*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) ($3.6 \times 10^4$ 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/

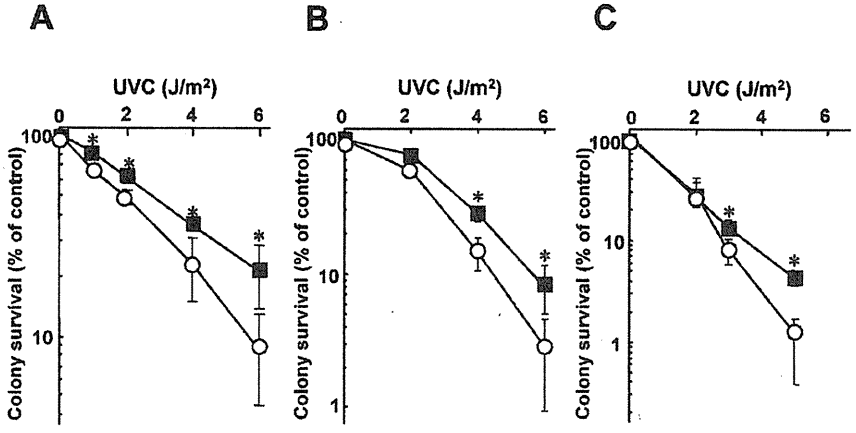


**FIG. 1.** Effects of rANX II supplementation in the culture medium on sensitivity to UVC-radiation lethality in CS cells. CSBB (panel A), CS3BES (panel B) and CS1ANS (panel C) cells were cultured in medium supplemented with rANX II (0.2 μg/ml) (■) and GST (0.007 μg/ml) (○) (panel A), rANX II (0.3 μg/ml) (■) 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

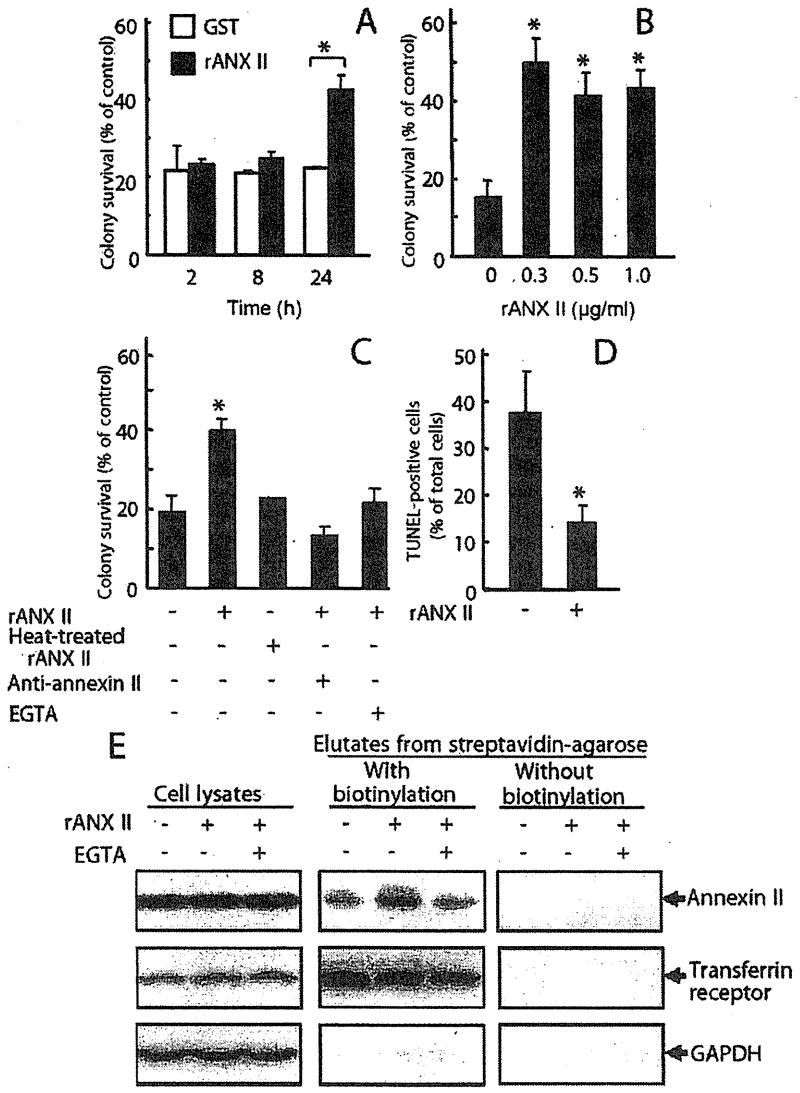


**FIG. 2.** Characterization of rANX II supplementation-induced UVC-radiation resistance in CS3BES cells. Cells were cultured in medium supplemented with rANX II (0.5 μg/ml) and GST (0.017 μg /ml) for the indicated time (panel A) and with rANX II at the indicated concentration for 24 h (panel B). Cells 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.

