levels were determined by RT-PCR as described in Materials and Methods. Values represent–fold increases compared to non-irradiated samples.

UVA (J/cm^2)		0	5	10	15
MMP-1	DE	1.0	1.5	2.4	5.6
	SE	1.0	1.8	7.7	10.2
TIMP-1	DE	1.0	1.2	1.2	1.4
	SE	1.0	1.1	1.5	1.6

Fig. 1. Maturation of equivalent models. Fibroblasts in dermal equivalents at early stage in a stellate morphology(a ; 24 hours after fabrication) transformed into bipolar shape at late stages of maturation (b ; 2 weeks).

Fig. 2. The elastin content(■) and collagenolytic activities(◆) in conditioned media from dermal equivalents. Dermal fibroblasts(5 x 10^5 cells) were seeded in collagen solution(2 ㎎/㎖ final concentration) and media conditioned for 24 hours were collected at each time point. Elastin content was measured by ELISA, and collagenolytic activity was assayed after activated by APMA using radiolabeled collagen as substrate. Data are presented as mean ± SD, n = 6.

Fig. 3. Acute effects of UVA on collagen biosynthesis in monolayer culture(◆) and dermal equivalent (■). Cultures were irradiated once with UVA at the dose shown and incubated in medium containing [^{3}H]-proline for 24 hours. Radioactivities in collagenase-sensitive protein in the conditioned media were measured. Data are presented as mean ± SD, n = 3.

Fig. 4. Effects of single exposure to UVA on collagenolytic activity. Total collagenolytic acitivities/unit cell number in dermal equivalents. Data are presented as mean ± SD, n = 3.

Fig. 5. Effects of repeated UVA irradiation(5 J/㎠ UVA five days a week) on elastin production and collagen biosynthesis in equivalent models. (a) The elastin concentration in conditioned media was measured by ELISA(■ : irradiated, ◆ : control). (b) Collagen biosynthesis was measured by the incorporation of [^{3}H]-proline into collagenous protein in dermal(◆) and skin(■) equivalents after repeated UVA irradiation. Data are presented as mean ± SD, n = 3.

Fig. 6. Effects of repeated UVA irradiation(5 J/㎠ UVA five days a week) on collagenolytic activities in dermal equivalent(■) and skin equivalent(▲ : irradiated, and ◆ : sham-irradiated) models. Collagenolytic activities were assayed by the degradation of radiolabeled fibrous collagen. Data are presented as mean ± SD, n = 3.

Fig.1

(a)

(b)

