Acta Med. Nagasaki 46 : 39−47

Establishment of Photoaging Mouse Model by Long-Term Repetitive UVA Irradiation

Fumihide OGAWA, Kazuhiro SHIMIZU, SANG JAE BAE, Youichiro HAMASAKI, Ichiro KATAYAMA

Department of Dermatology, Nagasaki University School of Medicine

To clarify the mechanism of long-term repetitive UVA irradiation in photoaging of the skin, we evaluated albino hairless (SKH: HR1) mice by the irradiation with UVA (300-420nm) for 12months. The output of irradiated UVA was 30J/cm²/day. Skin samples were taken from each group and examined at 0, 4, 8 and 12months. Morphologically, the structural changes of elastic fibers were observed in the dermis of irradiated group. In immunohistochemistry, tropoelastin, matrix metalloproteinase 2 (MMP2) and matrix metalloproteinase (MMP9) were expressed in the fibroblasts in the dermis of photoaged skin. The expressions of tropoelastin, MMP2 and MMP9 mRNA in cultured fibroblasts were analyzed by RT-PCR. The expression of MMP9 mRNA was increased cultured fibroblast derived from irradiated mice. These results suggest that MMP9 is an important mediator in the development of photoaged skin for one-year repetitive exposure to UVA.

ACTA MEDICA NAGASAKIENSIA 46 : 39−47, 2001

Key Words: UVA, photoaging, mouse model, matrix metalloproteinase, elastin, RT-PCR

Introduction

Along with the increase of average life span, the risk and problems of the cumulative amount of UV exposure have increased at terrestrial level.

Long-term exposure to UV irradiation results in various skin changes (Fig. 1), such as wrinkling, yellowing, uneven pigmentation, laxity or leathery dryness, termed photo skin aging, different from those in chronological aging. (1-3)

Alternations in photoaged skin are frequently found in dermal connective tissue (4, 5). Most conspicuous change in dermis is elastotic degeneration termed "solar elastosis" (Fig. 2), which is characterized by the increase of basophilic materials in the dermis, possibly derived from elastic fibers because of their reactivity with the anti-elastin antibody and their similarity of the amino acid composition to elastin. (6-9) Such symptoms appear clearly in the cutis rhomboidalis nuchae (Fig. 1). This ailment is sometimes encountered in aged people and outdoor workers under sunny climates. In contrast, chronologically aged skin is smooth, thin, unblemished and reduced in elasticity. Therefore, it is becoming very important to prevent skin from UV exposure in order to keep a good skin condition.

For this purpose, we established an animal model to analyze the cutaneous photoaging equivalent to that in human skin. Most of the previous animal studies adopted UVB as a source of UV(10-13), because UVB was thought to be the most effective waveband in the induction of photoaging and the role of UVB was extensively studied. In contrast, the role and significance of UVA in photoaged skin (14, 15) have not been fully examined although UVA is the most prevalent component of UV radiation in sunlight and penetrates more deeply in the skin than UVB. Therefore, the role of UVA causing photoaging in dermis has been the matter of discussion and paid an attention to investigators.

In this article, we reported the cutaneous changes in-

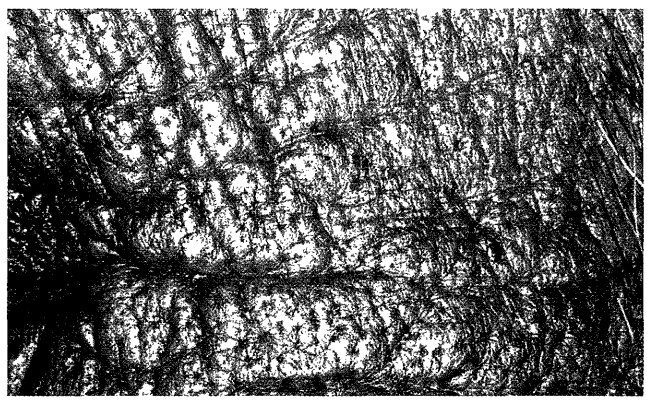

Fig. 1. Human photoagd skin. The skin lokked thickend and rough appearance, with course wrinkles and motted pigmentation.

Address Correspondence: Fumihide Ogawa M.D.
Department of Dermatoiogy, Nagasaki University School of Medicine 1-7-1 Sakamoto, Nagasaki 852-8501, JAPAN
TEL: +81-95-849-7333 FAX: +81-95-849-7335
E-mail: fogawa-ngs@umin.ac.jp

