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Identification of Skin Injury-related Genes Induced by Ionizing Radiation in Human Keratinocytes using cDNA Microarray

Manabu KOIKE*, Tomoe SHIOMI and Aki KOIKE

Apoptosis/ATF3/Keratinocyte/Skin/X-ray.

The skin is an external organ that is most frequently exposed to radiation. High-dose radiation initiates and promotes skin cancer and acute radiation injury. It is important to investigate the influence of high-dose radiation exposure on the skin at the molecular level to understand acute radiation injury. To identify genes that are associated with injury caused by high-dose radiation exposure of the skin, we used microarray technology to examine the effect of irradiation on approximately 1000 genes in normal human epidermal keratinocytes at 3 h postirradiation with a cytotoxic dose of X-ray (5 Gy). We found that 16 and 59 genes were up- and down-regulated respectively in the keratinocytes. Several apoptosis-related genes, for example, BAK and TSC-22, and anti-proliferative genes, for example, BTG-1 and BTG-3, were up-regulated. We focused on ATF3 because ATF3 is induced most strongly by X-irradiation, and its function in keratinocytes is unknown. The induction of the ATF3 mRNA and protein in keratinocytes following X-ray was confirmed by RT-PCR and western blot analysis. ATF3 was also induced and accumulated within the nuclei of keratinocytes after X-ray irradiation *in vivo* and *in vitro*. Exogenous EYFP-ATF3 also accumulated within the nuclei of keratinocytes. In the transient expression assay, EYFP-ATF3, but not EYFP, induced apoptosis in keratinocytes. Taken together, these results suggest that ATF3 plays a role in apoptosis in keratinocytes and is associated with skin injury caused by ionizing radiation.

INTRODUCTION

Ionizing radiation initiates and promotes cancer, apoptosis, aging, and immune suppression. The skin is an external organ that has most frequent opportunities exposed to radiation. High-dose radiation causes skin cancer and acute radiation injury to the skin. It is important to investigate the influence of high-dose radiation exposure on the skin at the molecular level to understand acute radiation injury and establish a therapeutic system for radiation injury to the skin. In addition, discovery of a biomarker for predicting a prognosis is expected, because the effects on the skin are dose-dependent and easily detectable by visual inspection.

The influence of high-dose X- or gamma-ray radiation exposure on human skin at the molecular level has been hardly analyzed due to the limited use of human materials. Clarification of the molecular signal transduction mechanisms in keratinocytes following high-dose radiation is necessary for medical treatment of acute radiation injury. To date, studies of the influence of irradiation on mouse skin and human cultured keratinocytes have been conducted as models of human skin. Parkinson *et al.* (1986) showed that the viability of irradiated (5 Gy) human skin keratinocytes is <5% of non-treated control.[1)] They also showed using gamma-ray that freshly isolated human skin keratinocytes are more radiosensitive than mouse skin keratinocytes,[1)] suggesting that radiation susceptibility differ between human and mouse keratinocytes.

Mitogen-activated protein kinase (MAPK) pathways are linked to the growth-factor-mediated regulation of diverse cellular events such as apoptosis, proliferation, and senescence. MAPK can also control cell fate, especially cell death, after irradiation in a cell-type-dependent manner. The p38 pathway plays an important role in the control of radiation-induced G_2/M arrest, which is protective; in certain cell types, it also plays a proapoptotic role via the induction of GADD transcription factors.[2)] Although activation of the Jun amino-terminal kinase (JNK) pathway was initially linked to toxic effects of radiation signaling, a recent study has shown that JNK can either protect or enhance radiation toxicity in a cell-type-dependent manner.[2)] This reports indicated that cellular radiation-toxic stress responses are complex and heterogeneous. Therefore, the JNK and p38 pathways appear to provide signals for both cell survival and cell death in a cell-type-specific manner. However, MAPK path-

*Corresponding author: Phone: +81-43-206-3139,
Fax: +81-43-206-3139,
E-mail: m_koike@nirs.go.jp
Radiation Hazards Research Group, National Institute of Radiological Sciences, 4-9-1 Anagawa, Inage-ku, Chiba 263-8555, Japan.

ways in human skin or normal keratinocytes are poorly described, but appear to be activated by high-energy radiation.

Recently, a microarray-based method for the high-throughput monitoring of gene expressions has been introduced. This technology has revolutionized gene expression studies by providing a method of measuring mRNA levels. The application of high-throughput screenings such as the cDNA microarray analysis will provide us with a clear concept of the stress-responsive pathway. In the fields of radiation biology, the pattern of gene expression in ML-1 human myeloid cells or Jurkat T cells exposed to gamma-ray was examined by DNA microarray analysis.[3, 4] The differentially expressed genes identified in these assay have been reported to function in various cellular processes such as cell cycle regulation, apoptotic response, carcinogenesis, and other cellular functions. Despite these analyses, our understanding underlying the mechanisms of acute radiation injury is not yet clarified. It is reported that radiation transcriptional responses varied widely in cell lines with different tissues of origin.[3] To date, a microarray analysis for identifying genes regulated in response to X- or gamma-ray irradiation of normal human keratinocytes has not been reported yet.

In this study, we searched and identified using microarray technology the genes that are associated with injury to the skin caused by high-dose radiation exposure.

MATERIALS AND METHODS

Cell lines and cultures

Normal human keratinocytes were obtained from Sanko Junyaku Corporation (Osaka, Japan) or Kurabo Industries Ltd. (Osaka, Japan). The cells were cultured in EpiLife Extended Life Span serum-free medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with bovine pituitary extract (BPE)-free keratinocyte medium supplement (Sigma-Aldrich) and an antibiotic. The cells were maintained in a humidified incubator at 37°C under 5% CO_2.

X-irradiation

Keratinocytes in the exponentially growing phase and the mice were exposed to various doses (5, 10, 20, 40 Gy) of X-ray at room temperature.[5,6] The X-rays were generated at 200kVp/20mA and filtered through 0.5 mm each of Cu and Al, using Pantak HF320S (Shimadzu, Kyoto, Japan).

