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Sunburn reaction in the dorsal skin of hypotrichotic WBN/ILA-*Ht* rats

T. Okada, S.M. Alberenque, A. Yasoshima, V. Malcotti, K. Katayama, K. Uetsuka, H. Nakayama and K. Doi
Department of Veterinary Pathology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo, Japan

Summary. The dorsal skin responses to a single irradiation with a high-dose of UVB (10kJ/m^2) were examined histologically and immunohistochemically in UVB-sensitive Wistar-derived hypotrichotic WBN/ILA-*Ht* rats (HtRs). Sunburn cells (SBCs) which were characterized by pyknotic nuclei and eosinophilic cytoplasm and had ultrastructual characteristics of apoptotic cells were first observed in the epidermis at 3 hours (h) after irradiation. The number peaked at 6 h, and then decreased rapidly. The expressions of p53 protein, which is known to be closely related to the formation of SBCs, and of p21 protein, which is one of the transcriptional target genes of p53, were immunohistochemically detected, and their labeling index (LI) in the epidermis peaked at 12 to 24 h (p53) or at 24h (p21) after irradiation. On the other hand, proliferating cell nuclear antigen (PCNA)-LI in keratinocytes was significantly lower than the control group at 6 h after irradiation and thereafter it increased and became significantly higher than the control group from 24 to 48 h. At 48 h, moderate hyperplasia with moderate numbers of mitotic keratinocytes was first observed in the epidermis. In the dermis, mild edema developed from 12 to 36 h and it accompanied mild lymphocyte infiltration at 36 h. Judging from the present results, it was suggested that some factors other than p53 might be involved in SBC formation, and that p53 might induce p21 protein and play an important role in cell growth arrest in keratinocytes after UVB irradiation.

Key words: Sunburn cell, WBN/ILA-*Ht* rat, PCNA, p53, p21

Introduction

Ultraviolet (UV), especially UVB (wave length: 290-320 nm), is known as an important environmental physical carcinogen. Acute exposure to UVB radiation induces sunburn reaction, and when chronically exposed, actinic keratoses and nonmelanoma skin cancer (squamous cell carcinoma and basal cell carcinoma) are induced (Gilchrest, 1984; de Gruijl et al., 1993). UVB is also known to influence the immune system (Nishigori et al., 1996a). Thus, human health is affected by UVB, but the detailed mechanisms of such UVB-induced disorders are still obscure.

As mentioned above, sunburn reaction is an acute skin lesion in response to a high-dose UVB irradiation. Apoptosis of keratinocytes (Danno and Horio, 1987; Iwasaki et al., 1996; Murphy et al., 2001), which is so-called sunburn cell (SBC) formation, and inflammation with edema in the dermis (Terui and Tagami, 2000) are characteristic histological features of the reaction. SBCs are easily recognized on hematoxylin and eosin-stained sections by their distinctive appearance, i.e. pyknotic nuclei and a shurunken and eosinophilic cytoplasm. UVB is thought to trigger SBC formation by direct injury of nuclear DNA in keratinocytes, mainly through the formation of several kinds of photoproducts at the dipyrimidine sites (Brash et al., 1987, 1996; Mitchell and Nairn, 1989). p53 protein has been said to play a pivotal role in SBC formation (Ziegler et al., 1994; Brash et al., 1996; Murphy et al., 2001). p53 expression was frequently detected in the nucleus of UVB-irradiated keratinocytes *in vivo* (Campbell et al., 1993), and Ziegler et al. (1994) revealed the importance of p53 in SBC formation by showing the significant decrease in UVB-induced SBCs in p53 knocked-out mice. On the other hand, p53 is also known to induce cell-cycle arrest at G1 phase at least partially through the up-regulation of its target gene, p21 (Ko and Prives, 1996; Levine, 1997).

In parallel with these changes, UVB is known to shift cell-mediated immune reaction from T helper (Th)1 to Th2 pattern (Beissert and Granstein, 1996; Nishigori et al., 1996b; Beissert and Schwarz, 1999). In fact, increased production of interleukin (IL)-10, Th2-related cytokine, was observed in cultured kerationocytes (Nishigori et al., 1996b) and epidermal cells in vivo (Shen et al., 1999) after UVB irradiation.