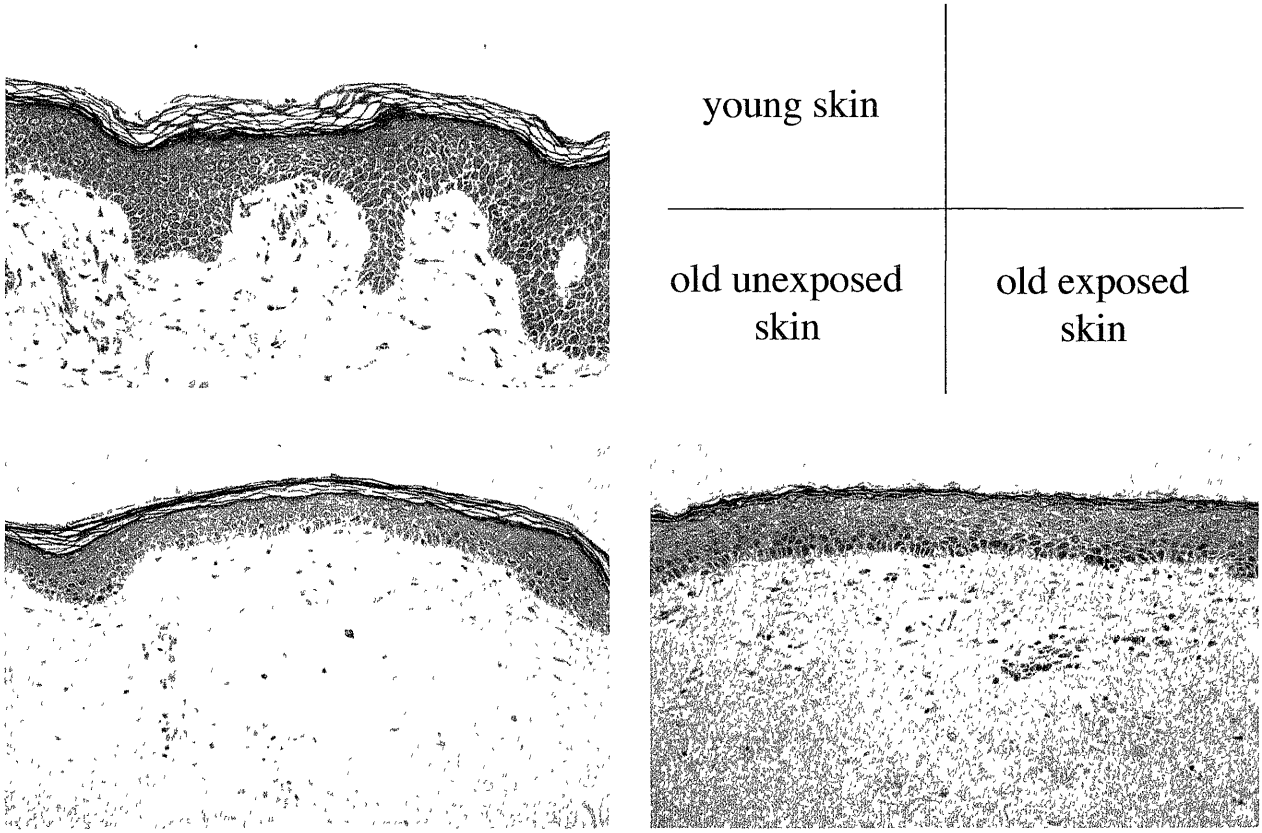

Fig. 2. Histlogic feratures of skin connective tissue are alterd in photoaged skin. Basophilic degeneration of dermal tissue was observed in old exposed skin.

duced in hairless mouse skin by repeated long-term UVA exposure, which is supposed to be equivalent to human photoaged skin observed in outdoor workers. Synthesis and degradation of elastic fibers during the photoaging were evaluated by immunohistochemical and RT-PCR analysis.

Materials and Methods

Animals and UVA irradiation

Albino hairless mice were purchased from the Charles River (SKH: HR1, female, 4weeks of age). Animals were housed in cages with wire mesh tops. The mice were 6 weeks old at the beginning of the experiment. The room was illuminated in a 12-h cycle (switched on at 7:00, and off at 19:00) and no daylight entered the room. The mice were irradiated from above, while being allowed to move freely in the cages. The source of UVA (FS20BLB; Toshiba, Japan) was mounted above the cage with 5mm glass filter, and this source was switched on and off automatically by programmed outlet (Power Key pro; Lavix, Japan) and computer. The daily irradiation time was 4-h and the mice were exposed from 9:00 to 13:00. Mice were divided into 7 groups of five individuals. Three groups were irradiated for 5 days per week for 4, 8 and 12 months. The other 3 groups were treated as age-matched controls and were non-irradiated for the same periods of time. Mice in the irradiated groups received a daily dose of 30J/cm^2/day, which resulted in cumulative doses of 2.4, 4.8 and 7.2KJ/cm^2 for 4, 8 and 12 months of irradiation, respectively. These doses did not cause any acute effects such as edema and erythema. The cages were rotated every week and their cutaneous appearances and weight were recorded every two weeks. Mice were sacrificed 7days after the final irradiation, and their dorsal skin was examined.

Histological analysis

Skin samples were fixed in 4% paraform aldehyde and processed for paraffin embedding. Paraffin tissue

sections (5㎛) were stained with hematoxyline-eosin stain and Weigert's resorcin-fuchsin stain.

Immunohistochemistry

The alkaline-phosphatase method was adopted.

Anti-mouse MMP-9 antibody and Anti-human MMP-2 antibody (goat immunoglobulin) were purchased from Santa Cruz Biotechnology. Inc. (sc-6841, sc-6838 respectively, CA, USA). The antibody (sc-6838) is polyclonal and cross-reacts with MMP-2 of mouse, rat and human origin (data from the manufacturer). Anti-bovine elastin (rabbit immunoglobulin) was purchased from LSL (LB-2187, Japan). The antibody (LB-2187) is polyclonal and cross-react with elastin of rat, mouse and human origin and not cross-react with type I, II, III, IV, V and VI collagen (data from the manufacturer).