Microarray analysis

Microarray analysis was performed by *IntelliGene Human CHIP 1K Set 1 version 1.0* (Takara Biomedicals, Shiga, Japan), onto which approximately 1000 cDNA fragments of known human genes had been spotted. GADD153, p21 and cyclin D1 were not included. Total RNA was extracted with Trizol (Life Technologies, Rockville, MD) from cells and mRNA was further purified by passing through an oligo-dT column (Oligotex-dT30, Takara). A fluorescent probe was synthesized by incorporating Cy3- or Cy5-dUTP using 1 μg of the above mRNA as a template and 50 U AMV reverse transcriptase. Cy3- and Cy5-labeled probes were prepared with the mRNAs isolated from the control cells and X-ray-treated cells, respectively. Hybridization and washing of the microarray were carried out according to manufacturer's instructions. The hybridized CHIP was visualized using the Affymetrix (Woburn, MA) 428 Array Scanner. Fluorescence intensity was processed, measured, and analysed by ImaGene software Ver.4 (BioDiscovery, Los Angels, CA) and data were imported into an Excel (Microsoft) database, with the corresponding gene names. Compensation of each fluorescence intensity was performed by the global normalization of the total fluorescence signal except the control signal. The analysis was performed once. The cut-off value for induction or repression was 1.5.

RNA extractions and RT-PCR

Total RNA was isolated by using SV total RNA isolation system (Promega, Madison, WI) according to the manufacture's protocol. RT-PCR was carried out using Qiagen One-Step RT-PCR kit (QIAGEN Inc, Chatworth, CA) according to the manufacture's protocol. Primers sequences were: sense 5'-CAACCTGGTTCAGCAGTTCA-3' and antisense 5'-CTGATTGCTGGGCACAAGTA-3' (450 bp) for human ATF1; sense 5'-ATGAAATTCAAGTTACATGTG-3' and antisense 5'-GAAACTGGTCTTTCCTTGATT-3' (1591 bp) for human ATF2; sense 5'-CTTTGTCAAGGAAGAGCTGAG-3' and antisense 5'-TTAGCTCTGCAATGTTCCTTC-3' (429 bp) for human ATF3; sense 5'- AACCGACAAAGACACCTTCG-3' and antisense 5'-GTGTCATCCAACGTGGTCAG -3' (415 bp) for human ATF4; sense 5'-GCTGAGCCGCGACTGTGATG-3' and antisense 5'-CCTGAGCGAGGCACAAGGGT-3' (306 bp) for human p21; and sense 5'-TGAAGGTCGGAGTCAACGGATTTGGT-3' and antisense 5'-CATGTGGGCCATGAGGTCCACCAC-3' (982 bp) for human glyceraldehyde 3-phosphate dehydrogenase (GAPDH).[7–10] Reaction products were separated on 2% agarose gel in Tris-acetate EDTA buffer, and stained with ethidium bromide.

Western blot analysis

The western blot analysis was performed as previously described.[11,12] In brief, total lysates from cells were boiled and cleared by centrifugation, and the supernatants were electrophoresed on 5–15% SDS-polyacrylamide gels. The fractionated products were electrotransferred onto Immobilon-P membranes (Millipore, Bedford, MA). After blocking nonspecific binding sites with 1% bovine serum albumin, the membranes were incubated with a rabbit anti-ATF3 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, Ca). The corresponding proteins were visualized using a

ProtoBlot western blot AP system (Promega) according to the manufacturer's instructions.

Immunofluorescence staining

Immunofuorescence staining was performed as previously described.[12] In brief, the cells were grown on culture glass slides (Falcon, Lincoln Park, NJ), washed with PBS, and fixed at room temperature. The fixed cells were first blocked for 30 min in a blocking solution and then incubated for 30 min at room temperature with a rabbit anti-ATF3 polyclonal antibody, a rabbit anti-PARP p85 fragment antibody, a rabbit anti-active-Caspase-3 antibody (Promega), or a mouse M30 CytoDEATH monoclonal antibody (Roche Molecular Biochemicals, Mannheim, Germany). After washing with PBS, antibody binding was detected by the application of Alexa fluor 568-conjugated secondary antibodies (Molecular Probes, OR, USA). DNA was stained with 0.025 μg/ml 4,6-diamino-2-phenylindole (DAPI) fluorescent dye (Boehringer Mannheim, Mannheim, Germany), and then the cells were examined under an Olympus IX 70 fluorescence microscope (Olympus, Tokyo, Japan) to determine localization.[12] Images were acquired with a Hamamatsu chilled 3-chip color charge-coupled-device camera (C5810-01) driven by IP lab (Signal Analitics Corp., Vienna, VA) imaging software.

Plasmid construction and transfection of DNA into normal human keratinocytes

Full-length human ATF3 cDNA was cloned in pEYFP-C1 (Clonetech, Palo Alto, CA) as previously described.[12] The junctions of a construct was verified by sequencing. Keratinocytes were plated on a 35-mm dish (Falcon) at a density of 2×10^5 cells/well the night before transfection. Transfection was performed in these cells using Effectene (QIAGEN Inc) as previously described.[12] Fluorescence in transfected cells was visualized as previously described.[13] In short, the cells were fixed in 0.2 M phosphate buffer (pH 7.4) with 4% paraformaldehyde (PFA). DNA was stained with DAPI fluorescent dye, and then the cells were examined under an Olympus IX 70 fluorescence microscope to determine localization.[12] Images were acquired as described above.

Transient transfection assays for effect of ATF3 on cell proliferation

The cell proliferation assay was performed according to the method of Mo and Dynan.[14] Keratinocytes were grown on a 35-mm dish (Falcon) the night before transfection. EYFP-ATF3 or EYFP vectors were introduced using Effectene (QIAGEN Inc.), as described above. The next day, the cells were replated on a glass slide (Falcon). After 1 or 2 days, yellow colonies were counted and categorized, on the basis of whether they contained one, two, or three or more cells. The percentage of colonies in each category, relative to the total number of colonies counted, was determined. Two hundred fluorescent clusters were counted in each experiment.