Wistar-derived hypotrichotic WBN/ILA-Ht rats (HtRs) are very sensitive to UVB, and are considered to

Offprint requests to: Dr. Kunio Doi, Department of Veterinary Pathology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan. Fax: +81-3-5841-8185. e-mail: akunio@mail.ecc.u-tokyo.ac.jp

be a useful experimental animal in the field of photodermatology (Kuroki et al., 2001; Malcotti et al., 2001a,b, 2002). In this study, the dorsal skin responses to a single irradiation with a high-dose of UVB (10kJ/m^2) were examined in HtRs, and interesting data about the relationship among apoptosis, proliferating activity, and expression of p53 and p21 in keratinocytes were obtained.

The protocol of this study was approved by the Animal Care and Use Committee of the Graduate School of Agricultural and Life Sciences, the University of Tokyo.

Materials and methods

Animals

A total of 24 7-week-old male WBN/ILA-*Ht* rats (HtRs) were obtained from Saitama Experimental Animal Co., Saitama, Japan. They were kept under controlled conditions (23±2 ºC and 55±5% relative humidity with 14-hour light and 10-hour dark cycle) using an isolator caging system (Niki Shoji Co., Ltd., Tokyo) and were fed commercial pellets (MF, Oriental Yeast Co. Ltd., Tokyo) and water *ad libitum* during the experimental period.

UVB irradiation

Twelve HtRs were exposed to an artificial UVB light (wavelength: 312nm; HP-6LM, ATTO Co., Tokyo, Japan) at 10 centimeters below the light source for 22.5 minutes (irradiation dose: 10 kJ/m^2). The dose was determined based on the preliminary dose-finding study, using UVB detector (ATR-3WX with sensor CX-312, ATTO Co., Tokyo, Japan).

Skin samples were obtained from 6 animals with a 6mm biopsy punch (Nagatoishi Co., Tokyo) under ether anesthesia at 1, 3, 6 and 12 hours (h) after UVB irradiation, respectively. Skin samples were also obtained from another 6 animals in the same way at 24, 36, 48 and 72 h after irradiation, respectively. In addition, skin samples were collected from 12 unirradiated animals in the same way and used as controls.

Half of the skin samples were subjected to histological and immunohistochemical examinations, and the remaining half to reverse transcription and polymerase chain reaction (RT-PCR) analysis.

Histology

Skin samples were fixed in 10% neutral-bufferd formalin, and 4-μm paraffin sections were stained with hematoxylin and eosin (HE) or toluidine blue for histological examinations. Epidermal cells showing intensely stained eosinophilic cytoplasm and pyknotic nuclei were considered to be sunburn cells (SBCs). The number of SBCs were counted on 6 HE-stained sections/animal at each point of examination, and SBC incidence was calculated per linear centimeter of the epidermis for each animal.

Electron microscopy

Small pieces of the collected skin were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), postfixed in 1% osmium tetroxide in the same buffer, and embedded in Epok 812 (Ohken Co. Ltd., Tokyo). Ultrathin sections were double-stained with uranyl acetate and lead citrate and observed under a JEM-1200 EX electron microscope (JEOL Co. Ltd., Tokyo).

Immunohistochemistry

Immunostaining for proliferating cell nuclear antigen (PCNA) was performed on 4-μm paraffin sections by avidin-biotin-peroxidase complex (ABC) method using ABC regent (Vector Laboratories, Burlingame, CA). Mouse anti-PCNA monoclonal antibody (clone PC 10; 1:100; Novocastra Laboratories, Newcastle, UK) was used as the primary antibody. For p53 and p21 immunostaining, 4-μm paraffin sections were deparaffinized, immersed in 10 mM citrate buffer (pH 6.0) and heated for 10 min at 121 °C by autoclave, and thereafter placed in methanol containing 0.3 % H_2O_2 for 30 min at room temperature to inactivate endogenous peroxidase. Then the sections were incubated in 8 % skimmed milk for 30 min at 37 ºC to reduce non-specific staining, and successively incubated in rabbit anti-p53 polyclonal antibody (FL 393; 1:300; Santa Cruz, CA) or mouse anti-p21 monoclonal antibody (clone SX118; 1:100; Pharmingen, SanDiego, CA) overnight at 4 °C. The sections were then incubated in EnVision+ polymer regent (Dako, Carpinteria, CA) for 30 min at room temperature. They were washed in tris-buffered saline for 5 min 3 times after each step, respectively. The positive signals were visualized by peroxidase-diaminobenzidine reaction, and the sections were counterstained with methylgreen.

