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Proteomic Study on X-irradiation-responsive Proteins and Ageing: Search for Responsible Proteins for Radiation Adaptive Response

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We investigated high- or low-dose irradiation-responsive proteins using proteomics on two-dimensional (2D) PAGE, and the effects of ageing on cell responses to radiation in variously aged rat astrocytes. After 5 Gy irradiation, the relative abundance of peroxiredoxin 2, an antioxidant enzyme, and latexin, an inhibitor of carboxypeptidase, increased. The induction of these proteins was suppressed by ageing, suggesting that the response to high-dose radiation decreased with ageing. The relative abundance of elongation factor 2 (EF-2) fragment increased 3 h and reduced 24 h after 0.1 Gy irradiation. Temporal enhancement of the EF-2 fragment due to low-dose irradiation was suppressed by ageing. Since radiation adaptive response in cultured astrocytes was observed 3 h but not 24 h after 0.1 Gy irradiation and suppressed by ageing as previously reported, alteration of the EF-2 fragment corresponded to the radiation adaptive response. We also examined phospho-protein profiles, resulting in the relative abundance of phospho-EF-1β and phospho-β-actin being altered by 0.1 Gy irradiation; however, ageing did not affect the alteration of phospho-EF-1β and phospho-β-actin, unlike the EF-2 fragment. The results suggested that the EF-2 fragment was a possible candidate for the protein responsible for the radiation adaptive response in cultured astrocytes.

Key words: adaptive response, ageing, oxidative stress, proteomics, radiation.

Abbreviations: 2D PAGE, 2-dimensional polyacrylamide gel electrophoresis; IEF, isoelectric focusing; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; MS, mass spectrometry; PRDX, peroxiredoxin; EF, elongation factor.

Proteomic analysis offers great potential for studies on cellular protein alterations in various diseases (*1–4*), ageing (*5–9*), oxidative stress (*10–15*) and radiation effects (*16–24*). Radiation effects on protein profiles of various biological systems have been investigated in order to explore the clinical biomarkers of radiotherapy (*18*, *24*) and the factors responsible for radiation resistance (*20*), and/or to clarify the radiation injury caused by radioactive xenobiotic exposure such as depleted uranium (*19*). In most cases, the purposes of these studies were the effects of high-dose irradiation, but not low dose. Low-dose radiation has been reported to induce various biological responses; for example, hormesis, which is a beneficial stimulant effect of chronic low-dose radiation (*25*, *26*), and adaptive response, which is a radioprotective effect of low-dose conditioning irradiation followed by subsequent high-dose challenging irradiation (*27*). In particular, the radiation adaptive response has been widely observed in various biological systems ranging from prokaryotes to eukaryotes, and in mammals it was reported to occur at both cell and whole-body levels (*28–31*); however, the molecular mechanisms that drive the adaptive response under these diverse conditions remain obscure.

Thus, to identify the cellular proteins responsible for the radiation adaptive response, we performed proteomic analysis on the effects of low-dose irradiation in rat astrocytes. X-irradiation is known as oxidative stress, since ionizing radiation induces hydroxyl radical, superoxide radical and hydrogen peroxide by means of ionizing and exciting water containing dissolved oxygen in organism. Furthermore, the central nervous system is susceptible to oxidative stress because of the high metabolic demand for oxygen by neuronal mitochondria, many oxidizable polyunsaturated fatty acids present in membrane lipids, and non-protein-bound irons in the cerebrospinal fluid, which produce highly reactive hydroxyl radicals (*32*). In the central nervous system, especially, astrocytes are known to respond to various stresses in diverse ways to protect neurons and their functions (*33–36*). Therefore, we have examined the radiation adaptive response in cultured astrocytes, and demonstrated that adaptive response in astrocytes was suppressed by ageing in the previous report (*29*).

Furthermore, ionizing radiation activates various signalling molecules, such as protein kinase C (PKC),

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mitogen-activated protein kinase (MAPK), p53, ataxia-telangiectasia mutated (ATM) and DNA-dependent kinase (DNA-PK), and this activation is mediated by phosphorylation (*37*). Low-dose radiation has been also reported to phosphorylate and activate several signalling molecules (*38*, *39*). Therefore, in the present study, we examined the alteration of protein expressions and protein phosphorylations due to low-dose irradiation using proteomics, and explored the potential proteins responsible for the radiation adaptive response. Since the cell response to radiation differs depending on the radiation dose, we also examined the alteration of protein expressions due to high-dose irradiation and compared with a low dose.