Immunohistochemistry

For immunohistochemical staining, freshly excised whole skin samples from C57BL/6J mice were frozen in an O.C.T. compound (Sakura Fine Technical Co., Ltd., Tokyo, Japan) in liquid nitrogen and sectioned with a cryostat microtome. Immunostaining was performed using the rabbit polyclonal anti-ATF3 antibody or anti-p53 antibody (NovaCastra, Newcastle upon Tyne, UK), and the DAKO Envision Plus HRP system (DAKO, Ely, Cambridgeshire, UK). PFA (4%)-fixed sections of mouse skin tissue were antigen-retrieved with heating using a microwave oven for 15 min in citrate buffer (pH 6.5), prior to peroxidase blocking (3%, v/v hydrogen peroxide). The primary antibody and horseradish peroxide-labelled polymer were used as per the DAKO Envision kit, followed by 3,3-diaminobenzidine treatment and counter-staining with haematoxylin before mounting.

RESULTS

Identification of radiation-induced gene in normal human epidermal keratinocytes

Skin is the largest organ in the body, whose outermost adult tissue is the epidermis. The epidermis is a stratified squamous epithelium composed primarily of keratinocytes and a few other cells. To identify radiation-injury-related genes in human keratinocytes, we first performed microarray analysis using Human CHIP 1K, in which about 1000 human genes including genes previously known to be radiation-responsible, *e.g.,* HDM2 and Topoisomerase II. The expression levels of an X-irradiated sample was compared with that of control keratinocytes by cDNA microarray assay. Primary human epidermal keratinocytes were grown on subconfuluence. We selected 5 Gy (dose rate, 0. 49 Gy/min) as a relatively lethal dose of X-ray,[1] to identify radiation injury-related genes induced by ionizing radiation. As shown in Table 1, the analysis of the hybridization signals revealed that 16 and 59 genes were up- and down-regulated at 3 h postirradiation. Among the transcripts changed by radiation treatment were some genes previously known to be radiation-inducible (*e.g.,* HDM2 and IL-1) and to be radiation down-regulative (*e.g.,* Topoisomerase I and Topoisomerase II) in human keratinocytes. The differentially expressed genes identified in this assay have been reported to function in various cellular processes such as apoptotic response (Bak and TSC-22), DNA repair (Xpg), carcinogenesis (APC and BRCA2), and anti-cell proliferation (BTG-1, BTG-3, and NME1), in addition to other cellular functions.

Radiation-induced ATF3 protein expression and subcellular localization in vitro and in vivo

As described above, we found the induction of several

Table 1. Induction (A) and repression (B) of genes in normal human keratinocytes upon treatment with X-ray for 3 h at 5 Gy

(A) Gene name	**Accession no.**	**Induction ratio**
activating transcription factor 3 (ATF3)	NM_004024	3.31
cytokine-inducible kinase (PLK3)	U56998	2.56
BTG family, member 3 (BTG-3)	AL049332	2.07
mouse double minute 2, human homolog of; p53-binding protein (HDM2)	M92424	1.82
diphtheria toxin receptor (heparin-binding epidermal growth factor-like growth factor) (DTR)	M60278	1.66
BCL2-antagonist/killer 1 (BAK)	Z93017	1.66
tumor necrosis factor receptor superfamily, member 10c (TNFRSF10c)	AF016267	1.65
interleukin 1, alpha	M28983	1.62
cytochrome c oxidase subunit VIa polypeptide 1 (COX6a1)	AL021546	1.60
unc119 (C.elegans) homolog	AF028789	1.57
B-cell translocation gene 1 (BTG-1)	X61123	1.56
transforming growth factor beta-stimulated protein TSC-22	AJ222700	1.54
prefoldin 5 (PFDN5)	D89667	1.54
ATP-binding cassette, sub-family D (ALD), member 4	Y14318	1.53
Pirin	Y07868	1.52
non-metastatic cells 1, protein (NM23A) expressed in (NME1)	X17620	1.50
(B) Gene name	**Accession no.**	**Induction ratio**
centromere protein F (350/400kD, mitosin)	U30872	0.25
centromere protein E (312kD)	Z15005	0.32
tight junction protein 1 (zona occludens 1)	L14837	0.37
chromodomain helicase DNA binding protein 1	AF006513	0.44
zinc finger protein 220	U47742	0.45
spectrin, alpha, non-erythrocytic 1 (alpha-fodrin)	J05243	0.45
myeloid/lymphoid or mixed-lineage leukemia (trithorax (Drosophila) homolog); translocated to, 4	AL161973	0.47
adenomatosis polyposis coli (APC)	M73548	0.5
mitogen-activated protein kinase kinase kinase 4 (MAPKKK4)	AF002715	0.5
zuotin related factor 1	AC004668	0.5
upstream regulatory element binding protein 1	AB002310	0.5
hyaluronan-mediated motility receptor (RHAMM)	AF032862	0.5
RAN binding protein 2	NM_006267	0.52
bullous pemphigoid antigen 1 (230/240kD)	M69225	0.53
SRY (sex-determining region Y)-box 9 (campomelic dysplasia, autosomal sex-reversal)	NM_000346	0.53
basonuclin	L03427	0.54
chromodomain helicase DNA binding protein 2	AF006514	0.54
topoisomerase (DNA) I (Topoisomerase I)	M60706	0.54
KIAA0054 gene product; Helicase	D29677	0.55
PDZ domain containing guanine nucleotide exchange factor(GEF)1	AB002311	0.56
dihydropyrimidinase-like 2	U97105	0.56