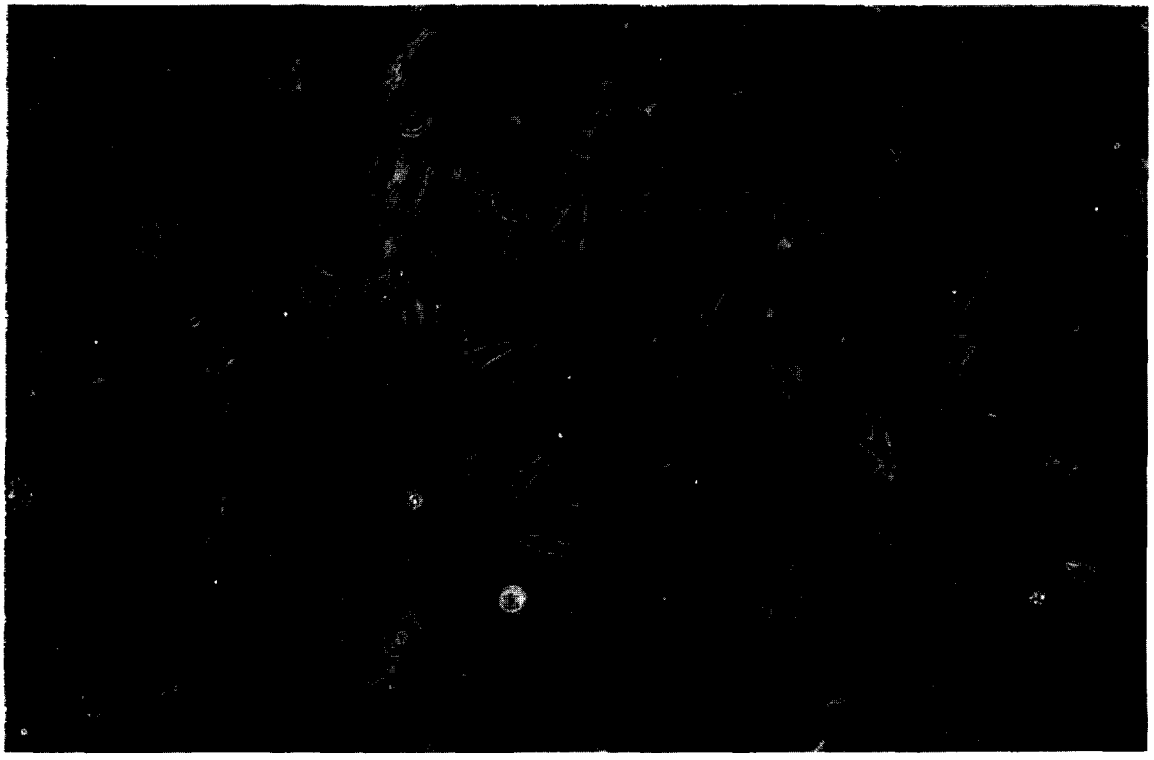


Fig. 2

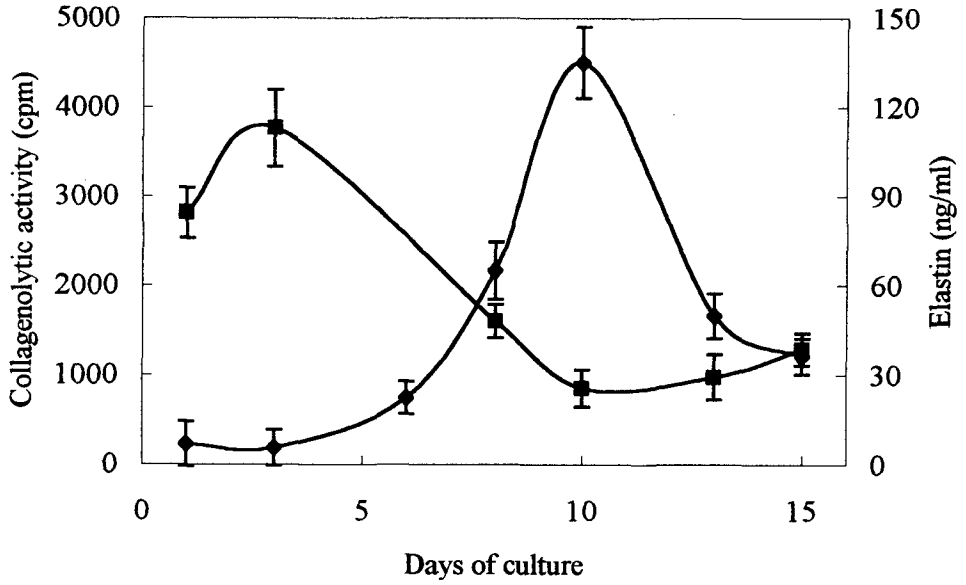

5000
4000
3000
2000
1000
0
Collagenolytic activity (cpm)
150
120
90
60
30
0
Elastin (ng/ml)
0
5
10
15
Days of culture