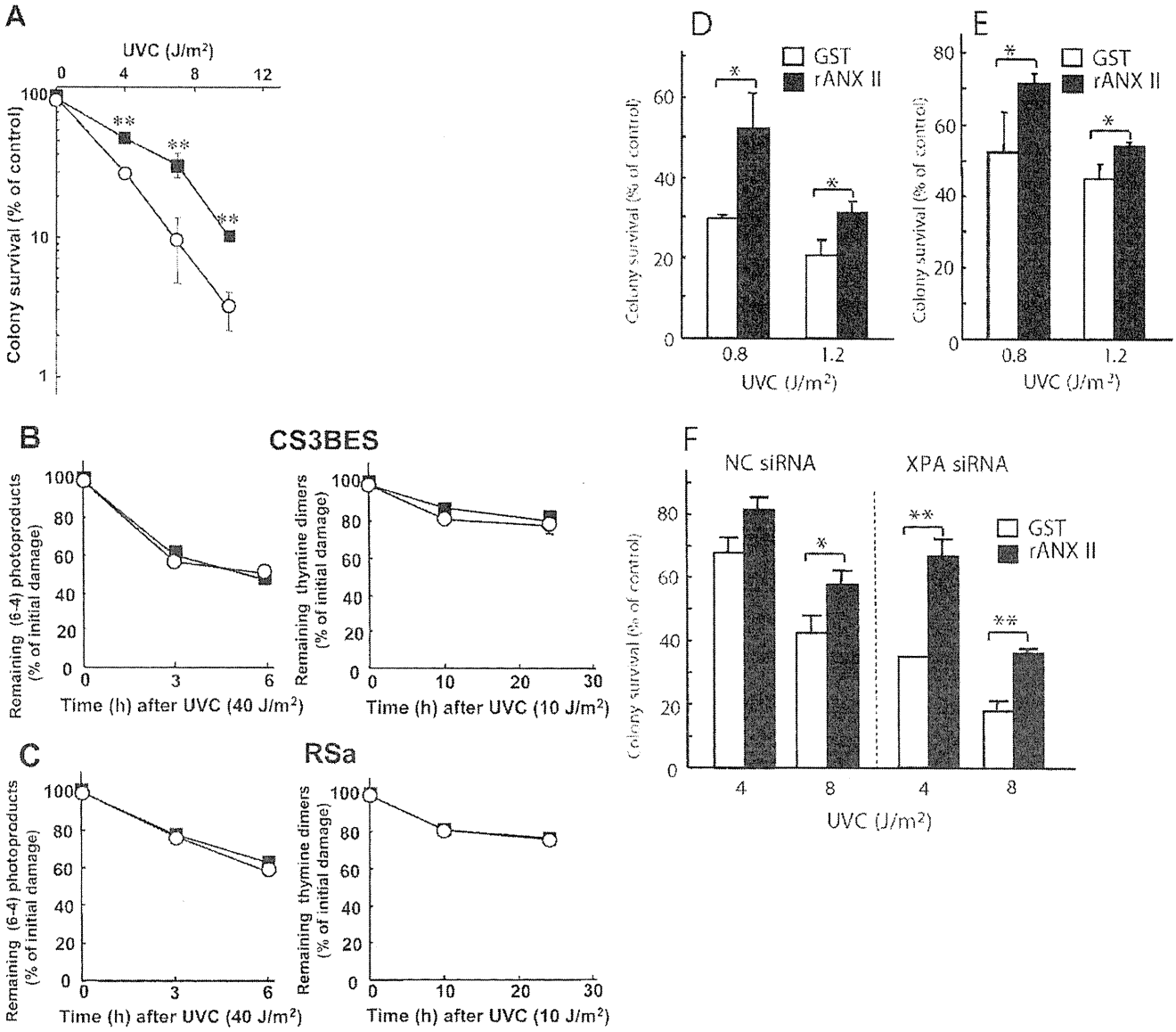


FIG. 3. Increased resistance to UVC-radiation lethality is independent of the removal of UVC-radiation-damaged DNA in rANX II-supplemented cells. RSa cells were cultured in the presence of rANX II (1.0 μg/ml) (■) and GST (0.033 μg/ml) (○) for 24 h followed by UVC irradiation and the colony survival assay, 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