Five micrometer sections of frozen tissue prepared for MMP-2 and 9 staining were immersed in 0.05M Tris-buffered saline (TBS) containing 10% normal rabbit serum (X0902, DAKO, Denmark) and sections prepared for staining elastin were immersed in 0.05M TBS containing 10% normal goat serum for 40 min. at room temperature to diminish the non-specific binding of the secondary antibody. After washing in 0.05M TBS for 5 min., each section was incubated overnight with anti-MMP2, MMP9 antibody (1:500) and anti-elastin antibody (1:500) at 4℃. After washing, the sections incubated with anti-elastin antibody were treated with anti-rabbit IgG labeled with alkaline-phosphatase (1:300) at room temperature for 1 h. purchased from ORGANON TEKNIKA Corp. (Durham, NC). The sections treated with anti MMP-2 and 9 antibodies were treated with anti-goat IgG labeled with alkaline-phosphatase (1:500) at room temperature for 1 h. purchased from CHEMICON International, Inc. (CA, USA). After washing, they were immersed in a prewarmed alkaline substrate buffer (ASB) containing 1mM levamisol for 5 min. to bring about conversion into substrate buffer and to block internal phosphatase activity, and subsequently they were treated at 37℃ with alkaline substrate buffer (ASB) (ASB consists of 0.1M Tris-HCl (pH9.5), 0.1M NaCl and 50 mM MgCl2) containing BCIP and NBT purchased from GIBCO BRL (Gaitherberg, MD). Negative control staining was obtained by replacement of the primary antibody with nonimmunized rabbit and goat serum.

RT-PCR analysis for tropoelastin, MMPs mRNA expression.

Total RNA was extracted from the cultured fibroblasts and by QIAshredder™ (QIAGEN, GERMANY) and RNeasy Mini Kit (QIAGEN, Japan). The synthesis of cDNA was performed using Superscript II kit (Gibco BRL, Rockville, MD) according to the method by the manufacturer. Each cDNA was subjected to PCR amplification using a DNA thermal cycler (PELKIN ELMER). DNA amplification was carried out in a standard 100㎕ PCR reaction that contained 2㎕ of the RT mixture. With the addition of the appropriate primer sets and Takara Taq™ (Takara Shuzo, Shiga, Japan), amplification was initiated with a 2 min initial melt at 95℃. This was followed by 35 cycles for MMPs and tropoelastin, 25 cycle for β-actin, at 95℃ for 1 min, 55℃ for 1'20", 72℃ for 1'40" in a thermal cycler.

The mouse tropoelastin PCR primers were synthesized as follows
5'-GGAGTTGGCATCCCGACATAT-3' (sense) and
5'-TTAGCAGCAGATTTAGCGGCA-3' (antisense).

The mouse MMP-2 PCR primers as follows
5'-AGGACAAGTGGTCCGCGTAAA-3' (sense) and
5'-TGTCATCATGGGATAATCGGAAGT-3' (antisense).

The mouse MMP-9 PCR primer as follows
5'-CTCTGAATAAAGACGACATAGACGGC-3' (sense) and
5'-AGAGAACTCCTTATCCACGCGAAT-3' (antisense).

The mouse β-actin PCR primers as follows
5'-TCCTCCCTGGAGAAGACTA-3' (sense) and
5'-AGTACTTGCGCTCAGGAGGA-3' (antisense).

The tropoelastin, MMP-2, MMP-9 and β-actin primers are predicted to amplify 469, 664, 733 and 313 bp DNA fragments, respectively. An aliquot of each amplification mixture was subjected to electrophoresis on a 1.8% agarose gel.

Each sequence of PCR product was confirmed.

After the electrophoresis, PCR products were visualized by ethidium bromide staining. The relative amount of each PCR product was determined by densitometric scanning (GeneTools, SYNGENE) and each value was normalized to that of β-actin.

Fibroblast culture.

Mouse dermal fibroblasts were derived from back skin of each group. The back skin was trimmed with scissors to remove excess fatty tissue, rinsed repeatedly with PBS, and diced into small fragments. The fragments were allowed to adhere to the bottom of the tissue culture plate in a humidified 5% CO_2 atmosphere at 37℃ for 1 hour, and covered with Dulbecco's modified Eagle's medium (DMEM) (Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) (Gibco BRL, Gaithersburg, MD) containing 100U of penicillin and 100㎍ of streptomycin (WAKO, Japan) per ml. The culture medium was changed every 3days, and the cells were subcultured at subconfluence at split ratio of about 1:2. After the fourth passage, the

cells were seeded at 1 x 10^5 cells/cm^2 in 75cm^2 culture bottle (NUNC™, Denmark) At confluence, the culture medium replaced with serum free fresh medium The cells were immediately lysed with lysis buffer and stored at 80℃ until RNA extraction

Statical analysis

Mann-Whitney U test was used for the statical analysis

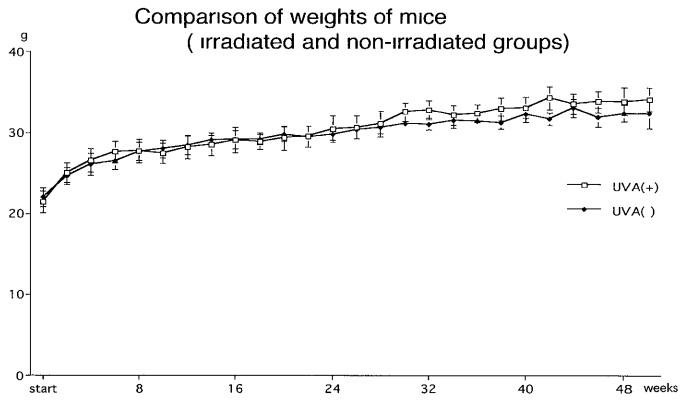

Fig. 3. Time course of the body weight of hairless mice The irradiated group is indicated by square symbols, and non irradiated group is by diamonds one Mice are weighed every two weeks for one year

