increased colony formation (*4*). By studying the mechanisms that underlie the HuIFN-induced UVC-radiation resistance, we recently identified chaperone proteins, such as heat-shock protein (HSP) 27, that are involved in this resistance (*5*). Additionally, we found that one of the HSP27-binding proteins, annexin II, is also involved in UVC-radiation resistance (*6, 7*).

Annexin II belongs to a family of calcium-dependent, phospholipid-binding proteins that are expressed in a diverse range of tissues and cell types (*8*). Initially identified as an intracellular molecule, annexin II has been implicated in the regulation of a variety of cellular processes, including exocytosis (*9*) and endocytosis (*10*). In addition to its intracellular functions, annexin II is also secreted into the extracellular environment in both soluble and membrane-bound forms (*11*). Although the functions of extracellular annexin II are not fully understood, annexin II is known to act as a cell surface receptor for extracellular ligands and has possible roles in the regulation of proteolytic cascades (*12*), signal transduction (*13*) and tumor invasion and metastasis (*14–17*). After the loss of anchorage (anoikis), a protective role of extracellularly supplemented annexin II against apoptosis has been reported (*18*). However, there are no reports investigating the ability of extracellular annexin II to modulate the survival capacity of human cells after UVC irradiation, particularly in cells with a high susceptibility to UVC-radiation-induced cell death. It is important to elucidate the roles of extracellular annexin II that can confer resistance to UVC radiation to human cells that are sensitive to UV radiation and the molecular mechanisms underlying these as a means to reduce cellular hypersensitivity to UV radiation in the cells of CS patients.

In this study, we examined whether extracellularly added recombinant annexin II (rANX II) affects the survival capacity of CS cells after UVC irradiation. We found that the cells precultured in medium supplemented with rANX II exhibited an increased resistance to UVC-radiation-induced cell death.

MATERIALS AND METHODS

Cells and Culture Conditions

Primary cultured cells from Cockayne syndrome (CS) patients, CSBB (*19*), and two immortal cell lines derived from CS patients, CS1AN-S3-G2 (CS1ANS) and CS3BE-S3-G1 (CS3BES) (*20*), were cultivated. CS1ANS and CS3BES have defective CS group B (CSB) and CS group A (CSA) genes, respectively, and were provided by Dr. A. R. Lehmann. The two CS cell lines were immortalized with the pSV3gpt plasmid, which contains the Simian virus 40 (SV40) early region encoding the T antigen and the bacterial gene xanthine-guanine phosphoribosyl transferase. UVC-radiation-sensitive human RSa cells, which were established from human embryo-derived fibroblastic cells by double transfection with SV40 and Rous sarcoma virus (*21*), human AP^r-1 cells, which were established from RSa cells by mutagenesis with ethyl methanesulfonate followed by UVC irradiation (*22*), and HeLa cells, which are a cervical cancer cell line (*23*), were used. The sensitivity of AP^r-1 cells and HeLa cells to UVC radiation is similar to and lower than that of normal human fibroblasts, respectively (*24*, our preliminary results).

XP6BES and XP2OS, two immortal cell lines derived from xeroderma pigmentosum (XP) patient, were also used (*25*). These two cell lines were immortalized by infection with SV40 and have defective XP group D (XPD) and XP group A (XPA) genes, respectively. The CSBB cells were cultured in Eagle's MEM (EMEM; Nissui, Tokyo, Japan) containing 10% (v/v) fetal bovine serum (Hyclone Laboratories, Inc.), and the other cells, including the immortalized cell lines from CS and XP patients, were cultured in EMEM containing 10% (v/v) calf serum (Gibco Invitrogen Corp., Grand Island, NY). All cells were cultured at 37°C in a humidified atmosphere containing 95% air/5% CO_2.

UVC Irradiation

UVC irradiation (200–280 nm) was performed as described previously (*5*). The intensity of the radiation was 0.4 $J/m^2/s$ for CS and XP cells and 1.0 $J/m^2/s$ for the other cells. The intensity was measured by a UVR-254 UV radiometer (Tokyo Kogaku Kikai Co., Tokyo, Japan). Mock-irradiated cells were treated in the same manner but were not irradiation.

Preparation of Recombinant Annexin II

Full-length human annexin II cDNA was prepared as described previously (*6*). The cDNA was fused to the 3′ end of the glutathione S-transferase (GST) gene in the pGEX-6P-1 vector plasmid (GE Healthcare UK Limited, Buckinghamshire, UK) using *Bam*HI and *Xho*I restriction sites. Expression of GST-fused annexin II (GST-ANX II) was induced by IPTG in *Escherichia coli* (*E. coli*) with XL-1 Blue. *E. coli* pellets expressing the proteins were lysed by sonication in a buffer containing PBS (pH 7.4), 1 m*M* dithiothreitol (DTT), 0.5 m*M* EDTA, 1.0% Triton X-100 and protease inhibitors including 0.5 m*M* phenylmethylsulfonyl fluoride (PMSF), 0.005 m*M* leupeptin, 0.005 m*M* pepstatin A, and 0.005 m*M* E64. Next, GST-ANX II in the lysates was bound to a glutathione (GSH) Sepharose 4 Fast Flow (GE Healthcare) column, and the recombinant annexin II (rANX II) was separated from the GST-ANX II by PreScission protease (GE Healthcare) digestion and eluted from the column according to the manufacturer's recommendations. GST was also eluted from the column after elution with glutathione and used as a control for the rANX II supplement. Eluates from the column were dialyzed against 20 m*M* Hepes, pH 7.4, containing 0.1 *M* NaCl, 10% glycerol, 0.2 m*M* DTT, and 0.5 m*M* PMSF, sterilized with a 0.20-μm filter, and then frozen at −80°C in small aliquots. Purity of the rANX II sample was estimated by CBB staining of an SDS gel followed by quantification of the signal intensity of the protein bands (Supplementary Fig. 1; http://dx.doi.org/10.1667/RR2561.1.S1). The rANX II sample (1.0 mg/ml) contained rANX II (0.97 mg/ml) and a small amount of GST (0.032 mg/ml).

rANX II Treatment

Cells were plated in 60-mm dishes (5×10^5 cells/dish) and incubated for 24 h to allow the cells to attach, and then rANX II was added to the medium at the indicated dose. After culturing in the presence of rANX II for the indicated time, the cells were used for each experiment.

Colony Survival Assay

Cells that were treated with rANX II were harvested and suspended in 0.72 m*M* $CaCl_2$-containing PBS (1.7×10^3 cells/ml). One milliliter of the cell suspension was spread out on 100-mm dishes, irradiated with UVC radiation, and cultured in 10 ml of medium for 2 weeks. Next, colonies were counted, as described previously (*5*). For the inhibitor experiment, LY294002 (50 μ*M*; Sigma-Aldrich Corp. St. Louis, MO), a phosphatidylinositol 3-kinase (PI3K) inhibitor, was added 30 min before the addition of rANX II, and cells were cultured for an additional 24 h in the presence of the inhibitor and rANX II and then harvested and used for the colony survival assay.

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Assay

DNA fragmentation was analyzed using the *In Situ* Cell Death Detection Kit, Fluorescein (Roche, Mannheim Germany). CS3BES cells were plated in a Lab-Tek II 4-well chamber slide (Thermo Fisher Scientific Inc., Rochester, NY) (3.6×10^4 cells/well). The cells were treated with rANX II (0.5 μg/ml) for 24 h followed by UVC irradiation (6 J/m^2). Twenty-four hours after irradiation, the cells were fixed in 4% paraformaldehyde in PBS, pH 7.4, for 1 h at 25°C and were treated with terminal deoxyribonucleotidyl transferase (TdT) enzyme buffer containing fluorescein dUTP for 1 h at 37°C in the dark. TUNEL-positive cells were visualized under a fluorescence microscope (IX71; Olympus Tokyo, Japan), and five different areas of each well were counted at 200× magnification. Cells in the same areas were also visualized under bright field, and approximately 730 cells and 830 cells were counted for the control cells and the rANX II-treated cells, respectively. As a negative control, cells were incubated with only the label solution instead of the TUNEL reaction mixture.

Measurement of Cell Cycle Phase Distribution

CS3BES cells that were treated with rANX II (0.5 μg/ml) for 24 h were harvested, and cell cycle phase distribution was analyzed with an Accuri C6 cytometer (Tomy Digital Biology, Encyclopedia Circle Fremont, CA). The data were analyzed with FlowJo software as described previously (*23*). For analysis of apoptosis, rANX II (0.5 μg/ml)-treated CS3BES cells were irradiated with UVC light (6 J/m^2), and the fraction of the population in the sub-G_1 phase (apoptotic fraction) was analyzed 24 h after irradiation.