Under light microscopy, the number of keratinocytes showing immunoreactivity for each antibody in the nucleus was counted on immunostained sections. PCNA-positive keratinocytes were found only in the basal layer, and PCNA-labeling index (LI) was calculated as the percentage of positive cells per all basal keratinocytes (at least 400 cells). On the other hand, although most of p53- and p21-positive cells were found in the basal layer, a few positive cells were also found in the spinous layer. Therefore, p53- or p21-LI was calculated as the percentage of positive cells per all basal and spinous keratinocytes (at least 400 cells).

RNA extraction and semi-quantitative RT-PCR

Total RNA was isolated from skin samples including dermis and epidermis with ISOGEN (Nippon Gene Co. Ltd., Toyama, Japan). To synthesize complimentary

DNA (cDNA), reverse transcription (RT) reaction was carried out using Oligo (dT) 12-18 primer and SUPERSCRIPT Preamplification System (Invitrogen, Rockville, MO) according to manufacturer's protocol.

To detect the level of *IL-10 and interferon (IFN)-γ* mRNA expression, polymerase chain reaction (PCR) was performed. The housekeeping gene *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* mRNA expression was used as an internal standard. Forward and reverse oligonucleotide primers for rat *IL-10, IFN-γ,* and *GAPDH* cDNA were designed and annealing temperature (Tm) for PCR was determined for each primer, respectively (Table 1). Chain reaction was carried out with 50 ml of reaction mixture which contained 5 ml of 10xPCR buffer, 200 μM of each dNTPs, 1.25 units of *Takara Taq*™ DNA polymerase (Takara Shuzo, Ohtsu, Japan), 50 pM of forward and reverse primers, and 1 μl of sample cDNA. First, PCR mixtures were preheated at 94 °C for 7 min, and amplification reaction (1cycle: 94 °C for 1 min, 60.0 °C for IFN-γ and 58.5 °C for others for 1 min, and 72 °C for 1 min) was performed for each cycle number, which were preliminarily determined for each gene (Table 1). Following this, samples were heated at 72 °C for 3 min and cooled at 4 °C. For all PCR procedures described above, Takara PCR Thermal Cycler SP (Takara Shuzo) was used. Four ml of each PCR product was electrophoresed on 1.2% agarose S (Nippon Gene Co. Ltd., Toyama, Japan) in 1 x Tris-borate EDTA (TBE) buffer, and stained with etidium bromide (Gibco BRL, Grand Island, NY). Bands were detected by Fluorescene gel imaging using UV-CCD video system Fas-III (Toyobo, Tokyo, Japan) and fluorescence intensity was quantified by pdi Quantity One® Software, respectively. From obtained results, relative ratio to GAPDH was calculated.

Statistical analysis

Results were represented as mean ± standard error (SE). To determine the difference between UVB and control group, unpaired Student's t-test or Man-Whitney's U test was performed (Statcel software package, Seiun-sha, Tokyo, Japan).

Results

Morphological findings

In the epidermis of the UVB group, SBCs which showed pyknotic nuclei and eosinophilic cytoplasm were first found at 3 h after irradiation (Figs. 1A, 2). The number of SBCs peaked at 6 h (Figs. 1B, 2), decreased rapidly at 12 h, and no SBCs were found at and after 24 h (Figs. 1C-F, 2). Ultrastructurally, SBCs showed condensation of nuclear chromatin and dark cytoplasm due to densely packed tonofilament bundles (Fig. 3), and these findings corresponded to the ultrastructual characteristics of apoptotic keratinocytes. In the control group, no SBCs were found during the experimental period. At 12 to 36 h after irradiation, intracellular edema was found in some basal and spinous keratinocytes (Fig. 1C-E). Until 36 h, mitotic keratinocytes were negligible. At 48 h after irradiation, moderate epidermal hyperplasia with moderate numbers of mitotic keratinocytes was found (Fig. 1F), and the epidermis showed a normal picture at 72 h (Fig. 1G).

In the dermis, slight edema developed from 12 to 36 h and it accompanied mild infiltration of lymphocytes at 36 h (Fig. 1C-E). The number of mast cells showed no changes after UVB irradiation and there was no difference between the UVB and the control group.