Results

Skin changes of UVA irradiated hairless mouse

The mice were weighed every two weeks There was no difference in body weight between irradiated and non irradiated group (Fig 3)

The skin changes were examined at 4, 8 and 12 months The skin was yellowish, sagging and thickened, and each wrinkle was thick and deepened on the neck and face at 12 months in the irradiated group (Fig 4)

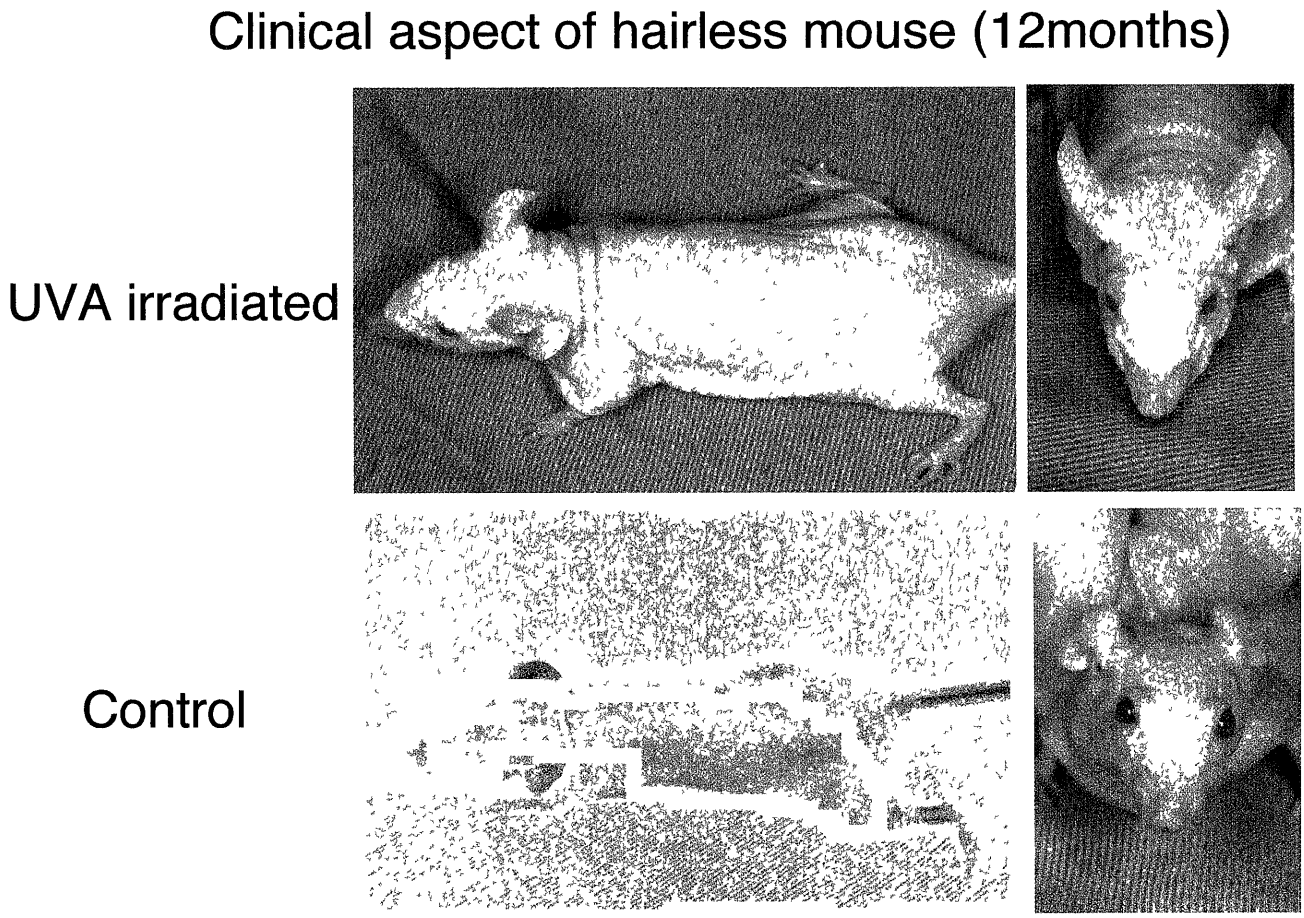

Fig. 4. Skin changes of hairless mouse at 12month Skin is yellowish, sagging and thickened and each wrinkle is thick and deepened on the nape and face in irradiated group as compared to non irradiated group