Biotinylation of Cell Surface Proteins and Pull-Down Assay

The biotinylation of cell surface proteins was performed as described previously (*26*). Briefly, CS3BES cells that were grown to subconfluence in 60-mm dishes were incubated with rANX II (2.0 μg/ml) for 1 h at room temperature to prevent internalization. After washing with ice-cold PBS at pH 8.0, the plates were incubated with 1 ml PBS at pH 8.0 containing 1 mg of Sulfo-NHS-LC-Biotin [sulfosuccinimidyl-6-(biotin-amido) hexanoate)] (Pierce Biotechnology, Rockford, IL) for 5 min at room temperature with mild agitation. After washing with ice-cold PBS at pH 8.0 containing 100 m*M* glycine, cells were lysed with a lysis buffer containing 50 m*M* Tris-HCl (pH 8.0), 150 m*M* NaCl, 0.5% (w/v) Triton-X 100, 0.5% (w/v) CHAPS (3-[(3-cholamidopropyl)dimethylammonio] propanesulfonate), 1 m*M* EDTA and protease inhibitors, including 0.5 m*M* PMSF, 0.005 m*M* leupeptin, 0.005 m*M* pepstatin A and 0.005 m*M* E64, and centrifuged at 15,000 rpm for 30 min. Equal amounts of protein (500 μg) from the supernatants (cell lysates) were incubated overnight at 4°C with 60 μl of streptavidin-agarose (Pierce Biotechnology). The resin was rinsed three times with the lysis buffer and once with PBS at pH 7.4 that contained protease inhibitors; biotinylated proteins were eluted in SDS sample buffer, and the cell-surface localization of annexin II was analyzed by immunoblotting using a mouse anti-annexin II monoclonal antibody (610068; BD Biosciences, Sparks, MD; 1:5,000 dilution). Immunoblotting analysis was performed as described previously (*5*). As a positive control for the cell surface-expressed proteins, the transferrin receptor was detected with a mouse anti-human transferrin receptor antibody (Zymed Laboratories Inc., 1:500 dilution). To determine possible biotinylation of intercellular proteins, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was detected with a goat anti-GAPDH antibody (sc-20357; Santa Cruz Biotechnology, Santa Cruz, CA).

Measurement of (6-4) Photoproducts and Cyclobutane Pyrimidine Dimers

Cells that were treated with rANX II were irradiated with UVC radiation, and, at the indicated time after UVC irradiation, the levels of (6-4) photoproducts and cyclobutane pyrimidine dimers were measured as described previously (*5*), with the following modifications: DNA was applied to the plate [200 ng/50 μl/well and 65 ng/50 μl/well for (6-4) photoproducts and cyclobutane pyrimidine dimers, respectively], the anti-(6-4) photoproducts antibody (MBL Medical & Biological Laboratories Co., Ltd., Nagoya, Japan; 1:3,000 dilution) and the MX-Thymine dimer monoclonal antibody (Kyowa Mede Co., Ltd, Tokyo, Japan; 1:3,000 dilution) were used as the primary antibodies, and substrate solution that contained 0.01% o-phenylene diamine and 0.005% H_2O_2 in citrate-phosphate buffer was used.

Immunoblotting Analysis of Bcl-xL and Bax

Cells that were treated with rANX II in the presence or absence of LY294002 (50 μ*M*) for 24 h were irradiated with UVC light. After UVC irradiation, cells were cultured for the indicated time, and whole cells were lysed with an SDS sampling buffer. Bcl-xL and Bax proteins were detected by immunoblotting analysis using a rabbit anti-Bcl-xL monoclonal antibody (54H6; Cell Signaling Technology, Beverly, MA; 1:1,000 dilution) and a mouse anti-Bax monoclonal antibody (sc-7480; Santa Cruz Biotechnology; 1:1,000 dilution), respectively. The protein levels of actin were also analyzed using a mouse anti-actin antibody (C4; ICN Biomedicals, Costa Mesa, CA; 1:10,000 dilution) as the loading control.

Knockdown of XPA and Bcl-xL

Small interfering RNA (siRNA) duplexes against human XPA and Bcl-xL mRNAs (XPA siRNA and Bcl-xL siRNA) were purchased from Santa Cruz Biotechnology (sc-36854 and sc-43630, respectively). The negative control duplex siRNA (NC siRNA) was also purchased from Santa Cruz Biotechnology. For the knockdown of XPA, the XPA siRNA (100 n*M*) and NC siRNA (100 n*M*) were transfected into AP^r-1 and HeLa cells for 5 h using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Twenty-four hours after transfection, the cells were replated and cultured with rANX II (1.0 μg/ml) for 24 h. After culturing in the presence of rANX II, the cells were used to examine UVC-radiation sensitivity by the colony survival assay. For the knockdown of Bcl-xL, the Bcl-xL siRNA (130 n*M*) and NC siRNA (130 n*M*) were transfected in to CS3BES and RSa cells using a method similar to that for the XPA knockdown, and the cells were cultured with rANX II (0.5 μg/ml for CS3BES cells and 1.0 μg/ml for RSa cells) for 24 h. The cells were subsequently analyzed for UVC-radiation sensitivity by the colony survival assay.

The suppressed expression of XPA and Bcl-xL in the siRNA-transfected cells was confirmed by immunoblotting. The XPA and Bcl-xL expression levels decreased 48 h after transfection, and the decreased levels were maintained until 72 after transfection (Supplementary Figs. 3 and 6, respectively; http://dx.doi.org/10.1667/RR2561.1.S1); thus we used a 48-h post-transfection time for UVC irradiation. In our preliminary analysis, the decreased protein levels of Bcl-xL and XPA appeared to continue at least 4 days after transfection of the respective siRNA (data not shown).

Statistical Analysis

Statistical analysis was performed using the Student's *t* test with StatView software (version 4.5; Abacus Concepts, Berkeley, CA).

RESULTS

Enhancement of Resistance of CS Cells to UVC-Radiation-Induced Cell Death by Preculture with rANX II

The purified rANX II sample contained a small amount of GST (Supplementary Fig. 1; http:dx.doi.org/10.1667/

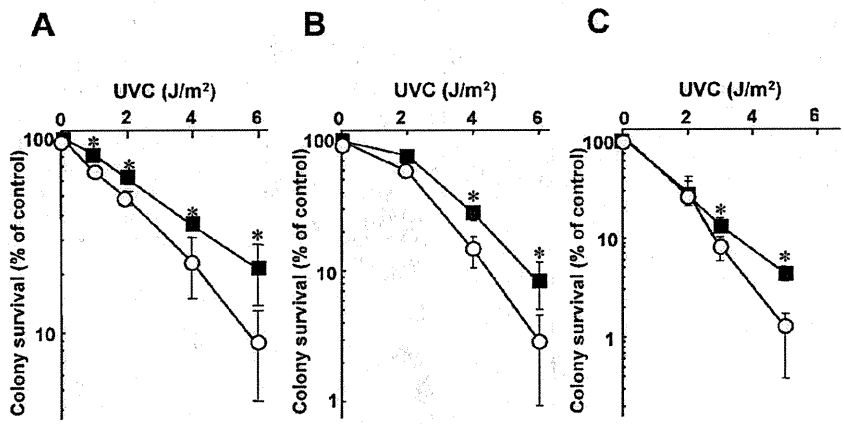

FIG. 1. Effects of rANX II supplementation in the culture medium on sensitivity to UVC-radiation lethality in CS cells. CSBB (panel A), CS3BES (panel B) and CS1ANS (panel C) cells were cultured in medium supplemented with rANX II (0.2 μg/ml) (■) and GST (0.007 μg/ml) (○) (panel A), rANX II (0.3 μg/ml) (■) and GST (0.01 μg/ml) (○) (panel B), and rANX II (0.5 μg/ml) (■) and GST (0.017 μg/ml) (○) (panel C), respectively, for 24 h. Cells were UVC-irradiated and assayed by the colony survival assay as described in the Materials and Methods. * indicates significant difference at $P < 0.05$ for rANX II addition compared to GST control.

RR2561.1.S1). The relative amount of GST to rANX II was estimated as approximately 1:30; therefore, as a control treatment for rANX II supplementation in medium, GST was added to the medium at 1/30th of the concentration of rANX II.

Primary CSBB cells that were precultured for 24 h in medium supplemented with rANX II (0.2 μg/ml) had a higher resistance to UVC-radiation-induced cell death than did the control cells treated with GST (Fig. 1A). Two immortalized cell lines, CS3BES and CS1ANS, were precultured with rANX II (0.3 μg/ml and 0.5 μg/ml, respectively) for 24 h and also had an increased resistance to UVC-radiation lethality (Fig. 1B and C).