MATERIALS AND METHODS

Materials—Immobiline DryStrip gel (pH 4–7, 18-cm long) and Pharmalyte (pH 3–10) were purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Tris, tricine, SDS, dithiothreitol (DTT), thiourea, ammonium bicarbonate and Triton X-100 were acquired from Sigma (St Louis, MO, USA), and molecular weight and pI marker proteins were obtained from Daiichi Pure Chemicals (Tokyo, Japan). SYPRO Ruby protein gel stain and Pro-Q® Diamond phosphoprotein gel stain were purchased from Genomic Solutions (Ann Arbor, MI, USA) and Molecular Probes (Eugene, OR, USA), respectively. Anti-glial fibrillary acidic protein (GFAP) monoclonal antibody (clone G-A-5), anti-elongation factor 2 (EF-2, H-118) polyclonal antibody, anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody and peroxidase-conjugated secondary antibodies were purchased from Chemicon International Inc. (Temecula, CA, USA), Santa Cruz Biotechnology (Santa Cruz, CA, USA), Chemicon International Inc. and Cell Signaling Technology (Beverly, MA, USA), respectively. ECL plus western blotting detection system was acquired from GE Healthcare. RNeasy Mini Kit was obtained from Qiagen Co. (Victoria, Australia). SuperScript™ III RNase H Reverse Transcriptase and KOD plus were purchased from Invitrogen (Carlsbad, CA, USA) and Toyobo Biochemicals (Osaka, Japan), respectively. TaqMan® Gene Expression Assays (primers of EF-2 and GAPDH, and TaqMan probes for each primer) were purchased from Applied Biosystems (Foster City, CA, USA). All other reagents were of the highest quality available.

Animals—Wistar rats (female, 1, 9, 22 and 24-months old) were obtained from the Laboratory Animal Facilities of the Tokyo Metropolitan Institute of Gerontology. The Animal Care and Use Committee of the Tokyo Metropolitan Institute of Gerontology approved all experimental procedures involving laboratory animals in this study.

Cell Cultures—Primary culturing of astrocytes taken from the rat hippocampus was carried out according to a previously described conventional procedure (*29*). Cells were cultured in MEM (Invitrogen) supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA) and 50 U/ml penicillin-50 μg/ml streptomycin (Invitrogen) at 37°C under a humidified atmosphere containing 5% CO_2/95% air. At confluence, the cells were trypsinized, collected, re-suspended and plated in 10 cm ϕdishes and used for the experiments. The cell cultures were >95% immunopositive against GFAP, a marker for astrocytes.

X-ray Irradiation—X-ray irradiation of cells was performed with a Hitachi X-ray irradiator (MBR-1505R2, Hitachi Medico, Co. Ltd, Tokyo, Japan) at room temperature. 5 Gy irradiation was performed with a single dose of 150 kV and 5-mA X-ray filtered with 0.1 mm copper and 0.5 mm aluminium, and 0.1-Gy irradiation was performed with 100 kV and 2 mA X-ray using 2.1-mm copper and 5.5-mm aluminium filters. The dose rates were 0.16 Gy/min in 5 Gy and 0.01 Gy/min in 0.1 Gy irradiation, respectively. The control cells treated with sham irradiation were left under air at room temperature during irradiation. Three rats of each age were used for experiments and one or two cell cultures per rat were carried out. Cells cultured from the same rat were divided into three groups; control, 3 and 24 h after irradiation, and exposed to X-irradiation simultaneously. After culturing under the conditions described above each time after irradiation, cells were washed twice with PBS and harvested using a plastic scraper.

Proteome Analysis—Protein extraction and 2D PAGE were performed as previously reported (*13*, *40*, *41*). Three cell groups irradiated at the same time were dealt with simultaneously from protein extraction to gel matching.