Histopathological analysis of UVA irradiated skin

The thickness of the whole skin on the nape and the back was measured at 0, 4, 8, and 12 months. In the irradiated group, a significant increase was observed in the thickness of the whole skin on nape, due to the proliferation and enlargement of dermal cysts. Four to five rows of keratinizing cysts in the subcutis were observed in the irradiated group, on the other hand, two or three rows of cysts were observed in the control. (Fig. 5) We next examined the thickness of the epidermis. The epidermis was thin in the control group, but the thickness of epidermis was equal to that at start point (6 weeks) in the irradiation group. In UVA irradiated skin, the atrophic changes recognized in chronological aging had not occurred. Therefore, there was a significant increase of epidermis at both nape and back in irradiated group as compared to non-irradiated group at 12 month. (Fig. 6)

We next examined the changes of elastic fiber by Weigert's resorcin-fuchsin stain. Thin and narrow elastic fibers were detected at the start point (6weeks). Elastic fibers were gradually thickened, but arrangement of fibers was still maintained in good order in the control group. On the other hand, elastic fibers were increased in number, getting thicker, and streams of fibers were out of order and their cross section looked like rain drop-shape in irradiated group. (Fig. 7)

Immunohistochemical analysis

The localizations of MMP2, MMP9 and tropoelastin protein were examined that were related to synthesis and digestions of elastic fiber were examined in the frozen tissue section. All were immunohistochemically confirmed in the fibroblast through the dermis, but not be obvious in the epidermis. (Fig. 8)

Tropoelastin, MMP2 and MMP9 mRNA expression in cultured fibroblasts

The each mRNA expression was investigated in cultured fibroblasts established from each mouse back skin. There was no significant difference in MMP2 mRNA expression between irradiated and control groups. The expression of tropoelastin mRNA tended to be high in the irradiation group at 12months. Although MMP9 mRNA expression decreased as compared to that at start point in non-irradiated group, the expression of MMP9 mRNA was significantly higher than that of in control group at 12month. (Fig. 9).

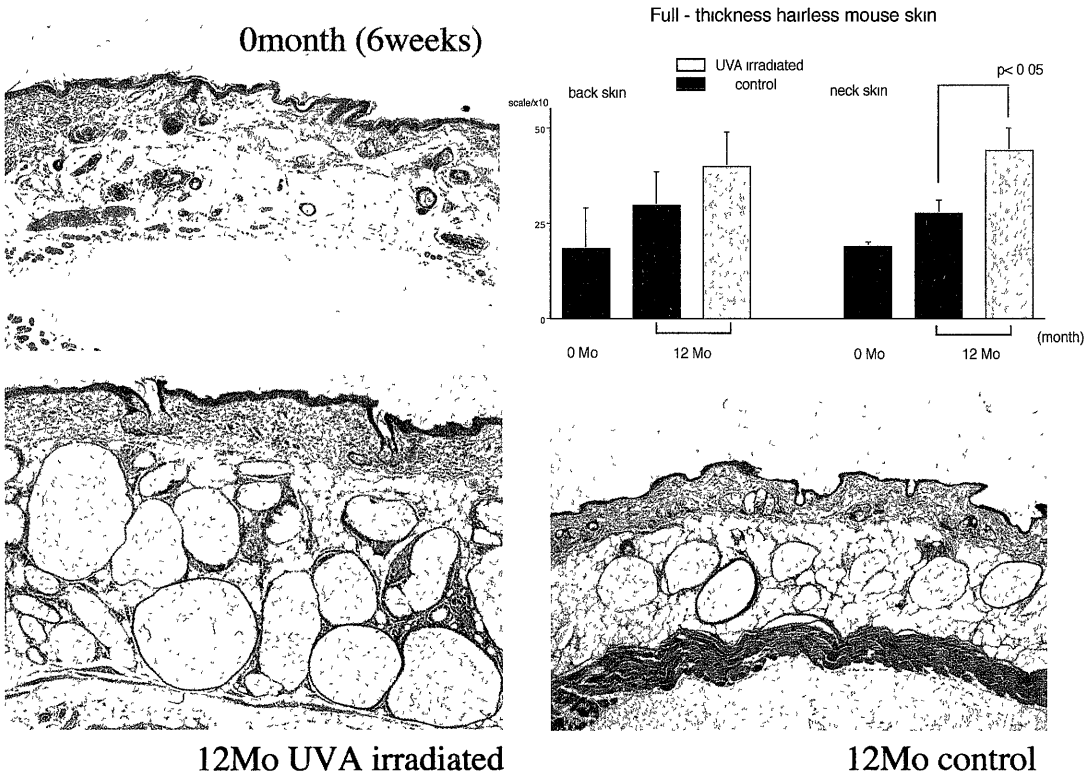

Fig. 5. The thickness of the skin. The thickness of the whole skin on the neck and back is examined at start point and 12months. A significant increase is observed in the width of the whole skin on the neck. A proliferation and enlargement of dermal cysts are observed in irradiated group.