Characterization of the rANX II Supplementation-Induced UVC-Radiation Resistance in CS3BES Cells

We characterized the rANX II supplementation-induced UVC-radiation resistance using CS3BES cells. The rANX II supplementation-induced resistance was observed after 24 h but not after 8 h or less (Fig. 2A) and occurred at rANX II concentrations greater than 0.3 μg/ml (Fig. 2B). After UVC irradiation at 4 J/m^2, the colony survival capacity increased approximately 2- to 3-fold relative to cells from the control treatment (Fig. 2A–C). Treatment with rANX II at 75°C for 10 min did not enhance the radiation resistance (Fig. 2C), suggesting that there may be heat-labile components (such as proteins) in the rANX II preparation that contribute to the UVC-radiation resistance. Suppression of this resistance by cotreatment with rANX II and an anti-annexin II antibody (Fig. 2C) suggests a strong involvement of annexin II in the mechanism of UVC-radiation resistance. Furthermore, the resistance was also suppressed by cotreatment with rANX II with EGTA (Fig. 2C).

To investigate whether rANX II supplementation-induced UVC-radiation resistance is associated with the suppression of apoptosis, DNA fragmentation was analyzed by a TUNEL assay (Supplementary Fig. 2; http://dx.doi.org/10.1667/RR2561.1.S1). Supplementation of rANX II significantly decreased the number of TUNEL-positive cells 24 h after UVC irradiation (6 J/m^2) relative to the control GST treatment (Fig. 2D). Supplementation of rANX II also decreased the fraction of the population in the sub-G_1 phase (apoptotic fraction) 24 h after UVC irradiation (6 J/m^2) relative to the control treatment; the apoptotic fraction decreased from 31% in the control-treated cells to 22% in the rANX II-treated cells. These results suggest that rANX II supplementation is involved in the suppression of UVC-radiation-induced apoptosis in CS3BES cells.

Without UVC irradiation, there was no significant difference between the cell proliferation rates (data not shown) or the cell cycle distribution between the rANX II-supplemented cells and the control cells. The fractions of the populations in G_1, S, G_2/M and sub-G_1 phases were 59.1, 24.5, 14.4 and 2.0%, respectively, in the rANX II-supplemented cells and 58.5, 24.0, 15.0 and 2.5%, respectively, in the control-treated cells.

Next, we compared the cell-surface localization of annexin II between CS3BES cells treated with control GST, rANX II or rANX II in the presence of EGTA by biotinylation of cell-surface proteins. The transferrin receptor, a cell surface protein, was biotinylated and strongly detected in the eluates from the avidin resin, while GAPDH, an intracellular protein, was not detected (Fig. 2E) in any of the cells. Under these conditions, annexin II was detected at low levels in the control cells, and the amount of the detectable annexin II increased in the rANX II-supplemented cells but did not increase in the cells cotreated with rANX II and EGTA relative to the control cells (Fig. 2E). The levels of annexin II, transferrin receptor and GAPDH proteins in total cell lysates were the same among the cells. This result, along with the EGTA-mediated

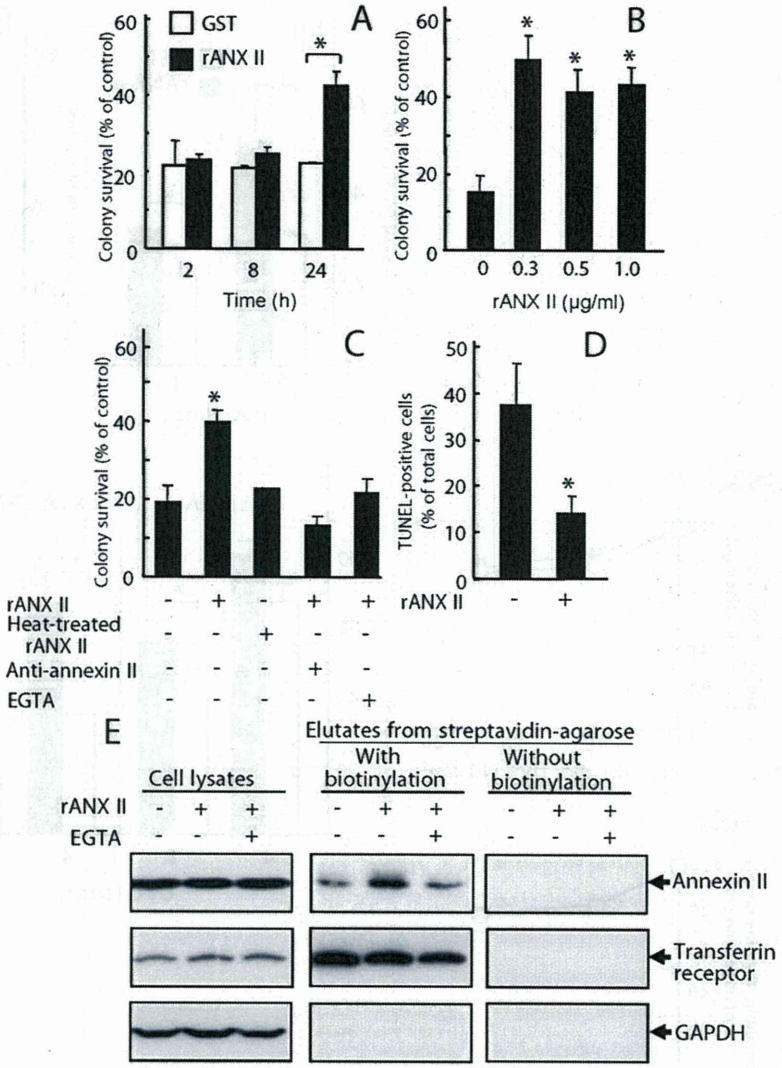

FIG. 2. Characterization of rANX II supplementation-induced UVC-radiation resistance in CS3BES cells. Cells were cultured in medium supplemented with rANX II (0.5 μg/ml) and GST (0.017 μg /ml) for the indicated time (panel A) and with rANX II at the indicated concentration for 24 h (panel B). Cells were cultured in medium supplemented with heat-treated (75°C, 10 min) rANX II (0.5 μg/ml), rANX II (0.5 μg/ml) that was preincubated with a rabbit anti-annexin II polyclonal antibody (sc-9061, Santa Cruz Biotechnology; 4 μg) for 30 min at 25°C, or rANX II (0.5 μg/ml) in the presence of EGTA (5 m*M*) (panel C). In panels B and C, GST (0.017 μg/ml) was added as a control for rANX II. After the preculture and UVC irradiation at 4 J/m^2, the colony survival assay was performed as described in the Materials and Methods. Cells were cultured in medium supplemented with rANX II (0.5 μg/ml) and control GST (0.017 μg/ml) for 24 h followed by UVC irradiation (6 J/m^2). Twenty-four hours after irradiation, TUNEL staining was performed and visualized using fluorescence microscopy (200×), and total cells were also visualized using bright-field microscopy in the same area, as shown in Supplementary Fig. 2 (http://dx.doi.org/10.1667/RR2561.1.S1). The percentage of TUNEL-positive cells is shown in panel D. In panels A–D, the data are presented as means ± SD. * indicates significant difference at $P < 0.05$ for rANX II addition with or without various treatments compared to GST control. Panel E: Cell surface localization of annexin II. CS3BES cells were incubated with GST (0.07 μg/ml), rANX II (2.0 μg/ml) or rANX II (2.0 μg/ml) in the presence of EGTA (5 m*M*) for 1 h at room temperature to prevent internalization; cells were incubated with and without Sulfo-NHS-LC-Biotin for 5 min at room temperature, and then biotinylated proteins were bound to streptavidin-agarose. Annexin II in total cell lysates and eluates from streptavidin-agarose were analyzed by immunoblotting, as described in the Materials and Methods. Transferrin receptor and GAPDH were also analyzed as a cell surface expressed protein and an intercellular protein, respectively.

suppression of the rANX II supplementation-induced UVC-radiation resistance (Fig. 2C), suggests that Ca^{2+}-dependent binding of rANX II to the cell surface is associated with the rANX II-induced UVC-radiation resistance.

The rANX II-Induced Resistance to UVC-Radiation is Independent of Removal of Radiation-Damaged DNA

Supplementation of rANX II also enhanced the UVC-radiation resistance of another sensitive cell line, RSa (Fig.