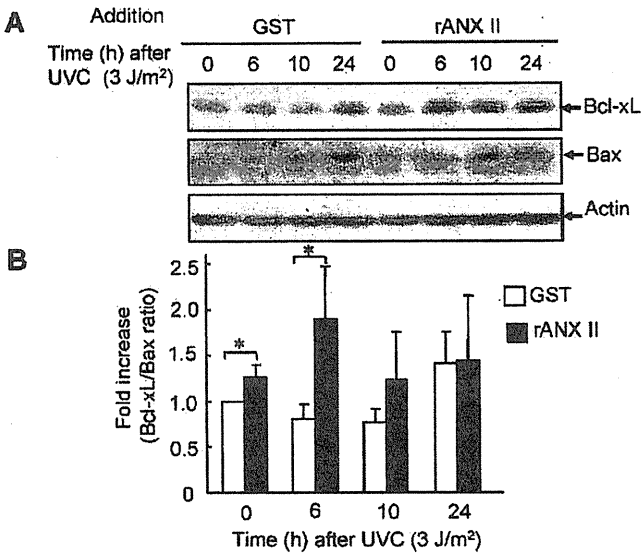


**FIG. 4.** Expression of Bcl-xL and Bax proteins after UVC irradiation in rANX II-supplemented CS3BES cells. CS3BES cells were cultured in the presence of rANX II (0.5 μg/ml) and GST (0.017 μg/ml) for 24 h and then irradiated with UVC light (3 J/m$^2$). At the indicated times after UVC irradiation, 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).

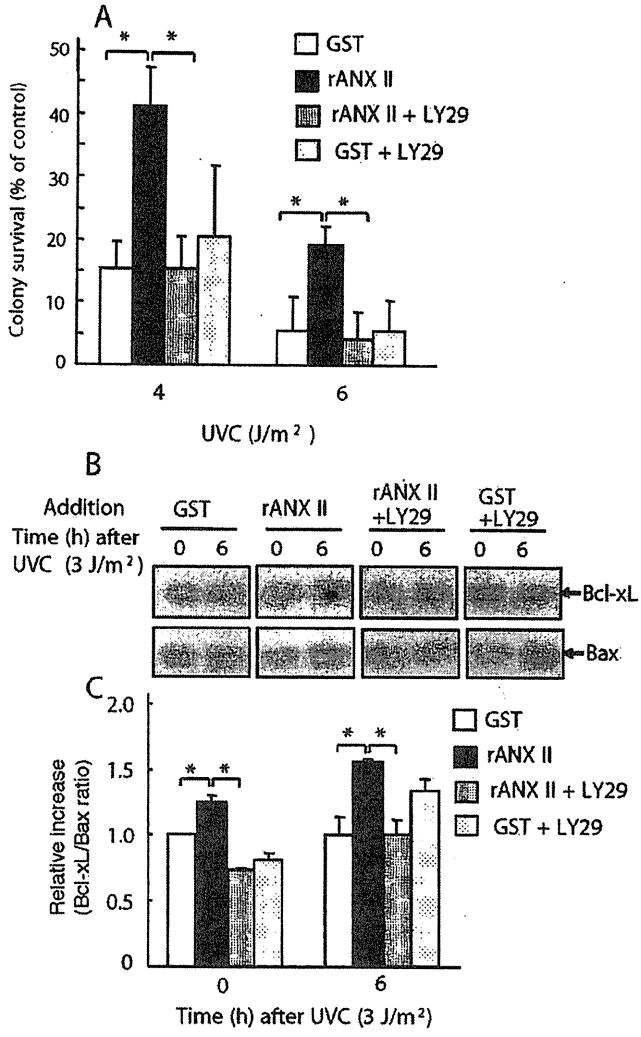


FIG. 5. Effects of the PI3K inhibitor on the rANX II supplementation-induced increase in the resistance to UVC radiation and Bcl-xL to Bax ratios. LY294002 (50 μ*M*) (LY29) was added to the medium 30 min before addition of rANX II (0.5 μg/ml), and CS3BES cells were cultured in the presence of rANX II 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