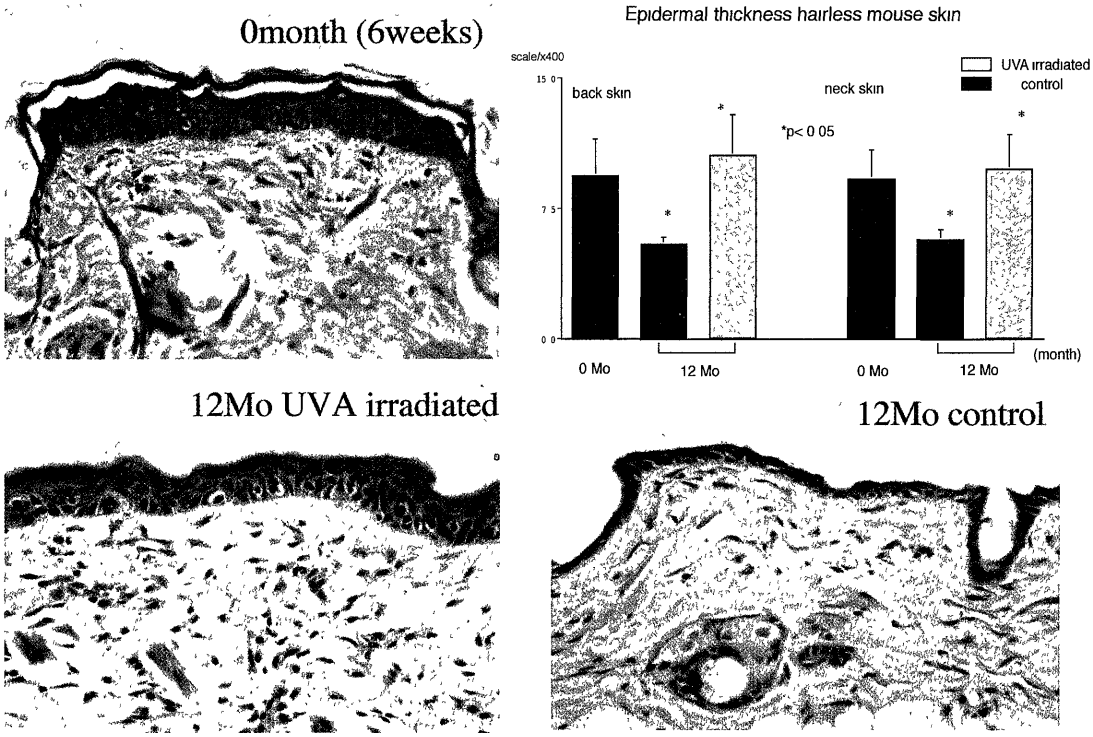

Fig. 6. The thickness of the epidermis on the neck and back is examined at start point and 12month. A significant increase of epidermis is detected at both neck and back in irradiated group.

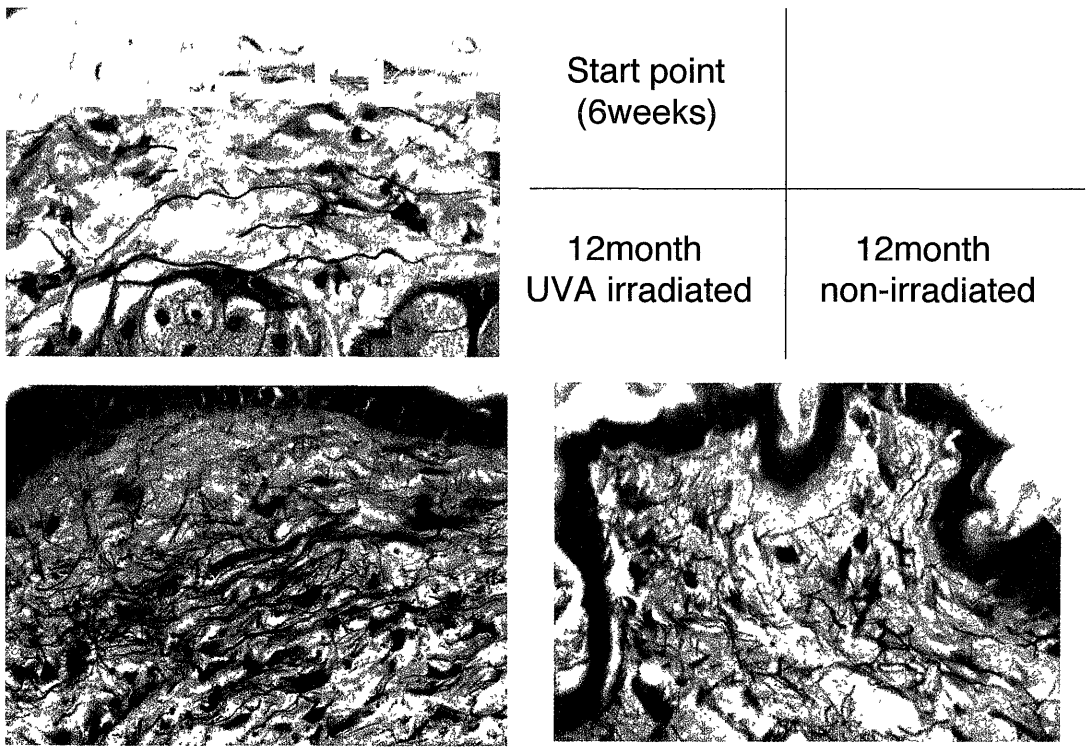

Fig. 7. Histological sections by Weigert's resorcin-fuchsin stain (x400). Start point: Unirradiated controls at start point. 12 months UVA irradiated: Irradiated group at 12months. Elastic fibers are thick and increased in number. Stream of fibers are out of order and its their cross sections look to be raindrop shaped. 12 months non-irradiated: Unirradiated group at 12months. Arrangement of elastic fibers were still in good order.