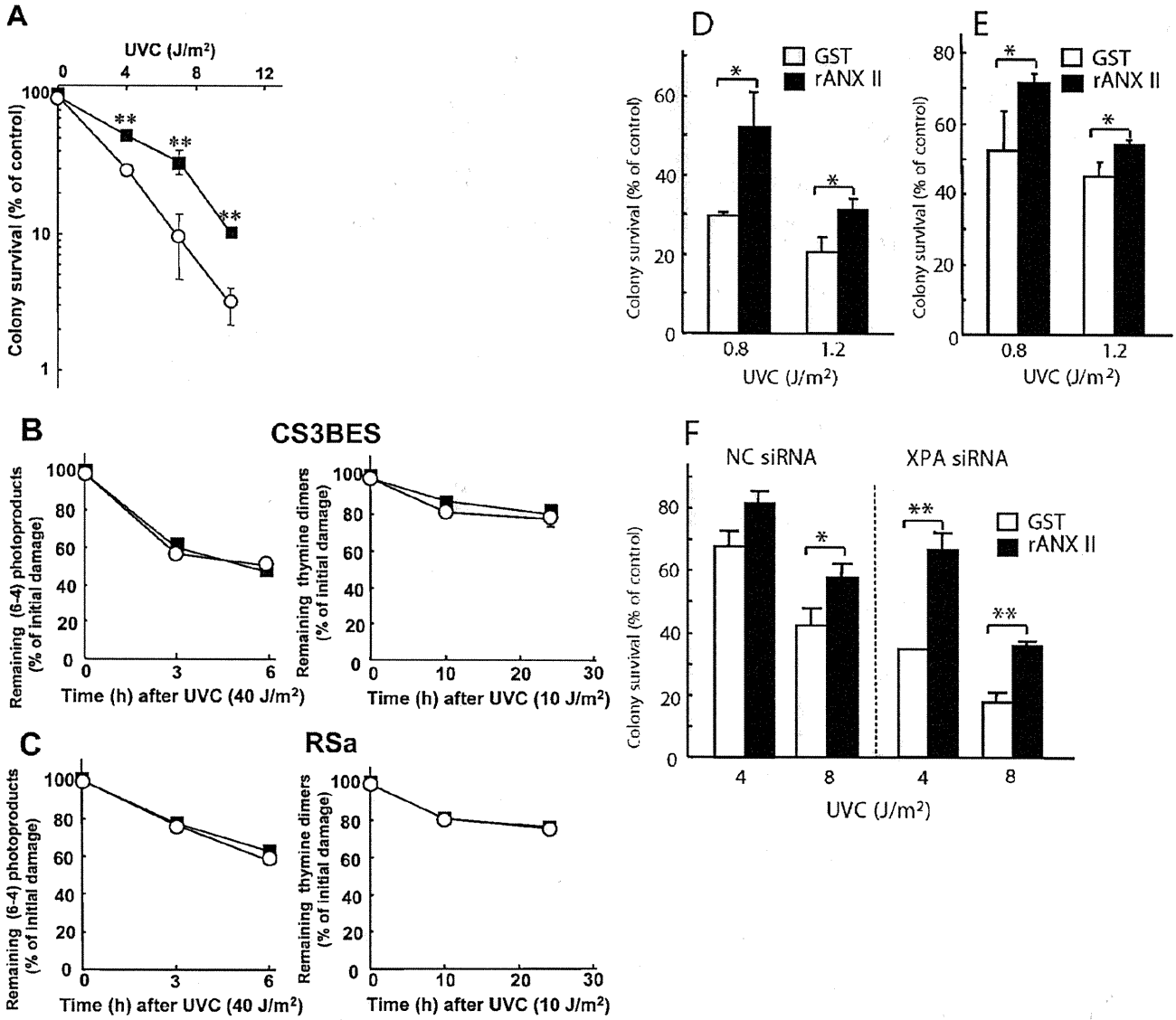

FIG. 3. Increased resistance to UVC-radiation lethality is independent of the removal of UVC-radiation-damaged DNA in rANX II-supplemented cells. RSa cells were cultured in the presence of rANX II (1.0 μg/ml) (■) and GST (0.033 μg/ml) (○) for 24 h followed by UVC irradiation and the colony survival assay, as described in the Materials and Methods (panel A). CS3BES (panel B) and RSa (panel C) cells were cultured in the presence of rANX II (0.5 μg/ml) (■) and GST (0.017 μg/ml) (○) (panel B) or rANX II (1.0 μg/ml) (■) and GST (0.033 μg/ml) (○) (panel C) for 24 h. The cells were irradiated with UVC light [40 J/m^2 for analysis of (6-4) photoproducts and 10 J/m^2 for analysis of cyclobutane pyrimidine dimers], and the removal of (6-4) photoproducts (left graphs) and cyclobutane pyrimidine dimers (right graphs) was measured, as described in the Materials and Methods. XP2OS (panel D) and XP6BES (panel E) cells were cultured in the presence of rANX II (0.3 μg/ml) and GST (0.01 μg/ml) for 24 h; the cells were then UVC-irradiated, and survival was analyzed by the colony survival assay, as described in Materials and Methods. AP^r-1 cells (panel F) were transfected with 100 n*M* XPA siRNA and NC siRNA, and 24 h after transfection, the cells were replated and cultured with rANX II (1.0 μg/ml) and GST (0.033 μg/ml) for 24 h. Sensitivity of the cells to UVC radiation was then analyzed by the colony survival assay. The data are presented as means ± SD. In panel A, ** indicates significant difference at $P < 0.005$ for rANX II addition compared to GST control. In panels D and E, * indicates significant difference at $P < 0.05$ for rANX II addition compared to GST control. In panel F, * indicates significant difference at $P < 0.05$ for rANX II addition compared to GST control in the NC siRNA-transfected AP^r-1 cells. ** indicates significant difference at $P < 0.005$ for rANX II addition compared to GST control in the XPA siRNA-transfected AP^r-1 cells.

3A), which has low levels of DNA repair activity (*5*). Thus we examined the effects of rANX II supplementation on the removal of (6-4) photoproducts and cyclobutane pyrimidine dimers in CS3BES and RSa cells. In CS3BES and RSa cells, approximately 50% and 40% of the initial (6-4) photoproducts were removed 6 h after UVC irradiation, respectively, and approximately 20% of the initial cyclobutane pyrimidine dimers were removed in both cell types 24 h after UVC irradiation (Fig. 3B and C). However, when cells were precultured with rANX II, neither cell type had

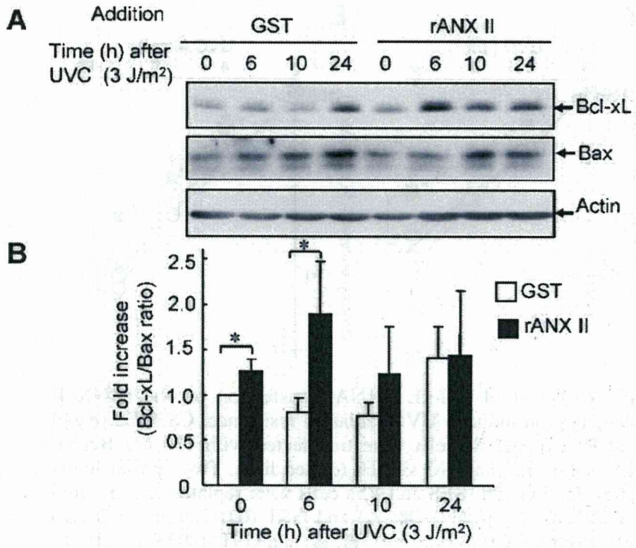

FIG. 4. Expression of Bcl-xL and Bax proteins after UVC irradiation in rANX II-supplemented CS3BES cells. CS3BES cells were cultured in the presence of rANX II (0.5 μg/ml) and GST (0.017 μg/ml) for 24 h and then irradiated with UVC light (3 J/m^2). At the indicated times after UVC irradiation, whole cell lysates were prepared, and the expression levels of Bcl-xL, Bax and actin proteins were analyzed by immunoblotting, as described in the Materials and Methods (panel A). The results shown are representative of findings from three independent experiments. Protein levels of Bcl-xL and Bax were normalized to actin levels and are presented as the ratios of Bcl-xL to Bax (panel B). The ratios immediately (time 0) after UVC irradiation in the control cells (GST alone) are designated as 1. The data are presented as means ± SD. * indicates significant difference at $P < 0.05$ for rANX II addition compared to GST control.

an increased ability to remove (6-4) photoproducts or cyclobutane pyrimidine dimers relative to their respective control cells (Fig. 3B and C). The amounts of (6-4) photoproducts and cyclobutane pyrimidine dimers immediately after irradiation (shown as time 0) were almost identical (data not shown) in the precultured and control cells.

Next, we examined the effects of a preculture with rANX II on XP cells, which are deficient in nucleotide excision repair (NER). The removal of (6-4) photoproducts and cyclobutane pyrimidine dimers was absent or low in the two XP cell lines, XP2OS and XP6BES (data not shown). After preculture with rANX II, both XP cell lines had an increased resistance to UVC-radiation-induced cell death (Fig. 3D and E). We subsequently examined the effect of XPA knockdown on the rANX II-induced radiation resistance of AP^r-1 cells, which have a higher resistance than do the CS3BES, RSa and XP cells. Knockdown of XPA (Supplementary Fig. 3A; http://dx.doi.org/10.1667/RR2561.1.S1) sensitized the AP^r-1 cells to UVC-radiation-induced cell death; this phenomenon was shown by a comparison between transfection with XPA siRNA and NC siRNA in AP^r-1 cells supplemented with control GST (Fig. 3F). There was a greater increase in resistance to UVC radiation by rANX II supplementation in the XPA-downregulated AP^r-1 cells than in the NC siRNA-transfected cells, but an increase was still observed in NC siRNA-transfected cells (Fig. 3F). However, an increase in resistance to UVC radiation by rANX II supplementation was observed in the XPA-siRNA treated HeLa cells but not in the NC siRNA-transfected cells (Supplementary Fig. 3A and B; http://dx.doi.org/10.1667/RR2561.1.S1).