continued		
zinc finger protein 262	AB007885	0.56
ERCC5 (human XPG)	X69978	0.56
breast cancer 2, early onset (BRCA2)	X95152	0.56
splicing factor, arginine/serine-rich 2, interacting protein	Y11251	0.57
E1A binding protein p300	U01877	0.57
tumor protein 53-binding protein, 1	AF078776	0.57
heterogeneous nuclear ribonucleoprotein U (scaffold attachment factor A)	AF068846	0.58
replication factor C (activator 1) 1 (145kD)	L23320	0.58
H. sapiens hbrm mRNA	X72889	0.58
eukaryotic translation initiation factor 4 gamma, 3	AF012072	0.60
KIAA0225 protein	D86978	0.60
myosin phosphatase, target subunit 1	D87930	0.60
neural precursor cell expressed, developmentally down-regulated 4	D42055	0.60
fragile X mental retardation 1	X69962	0.61
KIAA0210 gene product	D86965	0.61
CCAAT-box-binding transcription factor	M37197	0.61
endothelial differentiation, sphingolipid G-protein-coupled receptor, 1	M31210	0.62
topoisomerase (DNA) II alpha (170kD) (Topoisomerase II)	J04088	0.62
thrombospondin 1	X14787	0.62
kinesin 2 (60-70kD)	L04733	0.63
KIAA0326 protein	AB002324	0.63
Human methylenetetrahydrofolate dehydrogenase-methenyltetrahydrofolate cyclohydrolase-Formyltetrahydrofolate synthetase mRNA	J04031	0.63
RAN binding protein 2-like 1	AF012086	0.64
serine/threonine kinase 15	AF011468	0.64
inositol polyphosphate-4-phosphatase, type II, 105kD	U96922	0.65
zinc finger protein 146	X70394	0.65
cullin 2	U83410	0.65
CBF1 interacting corepressor	U03644	0.65
solute carrier family 26 (sulfate transporter), member 2	U14528	0.65
transcription factor AP-2 gamma (activating enhancer-binding protein 2 gamma)	U85658	0.65
rearranged L-myc fusion sequence	U22377	0.66
myosin VI	AB002387	0.66
insulin-like growth factor 1 receptor	NM_000875	0.66
zinc finger protein 148 (pHZ-52)	AJ236885	0.66
vesicle docking protein p115	D86326	0.66
radixin	L02320	0.66
host cell factor C1 (VP16-accessory protein)	U52112	0.66

Ratios of relative induction by x-ray compared to basal levels in normal human keratinocytes.

radiation-injury-related genes in keratinocytes after X-irradiation. As shown in Table 1, the ATF3 transcript was induced most strongly in the irradiated keratinocytes. First, the increase in ATF3 expression by irradiation was confirmed by RT-PCR analysis, using p21 as control (Fig. 1A), although there was some difference in the dose-dependency of these two genes: p21 reached the maximal level by 5 Gy, whereas ATF3 increased in a dose dependent manner. We also examined other genes of ATF family, namely, ATF1, ATF2 and ATF4. Although these genes have been shown to be stress- and/or extracellular signal-inducible, we found no significant induction of these genes by X-ray. Next, we confirmed the induction of the ATF3 protein in the irradiated keratinocytes by western blot analysis. Human keratinocytes were exposed to a high-dose of X-ray (20 Gy). The keratinocytes were collected at 4 h or 8 h after treatment and were analyzed for ATF3 protein expression. As shown in Fig. 1B, the cells exposed to X-ray exihibited a strong induction of the ATF3 protein compared with the unirradiated cells.

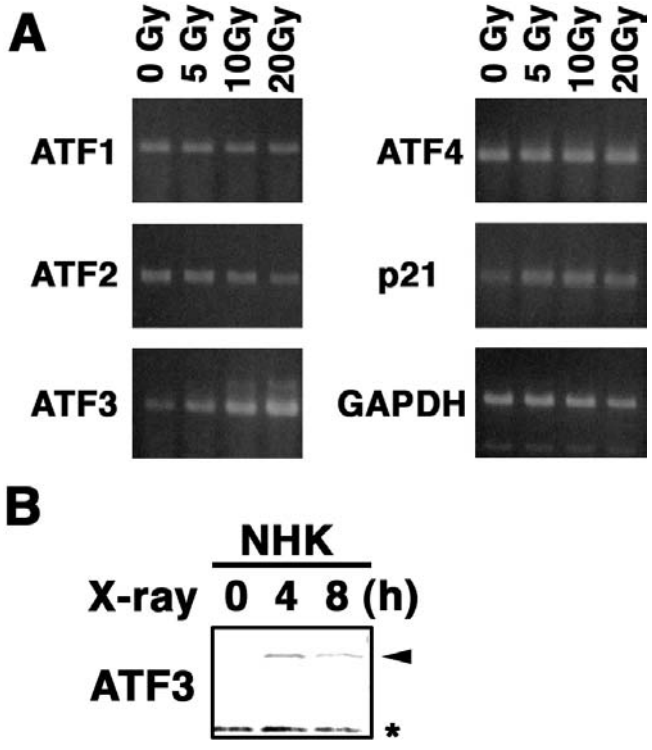

Fig. 1. Induction of ATF3-mRNA and -protein following ionizing radiation in human keratinocytes. (A) Induction of ATF3- and its family genes-mRNA following ionizing radiation in human keratinocytes. Cells were treated with IR by different doses. Total RNA was prepared at 3 h after treatment and analysed by RT-PCR analysis. (B) Normal human keratinocytes were nontreated (0 h), or irradiated with 20 Gy IR. Whole cell protein was prepared at 4 and 8 h after treatment. ATF3 protein levels were subsequently determined by western blot analysis. An asterisk indicates a non-specific band, which is a loading reference.

These results demonstrated that the ATF3 protein is induced by a high-dose of X-ray in normal human keratinocytes. ATF3 is a member of the ATF/CREB family of transcription factors.[15] Although several lines of evidence have indicated that ATF3 mRNA can be induced following genotoxic stress,[15] the target genes remains to be identified. In addition, the physiological function of ATF3 has been reported to have both protective and detrimental effects, depending on the cell type. On the other hand, the ATF3 function in normal human keratinocytes and the skin is unclear. Therefore, we focused our attention on ATF3.

To examine whether ATF3 induced by high-dose X-irradiation accumulates within the nuclei of keratinocytes in agreement with the function as a transcription factor, the expression of ATF3 was examined in normal human keratinocytes by immunofluorescence staining using an ATF3 antibody (Fig. 2A). In the unirradiated keratinocytes, ATF3 was not detected or was expressed at low levels. The keratinocytes exposed to high doses of X-ray (20 Gy) showed increased ATF3 levels. The staining for ATF3 was localized mainly within the nuclei of these cells. These results demonstrate that ATF3 is induced by high-dose X-ray in cultured human keratinocytes, and ATF3 induced by irradiation accumulates within the nuclei of keratinocytes.

X-ray activates both p38 MAPK and JNK in human cells.[16] Anisomycin is an inducer of JNK and p38 MAPK pathways.[17] It was reported that anisomycin increase the steady-state level of ATF3 mRNA in HeLa cells.[18] Thus, we examined whether the ATF3 protein is induced by anisomycin in normal human keratinocytes. We found that ATF3 was also induced in keratinocytes upon treatment with anisomycin, which is an activator of p38 MAP kinase/JNK and an apoptosis inducer (data not shown).