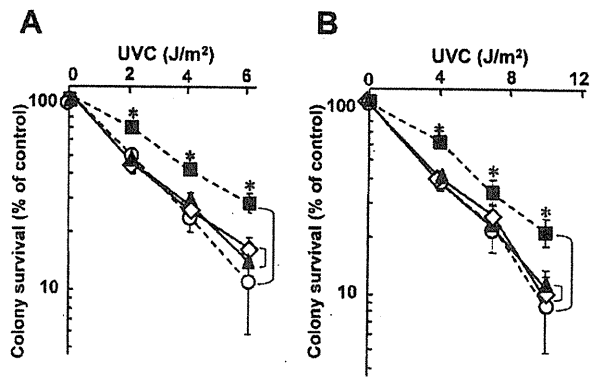


FIG. 6. Effect of Bcl-xL siRNA transfection on the rANX II supplementation-induced UVC-radiation resistance. CS3BES (panel A) and RSa (panel B) cells were transfected with 130 n*M* Bcl-xL siRNA (solid line) and NC siRNA (dotted line). Twenty-four hours after transfection, CS3BES and RSa cells were replated 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

We thank Dr. M. Zahed for technical support and R. Nobuhara for technical assistance. This work was supported in part by grants-in-aid from the following organizations: the Smoking Research Foundation, the Tokyu Foundation for a Better Environment, the Tsuchiya Foundation, the Goho Life Science International Foundation, the Kieikai Research Foundation, the Ministry of Health, Labor and Welfare for the Intractable Diseases Treatment Research Program and the Japan Society for the Promotion of Science (Japan).

Received: January 7, 2011; accepted September 7, 2011; published online: October 4, 2011

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Brain & Development 34 (2012) 124–127

BRAIN & DEVELOPMENT
Official Journal of the Japanese Society of Child Neurology

www.elsevier.com/locate/braindev

# Oxidative stress in patients with clinically mild encephalitis/encephalopathy with a reversible splenial lesion (MERS)

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Received 20 January 2011; received in revised form 27 March 2011; accepted 5 April 2011

**Abstract**

We examined oxidative stress markers, tau protein and cytokines in the cerebrospinal fluid (CSF) in six patients with clinically mild encephalitis/encephalopathy with a reversible splenial lesion (MERS). In the CSF, 8-hydroxy-2′-deoxyguanosine (8-OHdG) and hexanoyl-lysine adduct levels increased over the cutoff index in four and one out of six MERS patients, respectively. The CSF IL-6 and IL-10 levels were increased in three out of six patients, two of which had extended lesion of the cerebral white matter. The CSF value of tau protein, marker of the axonal damage, was not increased, and neuron specific enolase (NSE) in the CSF was not increased. The increased 8-OHdG levels in the CSF, DNA oxidative stress marker, in four MERS patients, suggesting involvement of oxidative stress in MERS. MERS is occasionally accompanied with hyponatremia, although our patients lacked hyponatremia. It is possible that the disequilibrium of systemic metabolism including electrolytes may lead to facilitation of oxidative stress and reversible white matter lesion in MERS. The increase of cytokine production seems to be involved in the distribution of lesions in MERS.


*Keywords:* Encephalitis; Encephalopathy; Corpus callosum; Splenium; Oxidative stress; Tau; Cytokine

## 1. Introduction

The patients with clinically mild encephalitis/encephalopathy with a reversible splenial lesion (MERS) showed the magnetic resonance imaging (MRI) findings of a reversible lesion in the central portion of the splenium of corpus callosum (SCC) with transient reduced diffusion, and had mild clinical courses and recovered completely without sequelae [1,2]. Intramyelinic edema, hyponatremia and axonal damage have been hypothesized for pathogenesis in MERS [2–4]. However, the detailed reasons for the transiently reduced diffusion are unknown. Levels of oxidative stress markers [5] and tau protein [6], a marker of axonal injuries, have been examined in the cerebral spinal fluids (CSF) in the patients with

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doi:10.1016/j.braindev.2011.04.004

developmental brain disorders. We reported the involvement of oxidative stress in a patient with limbic encephalopathy [7]. Here we examined the oxidative stress markers, tau proteins and neuron specific enolase (NSE) in CSF in six MERS patients to clarify the pathogenesis.