These results suggest that the rANX II-induced resistance to UVC radiation is independent of removal of radiation-damaged DNA.

Increased Ratios of Bcl-xL to Bax after rANX II Supplementation

The amounts of an anti-apoptotic protein, Bcl-xL, and a pro-apoptotic protein, Bax, were analyzed by immunoblotting in CS3BES cells that were precultured with rANX II for 24 h and irradiated with UVC light. An increase in the expression of the Bcl-xL protein was observed from 6 h until 24 h after UVC irradiation (3 J/m^2) in the rANX II (0.5 μg/ml)-supplemented cells (Fig. 4A). In the control cells supplemented with GST, no significant increase in the amount of Bcl-xL was detected for up to 10 h after UVC irradiation, but an increase was observed 24 h after UVC irradiation (Fig. 4A). The expression of the Bax protein increased after UVC irradiation in both GST- and rANX II-supplemented cells (Fig. 4A). In the rANX II-supplemented cells, the increase in the Bax expression was slower than that in Bcl-xL expression. When the Bcl-xL to Bax protein ratios were calculated as an index of survival activity, the ratio in the rANX II-supplemented cells 6 h after UVC irradiation increased approximately 2- to 2.5-fold relative to the ratio in the control cells (Fig. 4B). The ratio was also higher in the rANX II-supplemented cells immediately after UVC irradiation (time 0; Fig. 4B). The increase in the Bcl-xL to Bax protein ratios was also observed after UVC irradiation at 6 J/m^2 in the rANX II-supplemented CS3BES cells (data not shown). The increase in the Bcl-xL to Bax protein ratios after rANX II supplementation was also observed in RSa and AP^r-1 cells (Supplementary Fig. 4; http://dx.doi.org/10.1667/RR2561.1.S1); these cells also showed an increased resistance to UVC radiation after rANX II supplementation (Fig. 3A and F).

Suppression of the rANX II Supplementation-Induced UVC-Radiation Resistance is Associated with Decreased Bcl-x to Bax Ratios by a PI3K Inhibitor and Downregulation of Bcl-xL

We then examined whether signal transduction pathways are involved in the rANX II supplementation-induced resistance to UVC radiation in CS3BES cells. When the cells were precultured with rANX II (0.5 μg/ml) in the presence of the PI3K inhibitor LY294002 (50 μ*M*), the increase in resistance to UVC radiation induced by rANX II supplementation was completely suppressed (Fig. 5A).

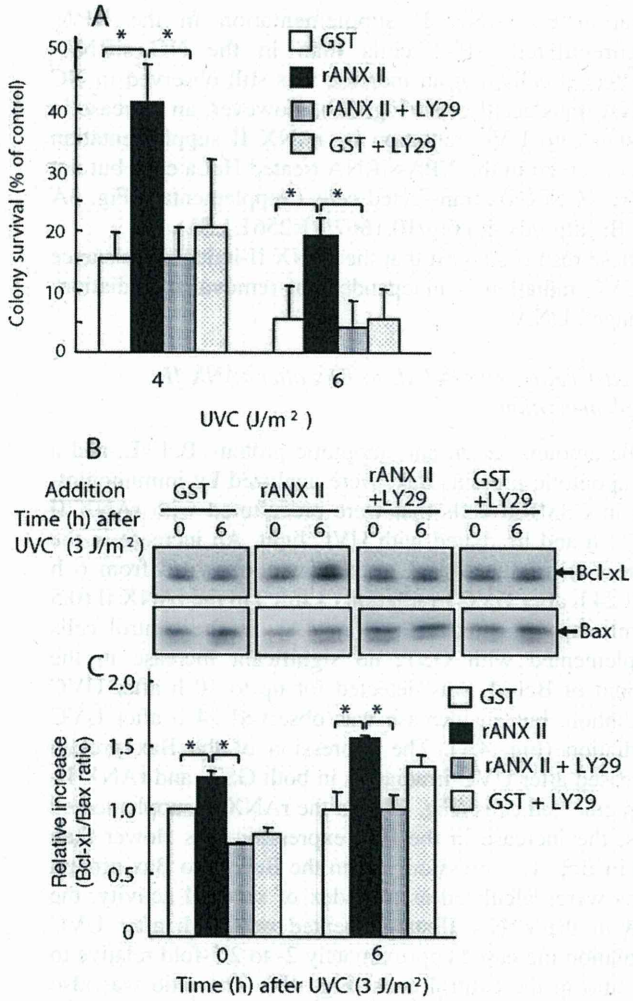

FIG. 5. Effects of the PI3K inhibitor on the rANX II supplementation-induced increase in the resistance to UVC radiation and Bcl-xL to Bax ratios. LY294002 (50 μM) (LY29) was added to the medium 30 min before addition of rANX II (0.5 μg/ml), and CS3BES cells were cultured in the presence of rANX II and LY29 for 24 h. After culturing, sensitivity to UVC-radiation-induced cell death at 4 J/m^2 and 6 J/m^2 was analyzed with the colony survival assay (panel A), and the expression levels of Bcl-xL and Bax proteins after UVC irradiation (3 J/m^2) were analyzed by immunoblotting (panel B), as described in the Materials and Methods. Bcl-xL and Bax protein levels were analyzed immediately (time 0) and 6 h after UVC irradiation, and the results are presented as the ratios of Bcl-xL to Bax (panel C). The ratios immediately (time 0) after UVC irradiation in the control cells (GST alone) are designated as 1. The data are presented as means ± SD. * indicates significant difference at $P < 0.05$ for rANX II addition compared to GST control or the combined addition of rANX with LY29004.

Control cells precultured with GST and LY29004 showed a slight but insignificant increase in survival activity after UVC irradiation relative to the control cells without LY29004 treatment (Fig. 5A).

In CS3BES cells treated with rANX II and LY29004, the expression levels of Bcl-xL and Bax were analyzed immediately (time 0) and 6 h after UVC irradiation because

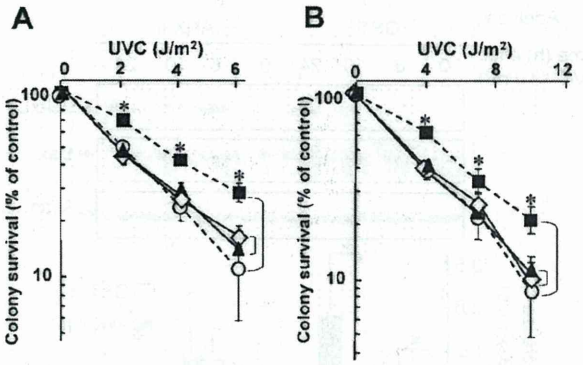

FIG. 6. Effect of Bcl-xL siRNA transfection on the rANX II supplementation-induced UVC-radiation resistance. CS3BES (panel A) and RSa (panel B) cells were transfected with 130 nM Bcl-xL siRNA (solid line) and NC siRNA (dotted line). Twenty-four hours after transfection, CS3BES and RSa cells were replated and cultured with rANX II (0.5 μg/ml) (■, ▲) and GST (0.017 μg/ml) (○, ◇) (panel A) or rANX II (1.0 μg/ml) (■, ▲) and GST (0.033 μg/ml) (○, ◇) (panel B) for 24 h, and sensitivity of the cells to UVC radiation was analyzed by the colony survival assay, as described in the Materials and Methods. The data are presented as means ± SD. * indicates significant difference at $P < 0.05$ for rANX II addition compared to GST control in the NC siRNA-transfected CE3BES and RSa cells.

the Bcl-xL to Bax ratios were significantly increased by rANX II supplementation at these times (Fig. 4B). LY29004 treatment suppressed the increased Bcl-xL to Bax ratios at both times (Fig. 5B and C). The control cells treated with LY29004 showed only a slight decrease in the Bcl-xL to Bax ratios at time 0 and an increase 6 h after UVC irradiation relative to cells that did not receive LY29004 treatment (Fig. 5C).