Proliferating activity of keratinocytes

Nuclear PCNA protein expression, which is observed during G1 to S phase of cell-cycle and represents cellular proliferating activity, was detected immunohistochemically. In the UVB group, PCNA-LI was significantly lower than that in the control group at 6 h after irradiation (Figs. 4, 5A,D), and thereafter it began to increase and kept a significantly higher level than that in the control group from 24 to 48 h (Figs. 4, 5B-D). At 72h, it returned to the control level (Fig. 4). In the control group, PCNA-LI showed no change during the experimental period (Figs. 4, 5A).

Expression of p53 and p21 protein in keratinocytes

Induction of p53 and p21 protein in keratinocytes after UVB irradiation was detected immuno-histochemically. Although most of p53- or p21- positive cells were found in the basal layer, a few positive signals were also found in the spinous layer. p53-LI first increased at 1 h after irradiation and showed a small peak at 3 h (Fig. 6). It showed the highest level from 12 to 24 h (Fig. 7A), and then decreased but thereafter kept significantly higher levels than those in the control group (Fig. 6). In the control group, p53-positive cells were rarely found (Figs. 6, 7B). On the other hand, p21-LI

Table 1. Oligonucleotide primers used in PCR.

GENE	FORWARD PRIMER (5' to 3')	REVERSE PRIMER (3' to 5')	CYCLES
IL-10	AACTGCACCCACTTCCCAGT	ATGACAGCGTCGCAGCTGTAT	36
IFN-γ	ACACGCCGCGTCTTGGTTTT	CGCTTCCTTAGGCTAGATTCTGG	38
GAPDH	GAGTATGTCGTGGAGTCTACTG	GCTTCACCACCTTCTTGATGTC	25