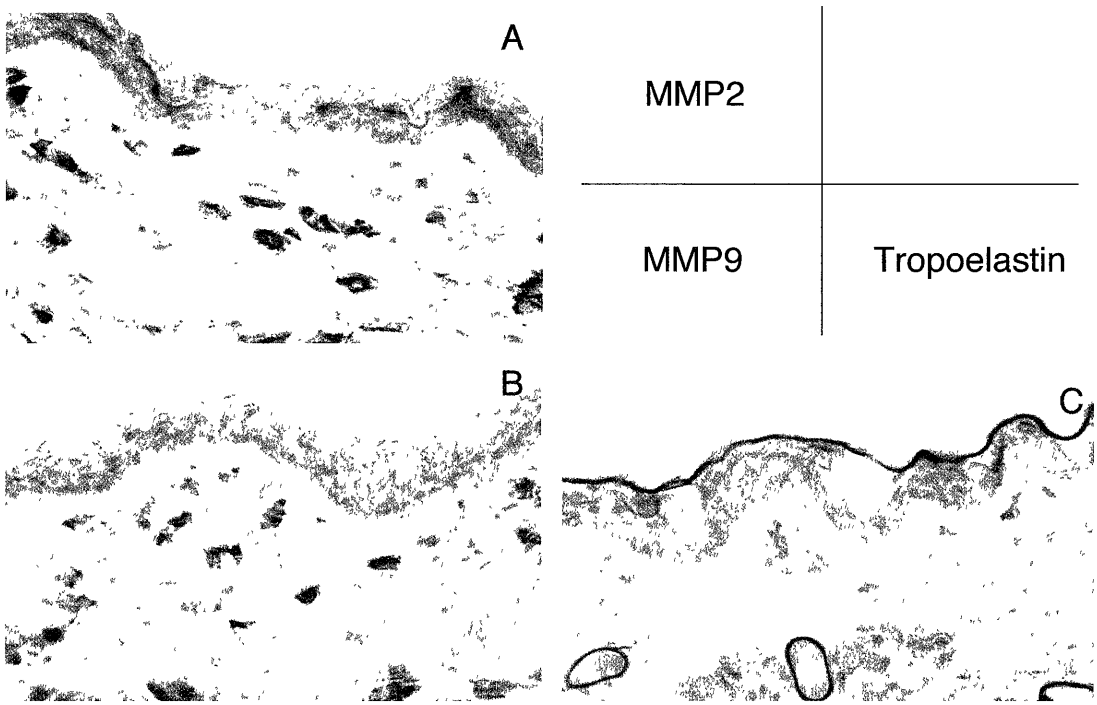

Fig. 8. Immunohistchemocal stain of frozen tissue at 12months in irradiated group. A: MMP2 (x400). B: MMP9 (x400). C: Tropoelastin (x400). All are localized in the fibroblasts in the dermis.

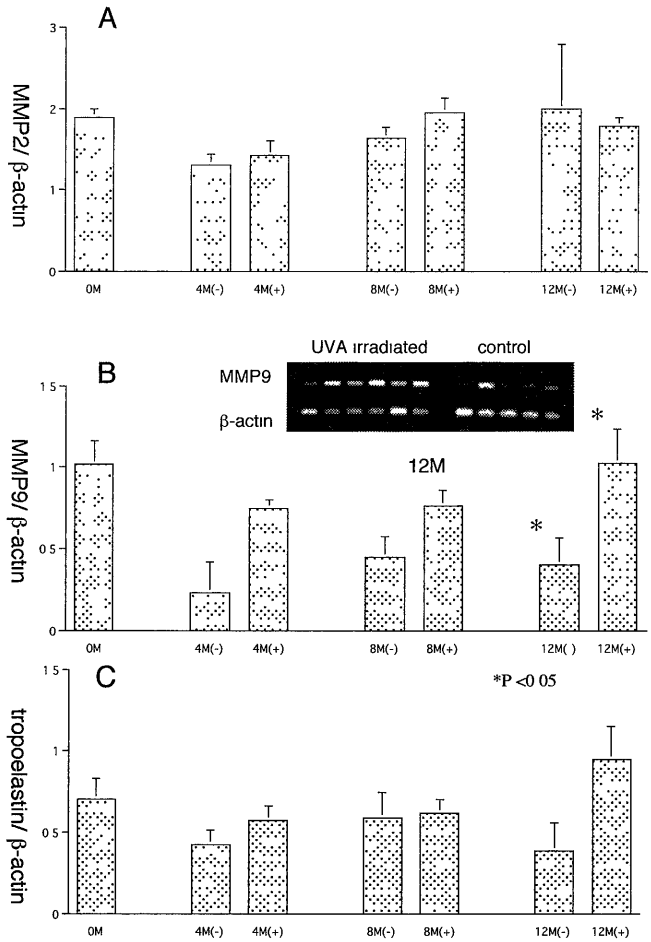

Fig. 9. Analysis of tropoelastin, MMP2 and MMP9 mRNA expression in cultured fibroblasts by RT-PCR. A: MMP2 mRNA expression. B: MMP9 mRNA expression. A significant increase of MMP9 mRNA expression was observed in irradiated group at 12months ($p<0.05$). Insert: Comparison of MMP9 mRNA expression between irradiated and non-irradiated group at 12month. C: Tropoelastin mRNA expression. The expression of mRNA is higher in irradiated group than non-irradiated control at 12month. No significant difference is noticed each bar point represents mean±SEM. *: $P<0.05$ (Mann-Whitney U-test)