The search for radiation-responsive protein spots and normalization of their relative abundance were performed as described previously (*13*). The abundance of spots was represented as parts per million of the total spots integrated using 'total quantity of valid spots' using PDQuest software (Bio-Rad Laboratories, Hercules, CA, USA). When the abundance of spots on 2D gels of irradiated cells changed >2-fold or less than one-half-fold compared with control cells, we regarded the spots as radiation-responsive proteins. The relative abundance of radiation-responsive protein spots was normalized against that of internal standard spots, which existed close to the spot and were expressed strongly and constantly. For analyses of phosphorylated protein profiles, protein spots on the gel were stained with Pro-Q® Diamond phosphoprotein gel stain, a specific stain for phosphoproteins (*42*).

In-gel protein digestion of selected gel spots and peptide mass fingerprinting (PMF) were also performed as described previously (*13*). Tryptic peptides were analysed using a MALDI-TOF mass spectrometer (AXIMA-CFR, Shimadzu Biothech, Kyoto, Japan). Internal calibration of the mass axis was carried out with trypsin autolysis ions. For protein identification, tryptic peptide mass data were sent to the Mascot search engine (Matrix science, London, UK' available at: http://www.matrixscience.com) as queries for searching the Swiss-Prot protein databases. Mascot search parameters were as follows: type of search, PMF; fixed modifications, carbamidomethyl (C); enzyme, trypsin; mass values, monoisotopic; peptide mass tolerance, ±0.2 kDa; max missed cleavage, 1. Identification was further confirmed using the MS-FIT search engine in ProteinProspector of the UCSF website (http://prospector.ucsf.edu/).

MALDI-QIT-TOF MS/MS Analysis—In-gel digestion was performed in a similar way to MALDI-TOF MS analysis, and the resulting peptides from gel spots were extracted by sequential extraction for 30 min at 37°C each in 100 µl aliquots of 50 mM ammonium bicarbonate and 30% acetonitrile. Combined extracts were concentrated in a Speed Vac to ~5 µl, re-dissolved in 30 µl of 0.2% trifluoroacetic acid and de-ionized using ZipTip (C18, Millipore Co. Billerica, MA, USA). Tryptic peptides were mixed with 2, 5-dihydroxy benzoic acid (DHB) as a matrix on a target plate and analysed using a MALDI-QIT-TOF mass spectrometer (AXIMA-QIT, Shimadzu Biotech). The peaks at *m/z* 1256.66, 1494.82, 2184.25 and 2220.21 were subjected to MS/MS analysis with collision-induced dissociation. Acquired MS/MS spectra were used for search the Swiss-Prot database with the Mascot search engine. Mascot search parameters were as follows: type of search, MS/MS ion search; fixed modifications, carbamidomethyl (C); enzyme, trypsin; mass values, monoisotopic; peptide mass tolerance, ±0.1 kDa; fragment mass tolerance, ±0.4 kDa.

Western Blot Analysis—Western blot analyses were performed as described previously (*13*). The membranes transferring the cellular proteins were incubated with primary antibodies against EF-2 (1:400) or GAPDH (1:500) overnight at 4°C, and with peroxidase-conjugated secondary antibodies (anti-rabbit IgG, 1:5000 or anti-mouse IgG, 1:5000) for 2 h at room temperature. The immunoreactive proteins were visualized using an ECL plus western blotting detection system (GE Healthcare) according to the manufacturer's instructions. The band intensities were determined using ImageJ version 1.33 u software.

Quantitative Real-time RT–PCR—Total RNA from irradiated or non-irradiated cells was isolated using RNeasy mini kit (Qiagen) according to the manufacturer's instructions. After reverse transcription (RT) with SuperScript™ III RNase H Reverse Transcriptase (Invitrogen), the resulting cDNA and RNA without RT of each sample were subjected to PCR assay (KOD plus) using GAPDH primers to confirm cDNA samples without contamination of genome DNA. For quantitative analysis of EF-2 mRNA expression, real-time PCR assay was performed using 7300 Real-Time PCR System (Applied Biosystems, Lincoln Centre Drive Foster City, CA, USA). Amplification was performed in a final volume of 20 µl containing TaqMan® Universal PCR Master Mix reagent (Applied Biosystems). As an internal standard, the mRNA level of GAPDH was determined in the real-time PCR assay for each cDNA sample.

Statistics—Data were compiled from several independent experiments. Student's *t*-test was used for statistical analysis, and for all cases $P < 0.05$ was considered to indicate statistical significance.