To confirm the nuclear localization of ATF3 in normal human keratinocytes, we constructed fusion genes of EYFP and human full-length ATF3 under the control of the immediate early promoter of human cytomegalovirus (CMV) (Fig. 3A). The expression vector was transfected into normal human keratinocytes. Two days after the transfection, the subcellular localization of the fusion protein was examined under a fluorescence microscope. The cells were also subjected to immunofluorescene analysis using an anti-ATF3 antibody (Figs. 3Ba'', b''), to confirm that the proteins visualized by EYFP were EYFP-ATF3 fusion proteins. We detected ATF3 in the EYFP-ATF3 transformants (Figs. 3Ba, a', a''). On the other hand, no ATF3 was detected in the EYFP transformants (Figs. 3Bb, b', b''). These results indicate that the EYFP-ATF3 fusion protein can be successfully expressed in normal human keratinocytes. EYFP-ATF3 fusion proteins were found in the nucleus (Fig. 3Ba), while EYFP alone was localized throughout the keratinocytes (Fig. 3Bb). These results suggest that the ATF3 protein accumulates within the nuclei of normal human keratinocytes. This agreed well with the results obtained by immunocytochem-

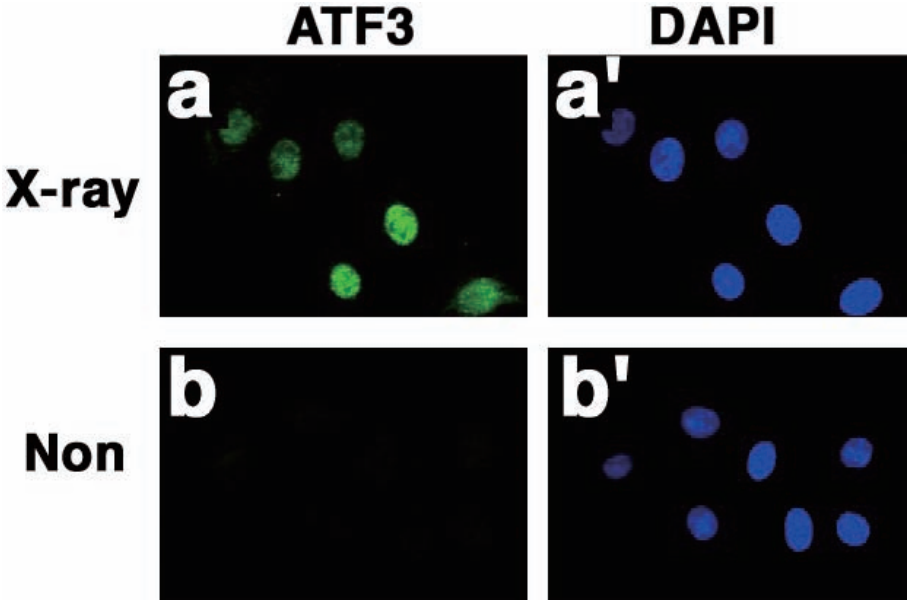

Fig. 2. The endogenous ATF3 protein accumulates within the nuclei of the irradiated human keratinocytes. Normal human keratinocytes were nontreated, or irradiated with 20 Gy IR and after 4 h subjected to immunofluorescene analysis with the anti-ATF3 antibody (a, b). Nuclei were visualized by DNA staining with DAPI (a', b').