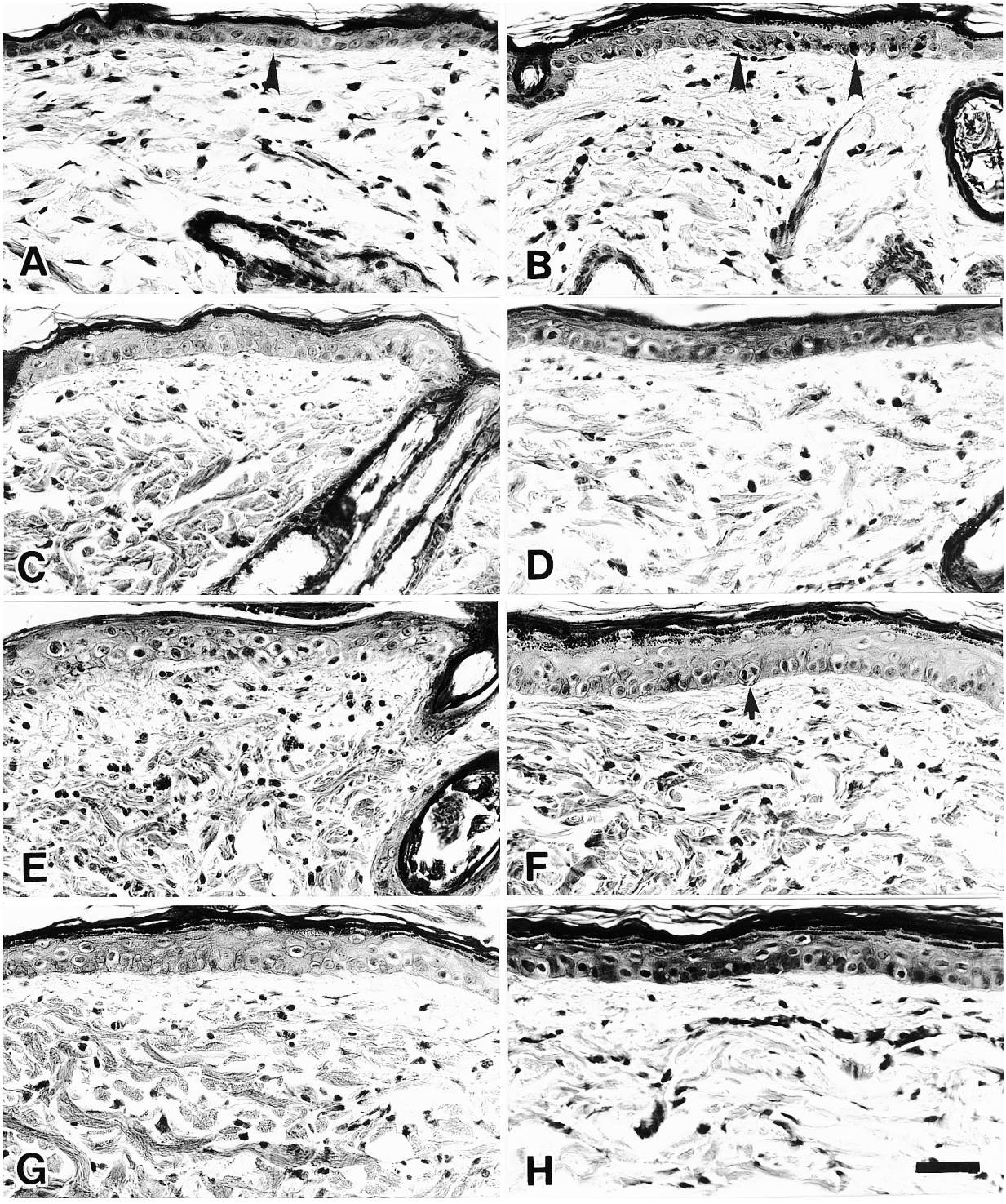

Fig. 1. Histological appearance of UVB-irradiated (**A:** 3 h; **B:** 6 h; **C:** 12 h; **D:** 24 h; **E:** 36 h; **F:** 48 h; **G:** 72 h) and control (**H:** 1 h) dorsal skin of HtRs. **A.** A few sunburn cells are found (arrowhead). **B.** Many sunburn cells are observed (arrowheads). **C and D.** Intracellular edema in epidermis and slight subepidermal edema are observed. **E.** Intracellular edema in epidermis and slight subepidermal edema with mild infiltration of lymphocytes are observed. **F.** Moderate epidermal hyperplasia with mitotic keratinocytes (arrow) is found. **G.** Normal picture. Bar: 50 μm.

started to increase significantly at 3 h after irradiation, peaked at 24 h, and, after that, decreased but kept significantly higher levels than those in the control group (Fig. 8A,B). In the control group, the level of p21-expression was low but constant throughout the experimental period (Figs. 8, 9B).

Expression of IFNγ and IL-10 mRNAs

IFN-γ mRNA expression increased slightly but significantly at 12 and 24 h after irradiation (Fig. 9). In contrast, there was no significant difference in IL-10 mRNA expression between the UVR and the control group during the experimental period.

Discussion

In this study, sunburn reaction in the dorsal skin of HtRs induced by irradiation with a single, high dose of UVB was investigated histologically and immunohistochemically.

SBCs were first observed at 3 h after irradiation, and the number peaked at 6 h, and then decreased rapidly in the epidermis. SBCs were considered to be keratinocytes in the process of apoptotic cell death, and showed ultrastructual characteristics of apoptotic keratinocytes (Kuroki et al., 2001; Malcotti et al., 2001b). As mentioned above, p53 is known to be closely related to the formation of keratinocyte apoptosis, i.e. SBC formation (Ziegler et al., 1994; Brash et al., 1996;

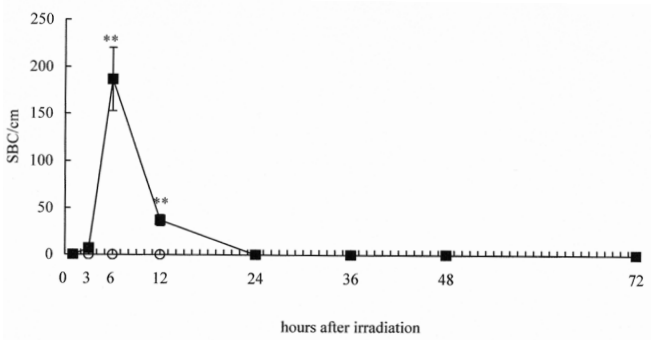

Fig. 2. Number of sunburn cells (SBCs) per linear centimeter of epidermis. black square: UVB group (n=6); white circle: control group (n=6). **p<0.01: Significantly different from control group at the same point (Mann Whitney's U-test).