These results suggest that the increase in the Bcl-xL to Bax ratios is involved in the enhancement of UVC-radiation resistance by rANX II supplementation. Therefore, we examined the effect of siRNA transfection-induced Bcl-xL downregulation on the rANX II-induced UVC-radiation resistance in CS3BES and RSa cells. After being transfected with Bcl-xL siRNA, both cell types showed the decreased Bcl-xL levels and the decreased Bcl-xL to Bax ratios relative to the cells transfected with NC siRNA (Supplementary Fig. 5; http://dx.doi.org/10.1667/RR2561.1.S1); the ratios did not increase upon rANX II supplementation (data not shown). The rANX II supplementation-induced UVC-radiation resistance was not observed in either cell type upon downregulation of Bcl-xL, but resistance was observed in both cell types transfected with NC siRNA (Fig. 6).

DISCUSSION

We previously reported that the overproduction of human annexin II induced by cDNA transfection in human UVC-radiation-sensitive RSa cells confers an increased resistance to UVC-radiation-induced cell death (*6*). We are now

investigating the mechanisms underlying the annexin II-involved UVC-radiation resistance. Annexin II might play both intracellular and extracellular roles in UVC-radiation resistance. We examined the possible roles of extracellular annexin II in UVC-radiation resistance by using rANX II in human UVC-radiation-sensitive cells and found that the addition of extracellular rANX II in the culture medium resulted in an increased resistance to UVC-radiation-induced cell death in CS and RSa cells. Supplementation of rANX II was involved in the suppression of UVC-radiation-induced apoptosis in CS3BES cells [Supplementary Fig. 2 (http://dx.doi.org/10.1667/RR2561.1.S1) and Fig. 2D].

The time dependence of the UVC-radiation resistance-enhancing effect of rANX II addition was slow in CS3BES cells (Fig. 2A). Supplemented rANX II appeared to bind to the surface of the CS3BES cells 1 h after rANX II addition (Fig. 2E). The rapid binding, as reported in human endothelial cells (*27*), suggests that certain slow events, such as changes at the transcriptional and/or translational levels, are possibly required for rANX II to exert the UVC-radiation resistance-enhancing effect. The concentrations of rANX II used in the present study ranged from approximately 6 n*M* to 30 n*M*. Suppression of the rANX II supplementation-induced UVC-radiation resistance (Fig. 2C) and binding of annexin II to the cell surface (Fig. 2E) by EGTA suggests that the binding of rANX II is calcium-dependent. Additionally, detection of annexin II on the surface of the control cells (Fig. 2E) suggests the existence of endogenous annexin II on the surface of CS3BES cells. Human umbilical vein endothelial cells (HUVECs) can bind annexin II in a calcium-dependent manner, and these cells, when washed with EGTA, bound annexin II in a dose-dependent manner at concentrations of up to 60 n*M* and were apparently saturated at concentrations of over 80 n*M* (*27*). The saturable concentration curve of rANX II in CS3BES cells, even at low doses of rANX II (Fig. 2B), may be due to interference by endogenous annexin II expressed on the surface of the CS3BES cells. Alternatively, factors other than rANX II, including S100A10 (*18*), might also be required for the UVC-radiation resistance-enhancing effect in the cells.

It has been reported that treatment with extracellular interferon (*4*), a platelet-activating factor, and serotonin receptor antagonists (*28*) accelerated the repair of UV-radiation-damaged DNA. In contrast, the extracellular addition of rANX II did not change the removal of (6-4) photoproducts and cyclobutane pyrimidine dimers in CS3BES and RSa cells (Fig. 3B and C). It is possible that the NER process does not play a main role in the rANX II supplementation-associated UVC-radiation resistance in the cells. This possibility is also supported by the increased resistance to UVC radiation in the rANX II-supplemented XP cells (Fig. 3D and E), which are deficient in NER, and in the XPA-downregulated AP^r-1 and HeLa cells (Fig. 3F and Supplementary Fig. 3B; http://dx.doi.org/10.1667/RR2561.1.S1). However, we cannot rule out the possibility that other repair pathways, for example translesion synthesis (TLS) (*29*), might play roles in the rANX II supplementation-associated UVC-radiation resistance.

In rANX II-supplemented CS3BES, RSa and AP^r-1 cells, the Bcl-xL to Bax expression ratios increased before and after UVC irradiation relative to the ratios in the respective control cells (Fig. 4 and Supplementary Fig. 4; http://dx.doi.org/10.1667/RR2561.1.S1). Bcl-xL is an anti-apoptotic protein, and Bax is a pro-apoptotic protein (*30*). The relative expression ratios of pro-apoptotic proteins to anti-apoptotic proteins have been reported to correlate with cellular sensitivity to the lethal effects of anti-cancer drugs (*23*). Intimate relationships have been reported between increased Bcl-xL expression levels and resistance to pro-apoptotic stimuli, such as anti-cancer drugs, hypoxia and matrix detachment (*31, 32*). Therefore, the increase in the Bcl-xL to Bax ratios found here may be related to the increased resistance to UVC-radiation-induced cell death in the rANX II-supplemented cells. In fact, the downregulation of Bcl-xL by siRNA transfection in CS3BES and RSa cells suppressed the increased UVC-radiation resistance by rANX II supplementation (Fig. 6). To our knowledge, this is the first report that the ratio of Bcl-xL to Bax is possibly increased by extracellular annexin II, thus leading to UVC-radiation resistance. The rANX II-induced change in the Bcl-xL/Bax ratios after UVC irradiation seemed to differ with the three cell types. In the UV-radiation-sensitive cells, CS3BES and RSa, the ratios peaked at 6 h after UVC irradiation but the increased ratios did not continue 6 h later, while in the UVC-radiation-resistant AP^r-1 cells, the rANX II-induced increase in the Bcl-xL/Bax ratios continued until 24 h. The difference might be related to the different sensitivity to UVC radiation; nevertheless, the details of the molecular mechanisms by which the ratios increase remain unclear.

In AP^r-1 cells, the Bcl-xL to Bax ratios increased greatly after UVC irradiation, even without the addition of rANX II, and the UVC-radiation resistance-increasing effect of rANX II was weak [Supplementary Fig. 4C and D (http://dx.doi.org/10.1667/RR2561.1.S1) and Fig. 3F]. In HeLa cells, a similar increase in the Bcl-xL to Bax ratios was not observed. The supplementation of rANX II did not increase the growth activity of cells that did not receive UVC radiation in any of the cell types studied here. Thus rANX II might confer a survival advantage after UVC irradiation in UVC-radiation-sensitive cells in which the surviving activity may be intrinsically weak. However, there is the possibility that the UVC-radiation resistance-increasing effect of rANX II, without an increase in NER activity, might lead to the induction of mutations; nevertheless, our preliminary experiments suggest that the frequency of UVC-radiation-induced mutation was not increased by rANX II supplementation in RSa cells (data not shown). Other repair pathways might function in the rANX II-supplemented cells.

Treatment with LY29004, a PI3K inhibitor, suppressed not only the enhancement of resistance to UVC radiation but also the increase in the Bcl-xL to Bax expression ratios by rANX II supplementation (Fig. 5A–C). The levels of phospho-Akt actually increased in the rANX II-supplemented cells relative to the phospho-Akt levels in the control cells, and this increase was suppressed by LY29004 (Supplementary Fig. 6; http://dx.doi.org/10.1667/RR2561.1.S1). Therefore, a PI3K/Akt-dependent pathway may be involved in the increase of the Bcl-xL to Bax ratios and the UVC-radiation resistance by rANX II supplementation. Annexin II is known to function as a cell surface receptor for ligands, including progastrin (PG) and gastrin peptides (*16*) and cathepsin B (*17*). Moreover, annexin II is thought to mediate the anti-apoptotic effect of PG in pancreatic cancer (*33*) and the urokinase type of plasminogen activator (uPA) signaling through interaction with cathepsin B (*17*). PG and uPA signaling that leads to protection against cell death has been shown to be mediated by PI3K-dependent pathways (*33, 34*). Furthermore, uPA elicits an enhancement of PI3K and MAPK activities and enhances the transcriptional activation of Bcl-xL expression (*34*). Based on these previous reports, the data shown here suggest that these ligands for annexin II might play roles in UVC-radiation resistance.

It is known that annexin II can be secreted into the extracellular compartment from human cells, such as HUVECs, that have received a temperature stress (*35*) or mouse epidermal cells that were exposed to low doses of radiation (*18*). In the latter cells, secreted annexin II was reported to be involved in the resistance to apoptosis; however, the molecular mechanisms underlying the secretion of annexin II are not well known. Therefore, a search for environmental compounds or bioactive substances that increase the extracellular release of annexin II and elucidating the mechanisms of annexin II release would supply useful information for reducing UV-radiation hypersensitivity in CS patients. A search for peptides that mimic extracellular annexin II is also important for reducing UV-radiation sensitivity.