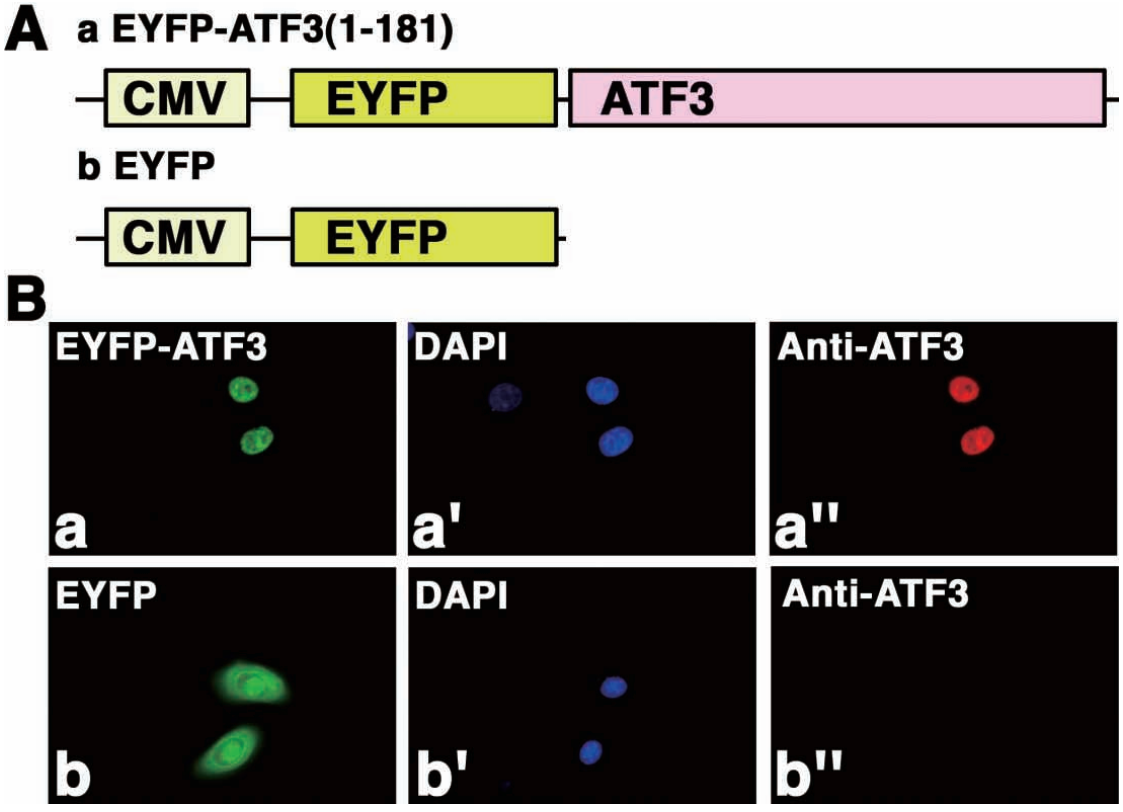

Fig. 3. The exogenous ATF3 protein accumulates within the nuclei of human keratinocytes. (A) Schematic diagrams of human EYFP-ATF3 (a) and EYFP (b) expression vectors used. (B) An expression vector of the fusion gene was transfected into normal human keratinocytes. Two days after transfection, the subcellular localization of the fusion protein was examined under a fluorescence microscope. The cells were subjected to immunofluorescene analysis with the anti-ATF3 antibody (a'', b'') to confirm the expression of the transfected ATF3. Nuclei were visualized by DNA staining with DAPI. For the same cells, EYFP-ATF3 (green; a) or EYFP image (green; b), anti-ATF3-Alexia-568 images (red; a'', b''), and DAPI-stained DNA images, (blue; a', b') are shown.

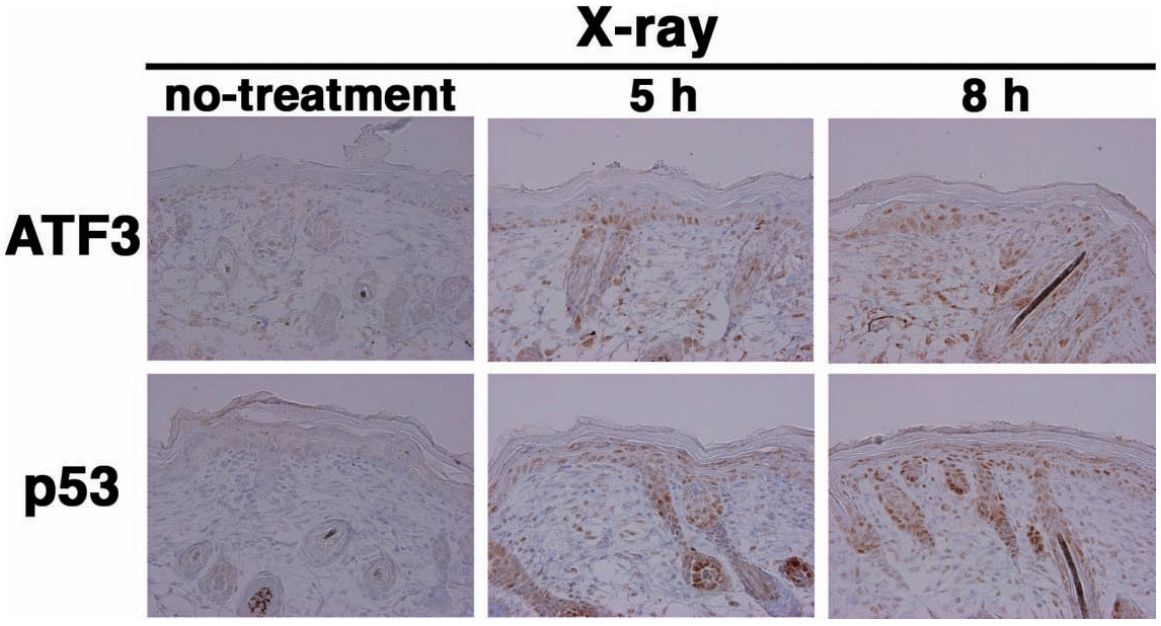

Fig. 4. ATF3 protein is induced by X-irradiation and is accumulated within the nuclei of keratinocytes in the skin of the irradiated mice. The sections of X-irradiated (40 Gy) mouse skin (5 h, 8 h) and control littermate mouse skin (non-treatment) were immuno-stained with antibodies to mouse p53 or ATF3 as indicated. Nuclei were visualized by counterstaining with haematoxylin before mounting.

ical analysis (Fig. 2).

To examine whether ATF3 is induced by high-dose X-ray *in vivo*, the expression of ATF3 was examined in cryostat sections of mouse skin by immunofluorescence staining using an ATF3 antibody (Fig. 4). In the unirradiated skin, ATF3, as well as p53 used as control, was not detected or was expressed at low levels. The skin samples exposed to high doses of X-ray (40 Gy) showed increased ATF3 and p53 levels. The staining for ATF3 at 5 h and 8 h after exposure to X-ray was found to be positive for both keratinocytes in the epidermis and fibroblasts in the dermis. Staining was localized mainly within the nuclei of these cells. These results demonstrate that ATF3 is induced by high-dose X-ray *in vivo*, and ATF3 induced by irradiation accumulates within the nuclei of keratinocytes and fibroblats in the skin of the irradiated mice. Conclusively, ATF3 was induced by high-dose X-ray in keratinocytes *in vitro* and *in vivo*.

Expression of ATF3 induces apoptosis in normal human keratinocytes

As described above, the ATF3 function in normal human keratinocytes and skin is unclear. On the other hand, to date, both the protective and detrimental effects of ATF3 expression have been reported in other cells. Rat ATF3 induces partial transformation in primary cells, suggesting that rat ATF3 per se possesses oncogenic potential.[19] In HeLa cells, the overexpression of ATF3 enhances etoposide- or camptothecin-induced apoptosis.[20] On the other hand, Fan *et al.* (2002) reported that the overexpression of the ATF3 protein moderately suppresses cell growth in HeLa cells.[21] Nobori