## 2. Materials and methods

Six patients (four female and two male, age 1–13 years) diagnosed as MERS with clinical course and MRI findings were included (Table 1). Six controls aged from 11 months to 8 years, in which the CSF was tested for the examination of fever, and no neurological symptoms such as convulsions were observed. Parent consent was obtained in all subjects in accordance with the Helsinki Declaration and all protocols were approved by the institutional ethics committee of the Tokyo Metropolitan Institute of Neuroscience. Viral infection was preceded in four out of six patients, and patient 5 demonstrated an increase in serum level of CRP (Table 1). The amount of DNA oxidative stress marker, 8-hydroxy-2′-deoxyguanosine (8-OHdG), and the early stage lipid peroxidation marker, hexanoyl-lysine adduct (HEL), were examined using ELISA kits (Japan Institute for the Aging, Shizuoka, Japan) [5]. Tau protein and NSE were determined using ELISA kit (BioSource International, Inc., CA) [6], and radioimmunoassay, respectively. Cytokine levels were evaluated by multiplex bead-based assay (BioPlex 200 system) (Bio-Rad Laboratories, CA). The comparison of averaged CSF values of each marker between the MERS patients and controls was analyzed by $t$-test, respectively, and $p < 0.05$ was evaluated as significant.

## 3. Results

There were no abnormalities in serum levels of sodium (136–141 mmol/l) and other electrolytes. Patient 5 showed delirium, whereas other five patients did not. Five patients except patient 6 had generalized tonic seizures, and anticonvulsants were given temporally. Patient 1 subsequently developed cluster of the similar convulsion, and continuous intravenous infusion of midazolam. Patients 2 and 4 were given antibiotics for a few days. Hyponatremia or hypoglycemia was not seen in patients. Patients 2 and 4 demonstrated the additional signal changes in the cerebral white matters on diffusion-weighted MRI (extended lesion). Patients 1, 2 and 4 showed transient mild increase of liver transaminase. In the CSF, no patients demonstrated abnormalities in protein level or cell count (Table 2). We calculated the mean + 2SD value in controls to set up a cutoff index for 8-OHdG, HEL, tau protein NSE and cytokines. In the CSF, 8-OHdG levels increased over the cutoff index in four out of six MERS patients. HEL level was increased over the cutoff index in patient 6, in which the 8-OHdG value was not increased. IL-6 and IL-10 were increased in three out of six patients, patients 2 and 4 of which had extended lesion of the cerebral white matter. Patient 6 showed increased levels of cytokines in the absence of extended lesion, but the distribution pattern of splenial lesions did not differ from that in patient 1, not showing increased levels of cytokines. There were no patients showing abnormal levels of tau protein and NSE. The CSF 8-OHdG values were significantly increased in the MERS patients ($p = 0.029$). There were no significant differences in CSF values of other markers between the patients and controls.

## 4. Discussion

The causes of transient and reduced diffusion in the SCC are unknown in MERS patients [2]. The SCC consists of commissural fibers from the occipital, temporal and parietal lobes, the changes in which tend to affect the SCC more frequently than the genu or body of corpus callosum. The difference in arterial vascularization and/or water content in the corpus callosum may lead to the frequent occurrence of SCC lesion [8,9]. Reversible diffusion changes in the SCC are also observed in patients with epilepsy receiving antiepileptic drugs, in which the involvement of focal cytotoxic edema occurring at the glial level is speculated by the absence of fiber interruption on diffusion tensor imaging [3]. However, no patients in this analysis had anticonvulsants before the onset of MERS. Intramyelinic edema may be hypothesized in pathogenesis of MERS, but the neonate showing an identical lesion in the SCC with incomplete myelination did not support such intramyelinic edema hypothesis [4]. Tau protein is reported to increase in

Table 1
Summary of clinical features. Abbreviations: GTCS, generalized tonic clonic seizure; DZP, diazepam; PB, phenobarbital; Extended, extended lesion of the cerebral white matter; GTS, generalized tonic seizure.

| Patient | Age/sex | Antecedent events | CRP at admission (mg/dl) | Convulsions | Anticonvulsants | Lesions on MRI |
|---|---|---|---|---|---|---|
| 1 | 1y/F | (Not determined) | 0.53 | Day 3, GTCS | DZP, PB, midazolam | Splenial |
| | | | | Cluster of GTCS | Midazolam | Splenial |
| 2 | 2y/F | Viral tonsilitis | 1.28 | Day 1, GTCS | DZP | Extended |
| 3 | 2y/M | Influenza A virus | 1.9 | Day 2, GTCS | DZP,PB | Splenial |
| 4 | 3y/F | (Not determined) | 3.5 | Day 4, GTCS | DZP,PB | Extended |
| 5 | 6y/M | RS virus | 8.527 | Day 6, GTS | (None) | Splenial |
| 6 | 13y/F | Influenza A virus | <0.3 | (None) | (None) | Splenial |