Fig. 3

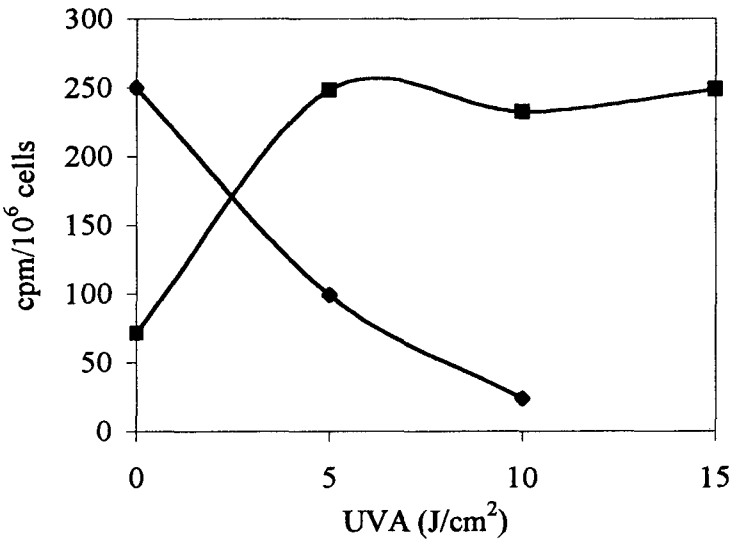


Fig. 4

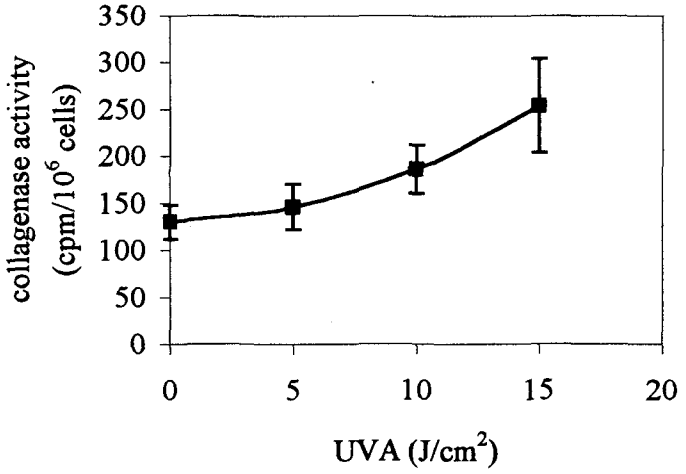
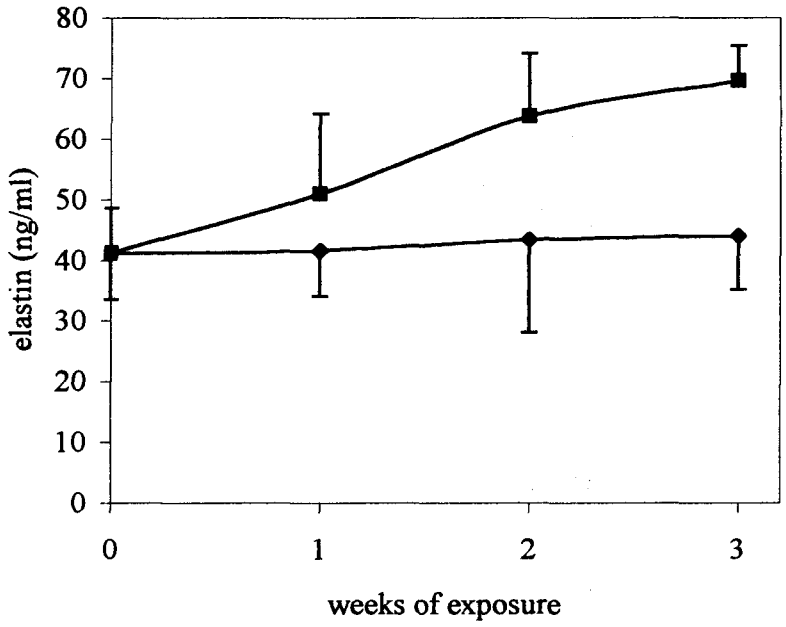

collagenase activity
(cpm/10^6 cells)
350
300
250
200
150
100
50
0
0
5
10
15
20
UVA (J/cm^2)

Fig. 5

(a)