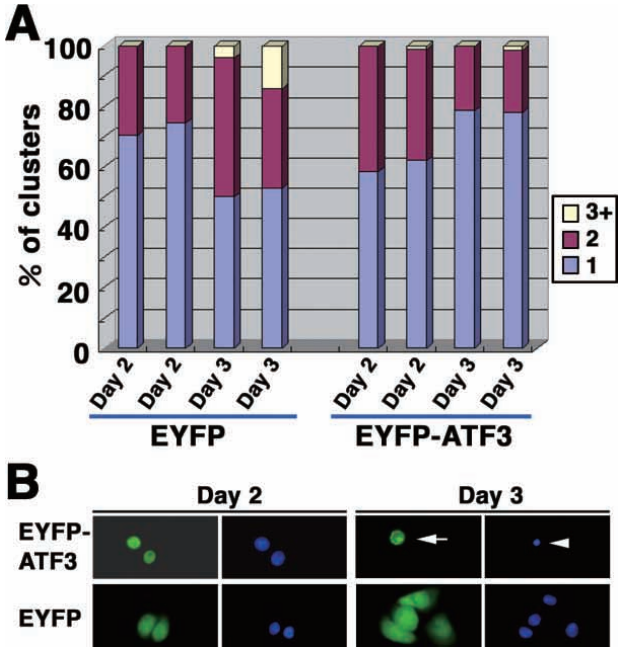

Fig. 5. Expression of ATF3 protein regulates cell proliferation in keratinocytes. (A) EYFP and EYFP-ATF3 were expressed in keratinocytes by transient transfection. Yellow fluorescent cell clusters were counted after 2 or 3 days. The graph shows the percentage of the fluorescent cluster containing one, two, or three or more cells. Two independent experiments are shown. Two hundred fluorescent clusters were counted in each experiment. (B) Typical appearance of fluorescent cell clusters in cultures transfected with EYFP or EYFP-ATF3. The cells were fixed and stained with DAPI (blue) on the indicated days. A condensed nucleus (arrowhead) was observed in ATF3-expressing cells (arrow) 3 days after transfection.

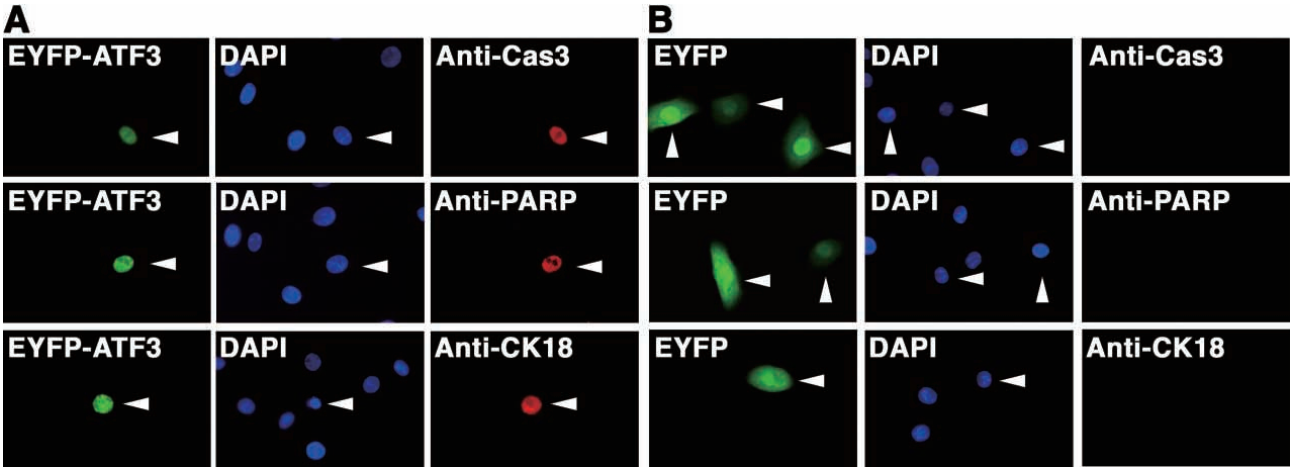

Fig. 6. EYFP-ATF3 protein induces apoptosis in keratinocytes in transient transfection assays. Normal human keratinocytes were transfected with EYFP-ATF3 (A) or EYFP (B). Two days following transfection, the cells were fixed and stained with three apoptotic marker antibodies (*i.e.*, an anti-active caspase-3 antibody (anti-Cas3), an anti-PARP p85 fragment antibody (Anti-PARP), or an M30 CytoDEATH antibody (Anti-CK18)) and DAPI. The subcellular localization (green) of the EYFP-tagged proteins, the cleaved active form of caspase-3 (red), the p85 fragment of caspase-3 cleaved PARP (red) or the caspase cleavage product of cytokeratin 18 (red), and DAPI (blue) was examined on the same cells by fluorescence microscopy. Arrowheads indicate the cells expressing the EYFP-tagged proteins.

et al. (2002) demonstrated that an anti-apoptotic role of ATF3 in cardiac monocytes.[22] Most recently, Hartman *et al.* (2004) have clearly demonstrated a proapoptotic role of ATF3 in beta cells.[23] To address this discrepancy, we were interested in determining whether the sustained expression of ATF3 affects cell proliferation and whether it results in cell death in normal human keratinocytes. We constructed a vector expressing ATF3 joined to EYFP as described above. This vector and a control vector expressing EYFP alone were introduced into normal human keratinocytes by transient transfection. Fluorescent cell clusters were scored after two or three days.[14] After two days, with EYFP-ATF3, approximately 40% of the fluorescent clusters consisted of two cells (Figs. 5A, B) In contrast, with EYFP alone, approximately 30% of clusters contained two cells (Figs. 5A, B). The expression of ATF3 may accelerate cell proliferation in normal human keratinocytes. On the other hand, after three days, with EYFP-ATF3, about 75% of the fluorescent clusters consisted of only a single cell and there was almost no cluster of three or more cells (Figs. 5A, B), suggesting that EYFP-ATF3 induces cell death between two and three days. In addition, the nuclei of many cells were condensed (Fig. 5B). Furthermore, EYFP-ATF3-positive cells shrank, rounded, and detached from the dish after four days (data not shown). In contrast, with EYFP alone, approximately 50% of clusters contained two, three, or more cells (Figs. 5A, B). Together, these results suggested that the expression of ATF3 causes cell death in normal human keratinocytes.