Table 2
Summary of data in patients and controls. Stars denote the values above the cutoff index. Abbreviations: 8-OHdG, 8-hydroxy-2'-deoxyguanosine; HEL, hexanoyl-lysine adduct; NSE, neuron specific enolase; IL, interleukin; n/a, not assessed.

| Patient | Cerebrospinal fluid | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Day | Cell count | Protein (mg/dl) | 8-OHdG (ng/ml) | HEL (nmol/l) | Tau protein (pg/ml) | NSE (ng/ml) | IL-6 (pg/ml) | IL-10 (pg/ml) |
| | Controls | | Mean ± SD | 0.043 ± 0.027 | 2.64 ± 1.72 | 450 ± 119 | 8.6 ± 4.1 | 3.5 ± 2.9 | 0.9 ± 0.8 |
| | | | (Cutoff) | 0.097 | 6.08 | 688 | 16.8 | 9.3 | 2.5 |
| 1 | 1 | 1.3 | 8 | 0.06 | 4.158 | 396 | 6.9 | 2.4 | 0.39 |
| | 13 | 2 | 19 | 0.06* | 3.355 | 637 | 16 | 0.76 | 0.03 |
| 2 | 2 | 0 | 13 | 0.173* | 2.6 | 422 | 5 | 9.41* | 6.11* |
| 3 | 3 | 3 | <10 | 0.174* | 4.99 | 489 | n/a | n/a | n/a |
| 4 | 4 | 0.3 | 32 | 0.374* | n/a | 48 | n/a | 13.96* | 7.15* |
| 5 | 3 | 1 | 24.9 | 0.328* | 1.72 | 320 | n/a | n/a | n/a |
| 6 | 2 | 0.3 | 21 | 0.041 | 7.37* | 281 | 5.1 | 15.56* | 2.79* |
| | | | Mean ± SD | 0.173 ± 0.134 | 4.03 ± 2 | 370 ± 184 | 8.3 ± 5.2 | 8.4 ± 6.7 | 3.3 ± 3.2 |
| | | t-test | (p value) | 0.018 | 0.275 | 0.194 | 0.457 | 0.101 | 0.086 |

the white matter diseases including leukodystrophy and encephalopathy with reduced diffusion in the cerebral white matter. The CSF values of tau protein were not increased in the MERS patients, indicating less possible involvement of axonal injury. The absence of severe axonal damage is in good accordance with the absence of fiber interruption on diffusion tensor imaging in the aforementioned epileptic patients with a similar SCC lesion [3]. It is noteworthy that the tau protein values increased from 396 to 637 pg/ml in patient 1 (Table 2), although the consecutive assay of CSF is not practical, given good prognosis of MERS.

Oxidative stress is reported to relate to some neurological diseases [5,7,10]. The CSF values of 8-OHdG and HEL increased in four and one MERS patients, respectively, and the increase of 8-OHdG values was significant (Table). The CSF 8-OHdG values increased in the patients with status convulsive [10]. In our study, the patient 1 with more severe epileptic seizure lacked abnormalities in the CSF oxidative stress markers. Oxidative stress is involved in infectious and/or inflammatory white matter diseases [11]. The MERS patients did not have any common predisposing episodes (Table 1). Patient 6 aged over 10 years and lack convulsion, and such clinical features may be related to increased level of HEL but not of 8-OHdG (Tables 1 and 2), however the exact reason of discrepancy between changes of 8-OHdG and HEL in each patient remains to be investigated. Five patients with the increased CSF values of 8-OHdG or HEL showed various inflammatory changes, in which the serum CRP levels ranged from < 0.3 to 8.527 mg/dl (Table 2). There was no direct relationship of oxidative stress with infection or inflammation. However, patients 2 and 4 with the extended lesion showed increased CSF values of IL-6 and IL-10, and altered cytokine production may be involved in the distribution of lesions in MERS. The involvement of oxidative stress has been reported in non-inflammatory myeline disorders such as pontine myelinolysis due to hypoglysemia [12]. It was reported in rats that a rapid rise in serum sodium following hyponatremia potentiated oxidative stress, which subsequently damaged myelin proteins [13]. MERS is occasionally accompanied with hyponatremia [2]. Although our patients lacked hyponatremia, the disequilibrium of systemic metabolism including electrolytes may lead to facilitation of oxidative stress and reversible white matter lesion in MERS.

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