ACKNOWLEDGMENTS

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第2回

GORLIN症候群シンポジウム

期日　平成24年1月21日（土）

会場　兵庫医療大学講堂

主催：厚生労働科学研究費補助金　難治性疾患克服研究事業
「Gorlin 症候群の病態解明と治療法確立のための臨床的研究」班
（課題番号 H22－難治－一般－120）

《プログラム》

13：00～14：00

I. はじめに……藤井 克則

II. シンポジウム……司会　塩浜 直

1. Gorlin 症候群について

千葉大学大学院医学研究院小児病態学　藤井 克則

2. Gorlin 症候群の疫学調査

千葉大学医学部附属病院小児科　内川 英紀

3. Gorlin 症候群と遺伝子変異

北里大学医学部分子遺伝学　宮下 俊之

4. ヘッジホッグシグナリング

兵庫医科大学遺伝学　中野 芳朗

休憩（15 分）

14：15～15: 30

5. Gorlin 症候群の診療……司会　水落 弘美

a) 皮膚疾患（基底細胞癌）

兵庫県立がんセンター皮膚科　村田 洋三

b) 歯科口腔外科疾患

兵庫医科大学歯科口腔外科　野口 一馬

c) 放射線環境と疾患

千葉大学教育学部基礎医科学　杉田 克生

d) 遺伝カウンセリング

東京女子医科大学附属遺伝子医療センター　斎藤加代子

e) 脳神経外科疾患

北里大学医学部脳神経外科　岡 秀宏

休憩（10 分）

15：40～16: 20

III. 質疑応答……司会　藤井 克則

a) 患者さんの立場から

b) Gorlin 症候群に関する質疑応答

c) Gorlin 症候群の今後について

IV. おわりに……宮下 俊之

はじめに

今から 17 年前（1995 年）に、私は千葉大学病院で 1 人の患者さんにお会いしました。たまたま抗生剤の副作用で小児科に入院されたのですが、特異的顔貌と肋骨奇形と大脳鎌石灰化があり、杉田克生先生のご助言により Gorlin 症候群と診断しました。この病気は 1960 年アメリカの Gorlin 博士によって報告された先天性奇形症候群で、肋骨奇形や手掌足底小陥凹等の先天奇形と基底細胞癌や髄芽腫等の高発癌性を特徴とする疾患です。ただこの時点では Gorlin 症候群の原因も不明で治療法もなく、病気を診断できたものの適切なアドバイスができず、大変歯がゆい思いをしたことを今でも覚えています。

しかしその後の医学の進歩はめざましいものでした。1996 年には Gorlin 症候群の責任遺伝子が *PTCH1* と同定され、多くの患者さんで *PTCH1* 遺伝子変異が報告されるようになりました。2002 年には阻害剤の cyclopamine が Gorlin 症候群で発生する髄芽腫に効果があることが Nature 誌に報告され、2009 年には新たな阻害剤 GDC-0049 のヒトの基底細胞癌と髄芽腫の治療効果が New England Journal of Medicine 誌に掲載されました。このように Gorlin 症候群に関わる新しい医療的知見は毎年のように一流誌に報告され、尽きることがありません。

このように高発癌性に対する新たな治療法が毎年のように提案されているにも関わらず、 日本国内では今まで Gorlin 症候群のまとまった調査が行われたことはありませんでした。 しかし 2009 年より厚生労働省難治性疾患克服研究事業が始まり、Gorlin 症候群はその一つとして初めて採択されました。初めての全国調査が 2009-2010 年に行われ、現在日本には Gorlin 症候群の患者さんが 310 名おられて、日本人人口 23 万人に 1 人の割合で日本国内に存在することが判明しました。また臨床症状の解析から基底細胞癌の発症率が欧米豪と比較して少ないことが明らかになりました。詳細な解析と病気の治療に対する実際の提言はこれからですが、これらの研究が日本国内の Gorlin 症候群研究の新たな一歩になればと考えています。

Gorlin 症候群は早期に診断して早期に対処すれば、健康被害を最少に抑えることが十分できる疾患です。また近い将来、市場に登場してくるであろう抗腫瘍薬を適切に使用することで、患者さ

んのQOLの改善や医療費の抑制も期待することができます。それにも関わらず現状ではGorlin症候群の社会的認知は必ずしも十分でなく、残念ながら診断も遅れがちです。昨年の東京・市ヶ谷に引き続き、関西・神戸で初めて行われるこの第2回Gorlin症候群シンポジウムでは、この病気の問題点をわかりやすく解説することで、医療関係者や患者さんご家族に病気を正しく理解していただき、新たな医療状況および患者関係の第一歩を踏み出せることを目標にしています。ぜひ活発な質疑応答をしていただき、Gorlin症候群への理解を深めていただければ幸いです。

このシンポジウムを開催するには今まで多難な道のりがありました。しかし多くの患者さんと関係する諸先生の協力があって初めてこの日を迎えることができました。試行錯誤の結果、Gorlin症候群のホームページ（http://gorlin.chibadai-shonika.net/）も作成されて情報発信の準備も整いました。まだまだ道半ばですが、Gorlin 症候群が私たち社会の中でさらに認知され、そして経年的に発生する腫瘍に対する治療が円滑に行われるようになるまで、この集まりを継続してゆけたらと願っています。

最後に、この年度末の忙しい時期にも関わらず今回のシンポジウムの開催にあたりご協力いただいた厚労省班会議のメンバーおよびシンポジストの方々、そして千葉大事務局の先生方の熱意と労力に深く感謝いたします。

平成24年1月21日

千葉大学大学院医学研究院小児病態学

藤井　克則

1. Gorlin 症候群について

藤井　克則

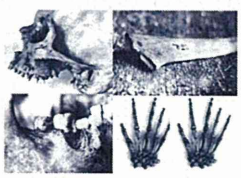
千葉大学大学院医学研究院 小児病態学

Gorlin症候群とは

Robert J Gorlin 博士
(1923-2006)

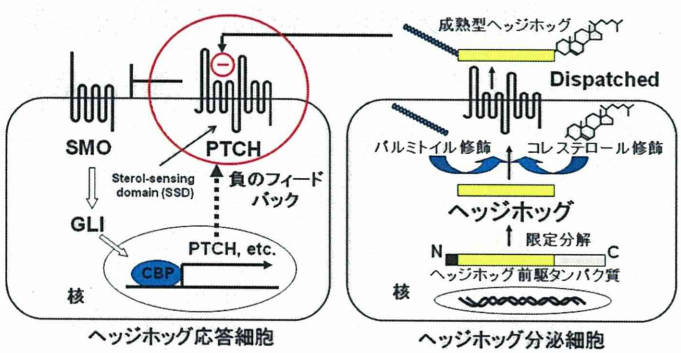
Gorlin症候群は、1960年Gorlin博士によって報告された先天性奇形症候群である。別名、基底細胞母斑症候群、母斑性基底細胞癌症候群と呼ばれる。発達上の奇形と高発癌性を特徴とする。

古代エジプトにもGorlin症候群は存在した



1

Gorlin症候群はなぜ起きる？

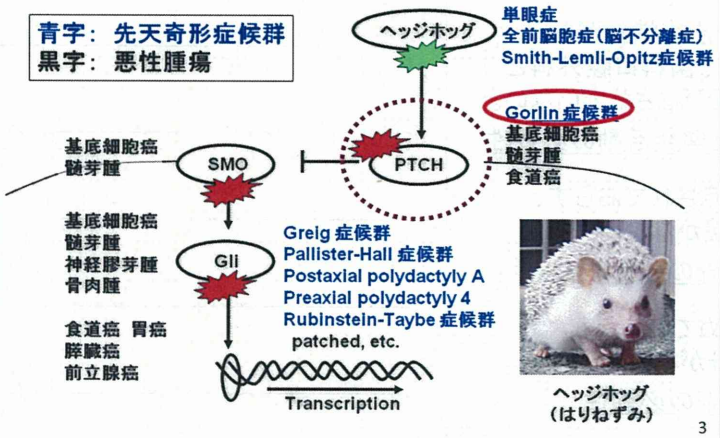


PTCH遺伝子異常によってSMO以下のシグナルが亢進して発症する

2

ヘッジホッグシグナルとヒト疾患

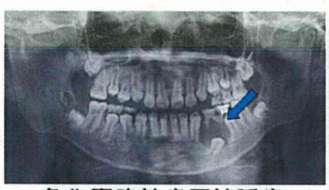
青字：先天奇形症候群
黒字：悪性腫瘍



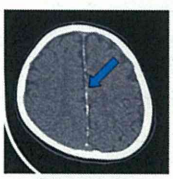
ヘッジホッグ
(はりねずみ)