The nuclei of transfected cells, as well as of irradiated keratinocytes, which were stained with DAPI, showed chromatin condensation that is typical of apoptotic cells (Fig. 5B and data not shown), suggesting that the cell death which is induced by EYFP-ATF3 is apoptosis in normal human keratinocytes. To confirm this, we examined using another test on apoptosis. The cells, which were stained with three well-characterized apoptotic marker antibodies, were examined by fluorescence microscopy. Using immunofluorescence staining and an antibody specific for the cleaved active form of caspase-3, the active form of caspase-3 was expressed in the nuclei of EYFP-ATF3-expressing cells (Fig. 6A), whereas no expression of active caspase-3 was detected in EYFP-expressed cells (Fig. 6B). In addition, positive signals were detected in the cells before causing morphological-changes during apoptosis such as nuclear fragmentation or condensation detected by staining cells with DAPI. These results support the idea that the EYFP-ATF3 induces apoptosis in normal human keratinocytes. Among other parameters, the cleavage of PARP to the apoptosis-specific 85-kDa fragment and the cleavage of cytokeratin 18 are also well characterized events of apoptosis in epithelial cells.[24,25] Using immunofluorescence staining and an antibody specific for the cleaved active form of PARP or cytokeratin 18, the active forms were expressed in the nuclei of EYFP-ATF3-expressing cells (Fig. 6A), whereas no expression of both the active forms were detected in EYFP-expressing cells (Fig. 6B). Together, these results indicate that ATF3 can induce caspase-3-dependent apoptosis in normal human keratinocytes.

DISCUSSION

The epidermis is a physiological barrier that protects an organism against pathogens and chemical or physical dam-

age. The degree of skin injuries caused by radiation influences the subsequent survival and prognosis. In the present study, we identified using microarray technology genes that are regulated by high-dose radiation exposure in normal human epidermal keratinocytes. We found that 16 and 59 genes are up- and down-regulated respectively in normal human keratinocytes. Furthermore, our results showed that ATF3 plays a role in the apoptosis pathway in radiation injuries caused by high-dose radiation exposure in human skin.

ATF3 encodes a member of the ATF/CREB family of transcription factors,[26,27] and its expression is induced in various tissues by different stress signals.[15,28] The induction of ATF3 correlates with cellular damage. However, to date, both the physiological significance of ATF3 induction and the target genes remain to be defined. Most recently, Hartman *et al.* (2004) have reported the generation of ATF3-deficient mice, and these ATF3-knockout mice have no lethality or obvious phenotypes,[23] supporting the concept that ATF3 is a stress-inducible gene and is not required under normal conditions. In addition, they have suggested that ATF3 is a novel regulator of stress-induced beta-cell apoptosis as determined using both loss-of-function and gain-of-function approaches. On the other hand, to date, both the protective and detrimental effects of ATF3 expression have been reported in other cells. In human umbilical vein endothelial cells, the overexpression of ATF3 suppresses TNF-alpha-induced apoptosis.[29] ATF3 has an anti-apoptotic role in cardiac monocytes.[22] These results suggest that ATF3 serves as an anti-apoptotic factor. In contrast, the overexpression of ATF3 enhances the ability of DNA damaging agents to induce apoptosis in HeLa cells.[20] Consistent with the results of transgenic mice expressing ATF3 in islets reported by Hartman *et al.* (2004),[23] other ATF3 transgenic mice expressing ATF3 have functional defects in the corresponding tissues: mice expressing ATF3 in the heart have conduction abnormalities and contractile disfunction,[30] mice expressing ATF3 in the liver and pancreatic ductal epithelium have liver disfunction and defects in endocrine pancreas development.[31,32] These results suggest that ATF3 serves as a proapoptotic factor. Possibilities that ATF3 has functions other than apoptosis has also been reported. In HeLa cells, the overexpression of ATF3 moderately suppresses cell growth, suggesting that ATF3 plays a negative role in the control of cell cycle progression.[21] On the other hand, Allan *et al*. (2001) showed that the overexpression of ATF3 induces DNA synthesis of up to 140% that of the control cells and the expression of cyclin D1 in hepatocytes,[33] suggesting that ATF3 plays a role in hepatpocyte proliferation. In addition, ATF3 expressing chick embryo fibroblasts proliferates faster than the control cells.[19] In this experiment, we have shown for the first time that EYFP-ATF3 induces apoptosis in normal human keratinocytes. We also observed that EYFP-ATF3 may enhance cell proliferation before the apoptosis. We speculate that the transient activation of ATF3 and its downstream genes enhances cell growth, while a persistent activity induced apoptosis in normal human keratinocytes. Studies of the target genes of ATF3 in this phenomena shed light on the physiological function of ATF3 in normal human keratinocytes. Furthermore, we presume that the results of the overexpression analysis of ATF3 by other researchers and us indicate that the physiological functions of ATF3 are dependent on cell type and derived tissues. Alternatively, we also presume that the duration and magnitude of ATF3 activation are the determinants of cell fate, although further studies are necessary to confirm this speculation.

The responses to ionizing radiation in human cells are dependent on cell type and derived tissues. Ionizing radiation initiates and promotes cancer in normal human cells. Moreover, such cells induce apoptosis, senescence and terminal differentiation in response to ionizing radiation. Generally, in the epidermis, the prevention of cancer progression is presumed to be mediated through the correction of mutations or alternatively, the elimination of damaged cells by driving them toward apoptosis, senescence, or terminal differentiation. As shown in Figs. 5 and 6, EYFP-ATF3 induced caspase-3-dependent apoptosis in normal human keratinocytes. In keratinocytes, ATF3, as well as other proapoptotic genes, *e.g.*, GADD45 and Noxa, is induced by ultraviolet B irradiation at a dose which induces apoptosis in approximately 20% of the cells.[34] In the epidermis, ATF3 may play a key role in the prevention of cancer progression by inducing apoptosis in keratinocytes whose abnormalities have produced to exposure ionizing radiation.

In conclusion, in keratinocytes the transcription factor ATF3 is induced by X-irradiation both *in vivo* and *in vitro*, and exogenous ATF3 induces apoptosis in normal human keratinocytes. Thus, ATF3 may play a role in the cell death pathway in radiation injury caused by high-dose radiation exposure in human skin. We speculate that the expression level of ATF3 is regulated dependent on the damage level of the cell, which is caused by X-irradiation, and that ATF3 may control the fate of human keratinocytes. To date, some target promoters for ATF3, *e.g.*, GADD153 and Cyclin D1, have been definitively identified *in vitro*, although molecular targets for ATF3 remained unclarified *in vivo*.[33,35] Further studies to elucidate the molecular mechanism underlying apoptosis mediated by ATF3 in keratinocytes will lead to a better understanding of not only the physiological functions of the ATF3 proteins but also intracellular signal transmission among cells, which is necessary to be known in treating acute radiation skin injury.

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