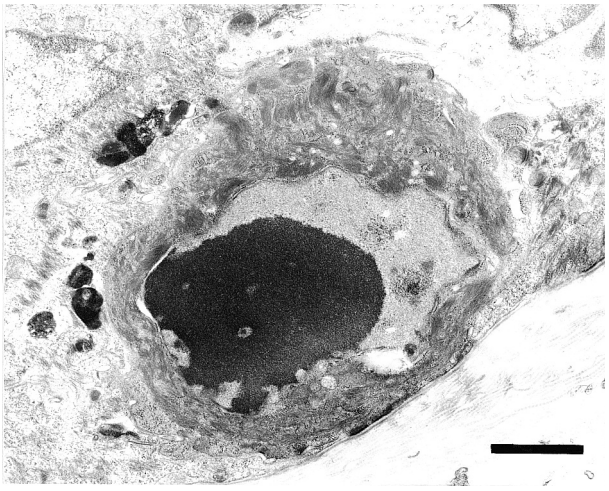

Fig. 3. Ultrastructual appearance of SBC in the epidermis at 6 h after irradiation. Condensation of nuclear chromatin and dark cytoplasm due to a densely packed tonofilament bundle are observed. Bar: 2 μm.

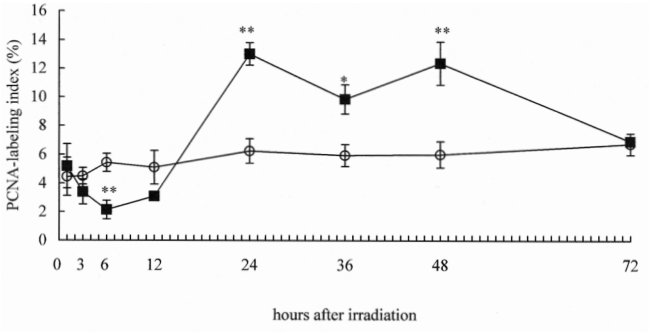

Fig. 4. Changes of PCNA-LI in keratinocytes. black square: UVB group (n=6); white circle: control group (n=6). * p<0.05 and **p< 0.01: Significantly different from control group at the same point (Student's t-test).

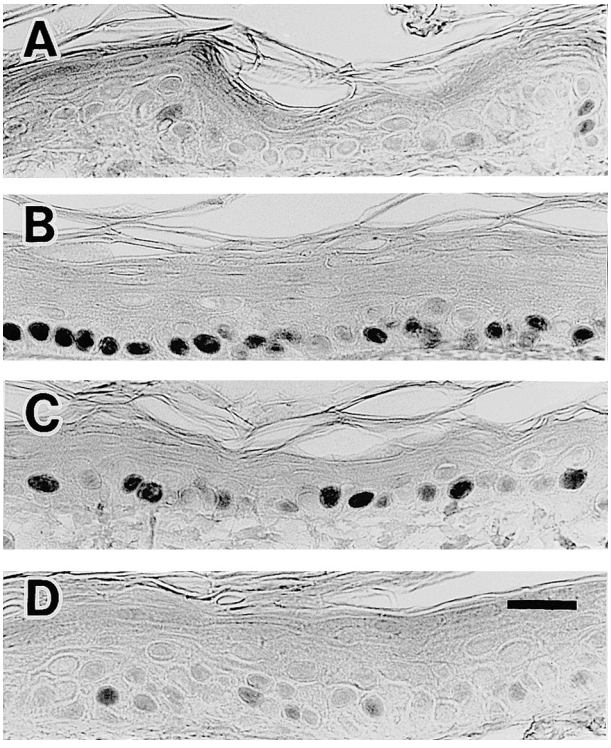

Fig. 5. Immunohistochemistry for PCNA protein. **A:** UVB 6 h; **B:** UVB 24 h: **C:** UVB 48 h; **D:** control 6 h. Bar: 30 μm.

Murphy et al., 2001). It is generally reported that p53-LI reaches its peak before the number of SBCs becomes maximum (Kawagishi et al., 1998; Ouhtit et al., 2000). However, in this experiment, p53-LI showed the highest level from 12 to 24 h following the peak of SBC expression at 6 h, although a significant but slight increase of p53-LI was detected at 1 and 3 h, prior to the peak of SBC incidence. This suggests that some factors other than p53 may be also involved in keratinocyte apoptosis after UVB irradiation in the present case. In this connection, it has recently been reported that UVB could induce keratinocyte apoptosis via a Fas-dependent pathway (Aragane et al., 1998; Takahashi et al., 2001). Also, a significant decrease in the number of SBCs was observed in Fas-ligand-deficient mice compared with wild-type mice (Hill et al., 1999). We are now examining the gene expression profiles in the epidermis of UVB-irradiated HtRs using laser capture microdissection and genome microarray methods.