Discussion

The many researches on photoaging by UV rays have been performed, but little has been observed over 1 year. It has been reported that skin damage produced by UVA might be different from that by UVB (15). Long-term repetitive UVA irradiation adopted in our study made significant cutaneous features and could cause photoaging, which was not consistent with the previous reports. Our experiment seemed to reflect many aspects of actual photoaging in human. We observed a significant hyperplasia of epidermis in the irradiated group as compared to that of non-irradiated group, which was neglected in previous studies. This discrepancy was made because our experiment was performed for 12 months, while most previous studies were at longest for 8 months. Epidermal hyperplasia seen in UVA-irradiated skin showed a striking contrast to epidermal atrophy in chronological skin aging, which might be induced by steady expression of MMP9 by repetitive UVA-irradiated fibroblasts or promoted by other factors under UVA irradiation.

We observed changes of elastic fiber by Weigert's resorcin-fuchsin stain. Berger et. al. reported that with filtered UVA tubes (peak emission 365nm) daily exposure of 16 hours for 8.5 months was needed to produce solar elastosis. (16) It was considered that the change of elastic fibers observed in our study was the early features of solar elastosis.

It is known that digesting enzymes play important roles in causing photoaging. (17) Starcher et. al. reported that elastase-deficient mice did not show UVR-induced elastosis. (18) In contrast to earlier reports indicating that MMP2 and MMP3 digested elastin (19, 20), recent report suggested that MMP1 and MMP3 did not digest elastin (21), while MMP2 and MMP9 did digest elastin (22, 23). Therefore, we chose MMP2 and MMP9 as representatives of digesting-enzymes of elasitn in our study.

Immunostaining for MMP2, MMP9 and tropoelastin was performed in skin samples and revealed that each of them was detected in fibroblast in the dermis and was not obvious in the epidermis. Fisher et. al. detected the gene expressions for collagenase, MMP9 and stromelysin induced by predominantly UVB irradiation only in the epidermis, although MMP proteins and their enzymatic activity were abundant in both the dermis and epidermis. (17) Therefore, they suggested that a substantial fraction of the MMPs synthesized in the epidermis was transported to the dermis. The differences between two experiments were due to the light sources used and irradiation protocols because they selected UVB as an exposure source.

As fibroblasts in the dermis were thought to be main source of MMP2 and 9 and tropoelastin, we examined mRNA expression of tropoelastin, MMP2 and MMP9 in cultured fibroblasts established from each mouse skin. The tropoelastin mRNA tended to be high in the irradiation group at 12months in the present study. The mRNA expression of elastin was reported to be increased in photoaged skin as compared to that in non-exposed skin from same individuals. (24) On the other hand, elastin mRNA levels were not affected by UVR (295-400nm) in dorsal skin of hairless mouse over 12weeks, and the hyperplasia of elastic fibers were suggested to be caused by post-transcriptional mechanism for elastin expression in another study (25). Schwartz et. al. also suggested that the increase in tropoelastin accumulation by UV irradiation may be caused by the altered taranslational efficiency, but not by the increase of steady-state level of mRNA. (26, 27) These works were performed using UVB source for a relatively short period. Furthermore, Kawaguchi et.al. reported that UVA irradiation did not affect elastin mRNA expression in vitro. (28) There was no previous study examining the expression of tropoelastin mRNA by long-term repetitive UVA irradiation. The expression of tropoelastin mRNA was slightly higher in irradiated group than in the control group at 12months in our study. A significant difference could be encountered if our study were performed for longer than 12months.

The expression of MMP9 mRNA was significantly higher in irradiated group than in non-irradiated group at 12month, which tended to increase in irradiated group as compared to control group at 4month. Otherwise, there was no significant difference in MMP2 mRNA expression between both groups. Previous study reported that MMP9 mRNA expression was increased by UVB irradiation, (29) and UV activation of AP-1 drived MMP9 induction in human skin in their study. Other studies reported that UVA irradiation in vitro resulted in an increase in MMP1 mRNA, but did not stimulate MMP2 (30), because of the activation of AP-1 in human keratinocytes. (31) The upstream region of mouse MMP9 was found to contain several common promoter elements including a AP-1-like binding sites. (32) The continuous activation of AP-1 by UVA exposure might drive MMP9 induction in our experiment.

We observed hyperplasia of elastic fiber in irradiated group in spite of high expression of mRNA of MMP9. Fisher et. al. sugg0ested that the dermal degradation followed by imperfect repair was repeated with each intermittent exposure to UV irradiation, leading to accumulation of solar scarring, and ultimately visible photoaging. (33) Our data suggested their data in the

point that the imperfect repair would be also repeated in the present study.

Coussens et.al. (34) reported that decreasing the incidence of carcinomas and restriction of keratinocyte hyperproliferation in MMP9-deficient/HPV16 mice. It was suggested that MMP9 expression affected the hyperplasia of epidermis directly or indirectly in photoaged skin in our study.

In conclusion, we demonstrated that photoaging can be induced in hairless mouse by the long-term repetitive UVA irradiation. The fibroblast of the dermis was deeply concerned with these changes, and the increase of MMP9 mRNA expression was considered to play an important role in the accumulation of elastin produced by the repeated, imperfect repair. This animal model provides new clues for the prophylaxis of photoaging.

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