3

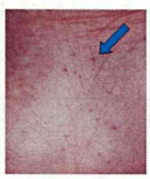
Gorlin症候群の臨床症状



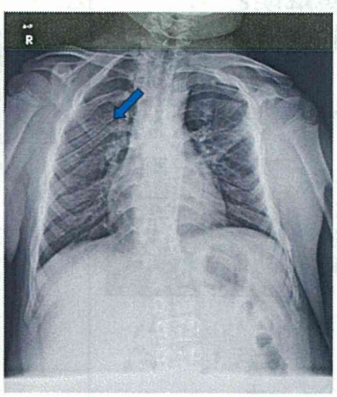
角化嚢胞性歯原性腫瘍



大脳鎌石灰化



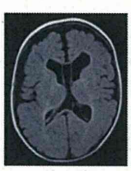
手掌小陥凹



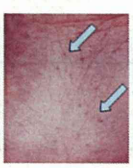
二分肋骨・癒合肋骨

4

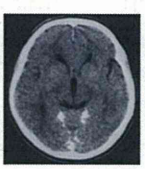
Gorlin 症候群の臨床経過



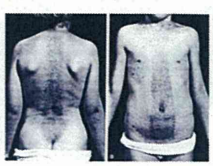
大頭症



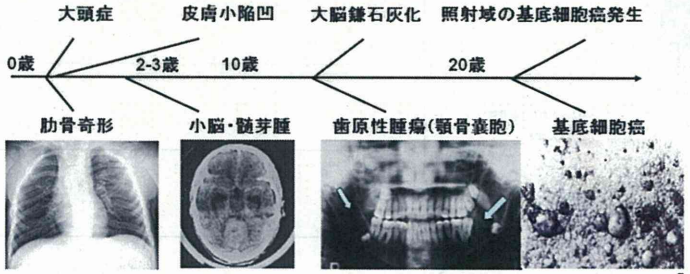
皮膚小陥凹



大脳鎌石灰化



照射域の基底細胞癌発生



5

Gorlin 症候群の社会的問題点

1. 高発癌性遺伝疾患でありながら治療指針がない。
   診療が小児科、脳外科、皮膚科、歯科口腔外科と
   多岐にわたるため系統的な治療がなされていない。

→ 日本の診断・治療指針の作成と啓発活動の必要性

2. 特定疾患や小児慢性疾患に認定されておらず、
   患者やその家族に経済的な支援がない。

→ 国内の患者数把握と医療費調査の必要性

3. 分子標的治療薬が市場に出始めているが、
   その適否を判断する機関や指針がない。

→ 国内の患者数把握と医療費調査の必要性

2009-2011 厚生労働省科学研究費 難治性疾患克服研究事業

「Gorlin 症候群における病態解明と治療法確立のための臨床的研究」班

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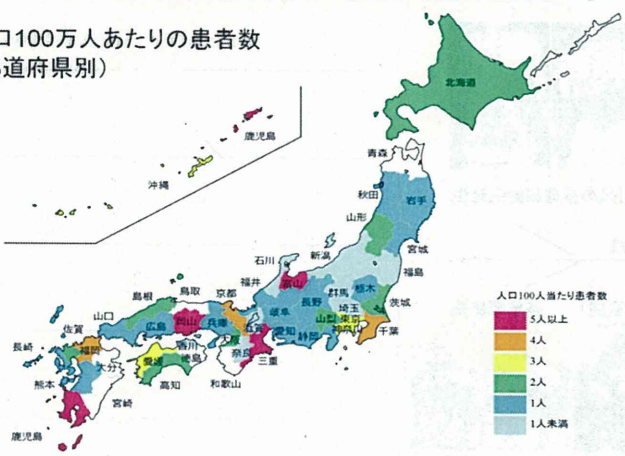
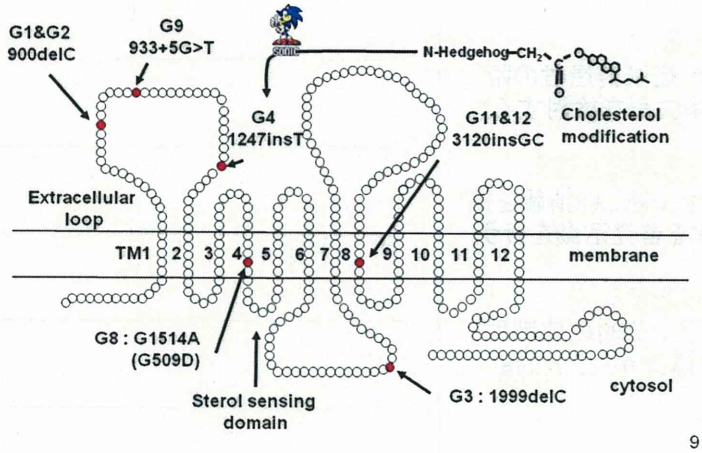
Gorlin症候群の国内患者分布

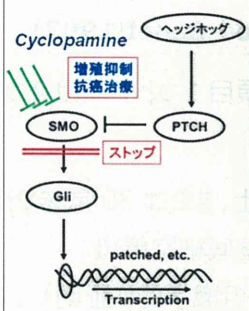
人口100万人あたりの患者数
（都道府県別）

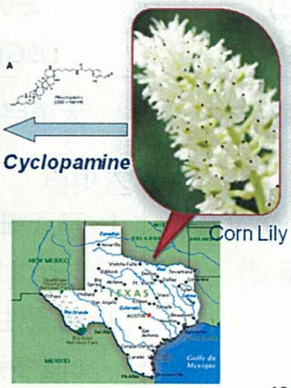


Gorlin症候群のPTCH1遺伝子解析



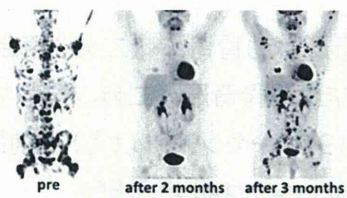
Gorlin症候群の腫瘍治療
—阻害剤の発見—





アメリカ西部に
一つ目の羊が
誕生していた

Gorlin症候群の腫瘍治療
—阻害剤の臨床応用—

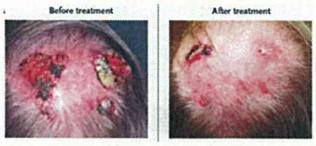


FDG-PET scans

extensive
metastases

髄芽腫への
治療効果

N Engl J Med. 2009 Sep 17;361(12):1173-8



基底細胞癌への
治療効果

N Engl J Med 2009;361:1164-72.

Gorlin 症候群班研究の方向性

1. 日本人患者の臨床像を明らかにする
全国2次調査における157名のGorlin 症候群患者の臨床像を検討し、本邦の診断および治療方針を検討する

2. Gorlin症候群の医療的・社会的啓発活動を行う
Gorlin症候群のホームページを作成して新しい情報を掲載するとともに、パンフレット作成による啓発活動を行う。

3. Gorlin症候群への医療経済的助成を目指す
Gorlin症候群は高発癌性にも関わらず、公的助成制度の適応はない。患者数把握と医療費調査から、公的助成対象になるよう働きかけを行う。

12

【参考資料】

Kimonis の診断基準（米国） (Kimonis; Am J Med Genet 1997)	Evans の診断基準（英国） (DGR Evans; J Med Genet 1993)
大項目２つ、または大項目１つと小項目２つ	大項目２つ、または大項目１つと小項目２つ
大項目 1. 基底細胞癌（２つ以上、または 20 歳未満） 2. 歯原性角化性嚢胞（組織学的に証明） 3. 手掌または足底小陥凹（３つ以上） 4. 大脳鎌石灰化 5. 肋骨奇形 （二分肋骨、癒合肋骨、著明な扁平肋骨） 6. 家族歴（１親等以内）	大項目 1. 基底細胞癌（3 個以上、または 30 歳未満）、あるいは 10 歳以降の基底細胞母斑 2. 歯原性角化性嚢胞（組織学的に証明）、あるいは多骨性骨嚢胞 3. 手掌足底小陥凹（3 つ以上） 4. 異所性石灰化：大脳鎌石灰化（層状または 20 歳未満） 5. 家族歴
小項目：以下のどれか１つを満たすもの 1. 大頭症（身長補正したもの） 2. 先天奇形：口蓋裂あるいは口唇裂、前額突出、粗野顔貌、中等度から重度の眼間乖離 3. その他の骨奇形：スプレンゲル変形、胸郭変形、著明な合指症 4. 放射線学的異常：トルコ鞍の骨性架橋、椎骨奇形（半椎体、癒合/延長椎体）、手足のモデリング変形、手足の火焔様透過像、 5. 卵巣線維腫 6. 髄芽腫	小項目 1. 先天骨奇形：肋骨奇形（二分、癒合、扁平、欠損）または椎骨奇形（二分、楔状、癒合） 2. 大頭症（97 パーセンタイル以上）、前額突出 3. 心臓線維腫、または卵巣線維腫 4. 髄芽腫 5. 腸管膜リンパ嚢胞 6. 先天奇形：口唇裂/口蓋裂、多指症、眼球異常（白内障、眼球欠損、小眼球症）