On the other hand, p53 is also known to activate one of its target genes, p21, and induce cell-cycle arrest. p21 protein is considered to act as a cyclin-dependent kinase inhibitor, and a partially intermediate p53-dependent cell-cycle arrest (Ko and Prives, 1996; Levine, 1997). In the present study, p21-LI peaked at 24 h after irradiation, while p53-LI showed the highest level from 12 to 24 h. Therefore, in this study, it is suggested that p53 itself might induce p21 protein expression, and play a more important role in cell growth arrest rather than in apoptosis in keratinocytes after UVB irradiation. How

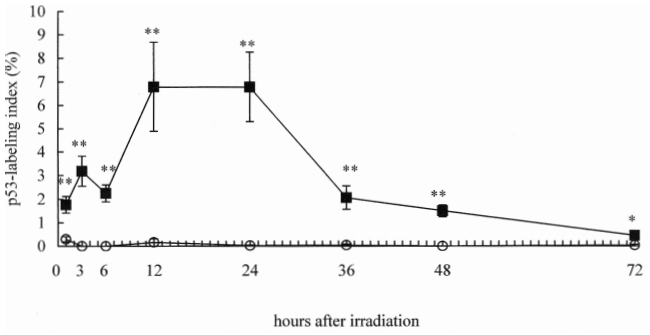

Fig. 6. Changes in p53-LI in keratinocytes. black square: UVB group (n=6); white circle: control group (n=6). * p<0.05 and ** p<0.01: Significantly different from control group at the same point (1 h: Student's t-test; other points: Mann Whitney's U-test).

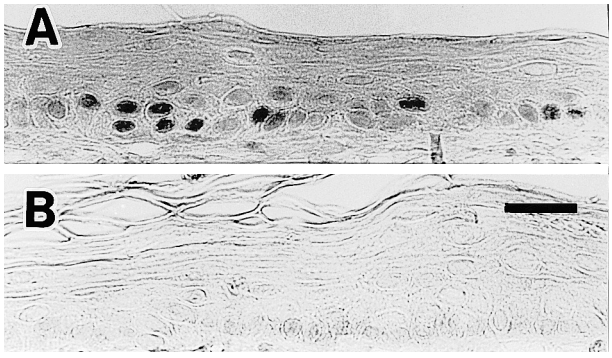

Fig. 7. Immunohistochemistry for p53 protein. **A:** UVB 24h; **B:** control 24h. Bar: 30 μm.

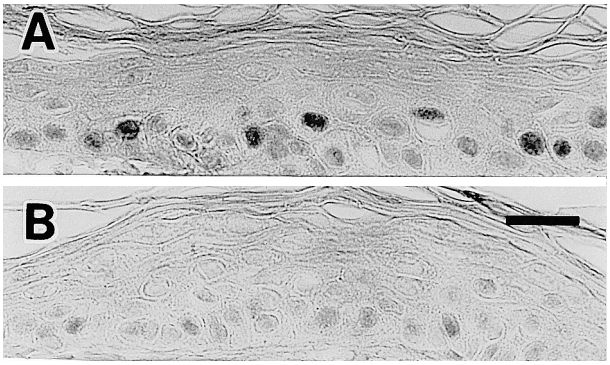

Fig. 9. Immunohistochemistry for p21 protein. A: UVB 24h; B: control 24h. Bar: 30 μm.

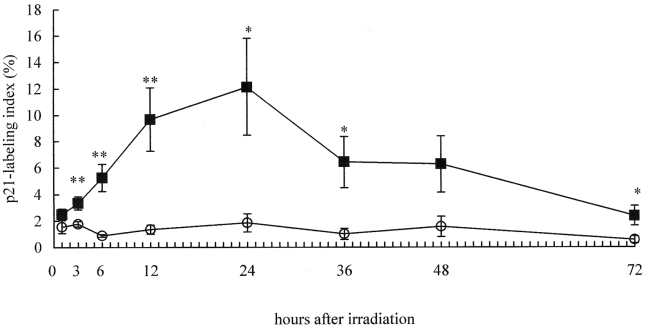

Fig. 8. Changes in p21-LI in keratinocytes. black square: UVB group (n=6); white circle: control group (n=6). * p < 0.05 and ** p < 0.01: Significantly different from control group at the same point (Student's t-test).