80
70
60
50
40
30
20
10
0
elastin (ng/ml)
0
1
2
3
weeks of exposure

(b)

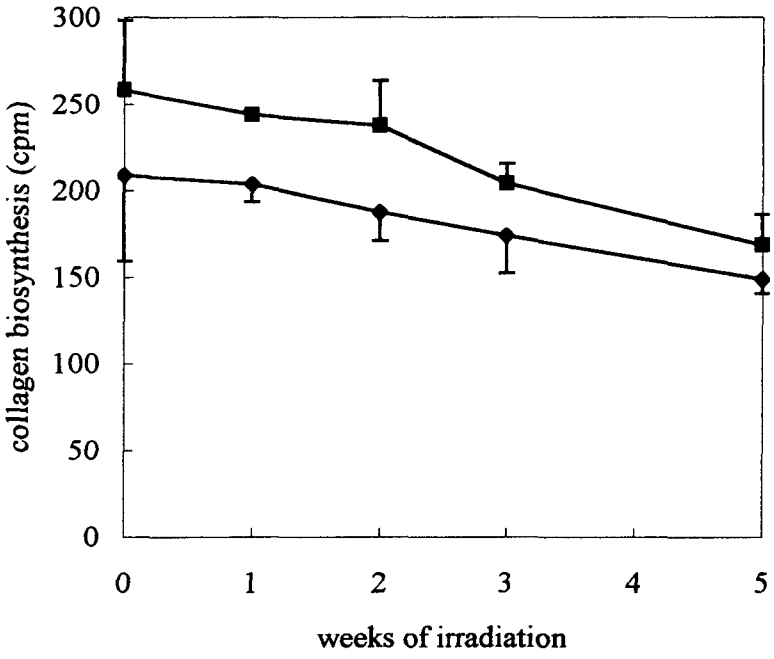
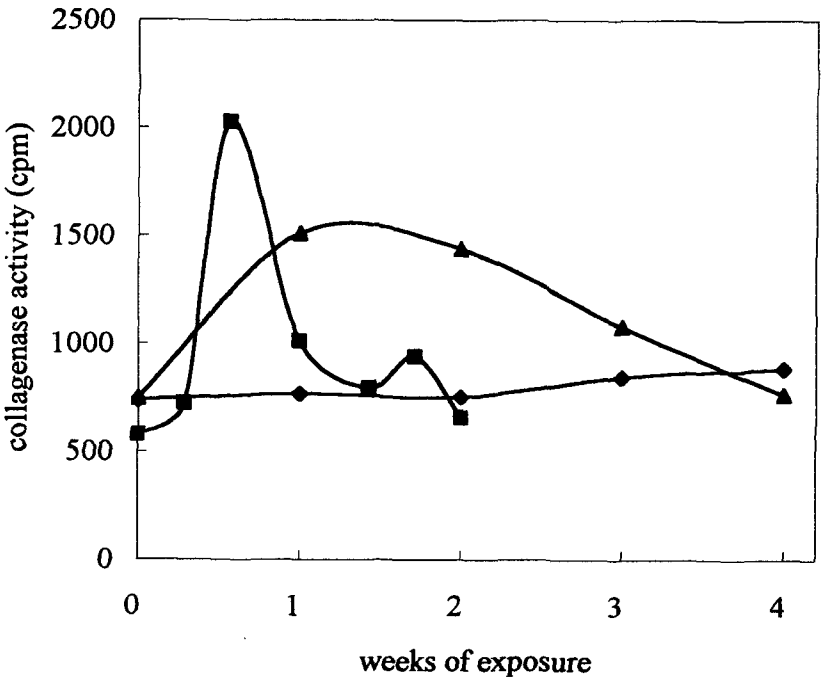

300
250
200
150
100
50
0
collagen biosynthesis (cpm)
0
1
2
3
4
5
weeks of irradiation

Fig. 6


2500
2000
1500
1000
500
0
collagenase activity (cpm)
0
1
2
3
4
weeks of exposure