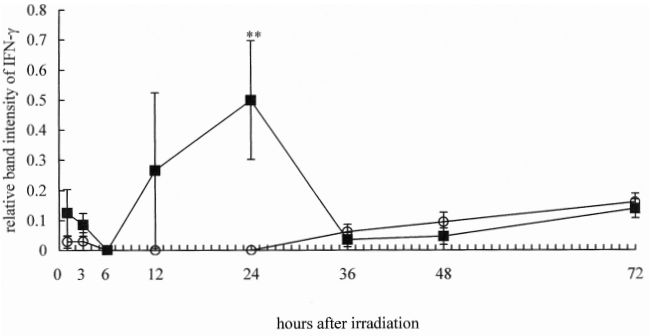

Fig. 10. Changes in *IFN-γ* mRNA expression detected by RT-PCR. black square UVB group (n=6); white circle: control group (n=6). **p < 0.01: Significantly different from control group at the same point (Mann Whitney's U-test).

p53 protein can choose apoptosis or cell-cycle arrest in UVB-irradiated keratinocytes is an interesting problem. With regard to this, Toren et al. (1998) showed that p53-regulated keratinocyte apoptosis by UVB irradiation is dependent on keratinocyte differentiation in vitro and they inferred that p53 alternative splicing variant may be involved in this mechanism. To clarify this point, further studies are needed.

PCNA-LI in keratinocytes was significantly lower than in the control group at 6 h after irradiation, when SBC incidence showed its peak level. Thereafter PCNA-LI increased and became significantly higher than in the control group from 24 to 48 h, and finally returned to the control level at 72 h. The early increase in PCNA-LI was considered to be a regenerative reaction following UVB-induced keratinocyte apoptosis. On the other hand, taken together with the above-mentioned changes in p53- and p21-LI, there is a possibility that cell-cycle might be arrested at the late G1 or S phase at least until 24 h and it might begin to rotate again at 36h, when p53- and p21-LI decreased. After that, moderate epidermal hyperplasia with increased mitotic keratinocytes developed at 48h, when PCNA-LI still kept the peak level. Mitosis of keratinocytes was negligible until 36 h. In this concern, Berg et al. (1996) reported that, after UVB irradiation, BrdU-LI (ratio of cells in S phase) had peaked at 24 h after the peak of p53-LI, and Ouhit et al. (2000) reported that the PCNA protein expression in the epidermis had peaked at 36 or 24h after the peak of p53 or p21 protein expression by Western blot and immunohistochemical analysis.

On the other hand, in the dermis of UVB-irradiated dorsal skin of HtRs, slight edema developed from 12h to 36h after irradiation, and it accompanied mild infiltration of lymphocytes at 36h. We tried to differentiate $CD4^+$ and $CD8^+$ lymphocytes immunohistochemically, and there was no difference between the UVB and the control group (data not shown), although there was a report that suggested the involvement of the $CD4^+$ and $CD8^+$ lymphocytes in UVB-induced inflammation (Terui and Tagami, 2000). In addition, there was no difference in the number of infiltrating mast cells in the dermis between the UVB and the control group throughout the experimental period. Along with inflammatory changes, it has been reported that the expression of *IL-10* (Th2-related cytokine) mRNA expression increased in UVB-irradiated skin (Nishigori et al., 1996b; Beissert and Schwarz, 1999; Shen et al., 1999), but no significant increase in the expression of IL-10 mRNA was observed in the present study. In contrast, the expression of *IFN-γ* (Th1-related cytokine) mRNA was significantly but slightly increased from 12 to 24h, when inflammatory edema was found.

As mentioned above, in the present study an interesting relationship among the kinetics of apoptotic cell death, cell proliferation, and cell-cycle arrest was found. Further investigations on the mechanisms of UVB-induced apoptosis, cell-cycle arrest and proliferation in keratinocytes in HtRs are now in progress using laser capture microdissection and genome microarrray methods.

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