RESULTS

Radiation-responsive Proteins in Rat Astrocytes and Effects of Ageing—Figure 1A shows a typical protein profile of rat astrocytes by 2D PAGE. About 1200 protein

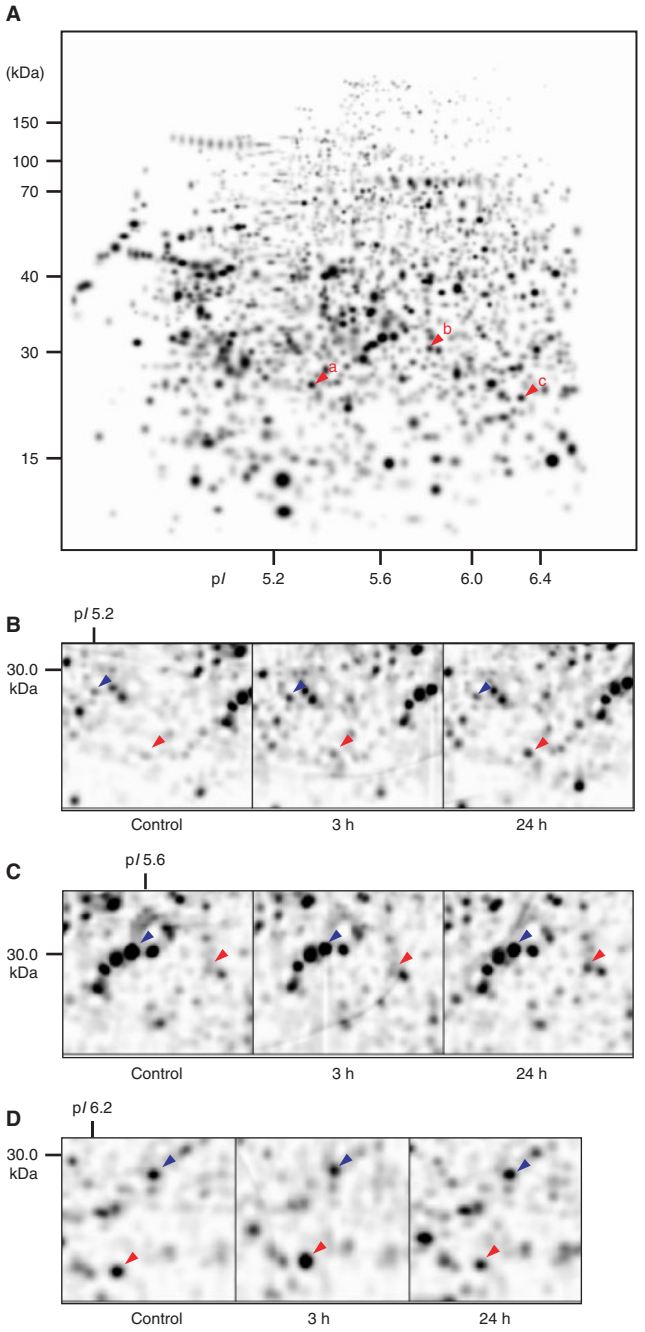

Fig. 1. **Typical protein map of 2D PAGE for young rat astrocytes.** 2D gel was stained with silver. (**A**) Spots indicated by red arrowheads represent radiation-responsive proteins. Spot 'a' and 'b' were altered by 5 Gy irradiation, and spot 'c' was altered by 0.1 Gy irradiation. Vertical axes are designated as molecular mass (kDa) and horizontal axes as p*I*. (**B**, **C** and **D**) Regions around spots 'a', 'b' and 'c' are enlarged to facilitate the identification of each spot indicated by red and blue arrowheads. Panels represent 2D gels of cell extracts from control, 3 h and 24 h after 5 Gy (B and C) or 0.1 Gy irradiation (D). Spots indicated by red arrowheads in (B), (C) and (D) represent spots 'a', 'b' and 'c', respectively, and blue arrowheads represent internal standard as ATP synthase beta chain, mitochondrial precursor (B), proteasome activator complex subunit 2 (C), hypoxanthine-guanine phosphoribosyl transferase (D).

Table 1.

Spot No.	Protein	Experimental mass (kDa)/p*I*[a]	Theoretical mass (kDa)/p*I*	Score[b]	Peptide matched[b]
a	Peroxiredoxin 2	22.6/5.3	21.8/5.3	74	6
b	Latexin	29.3/5.8	25.6/5.8	80	6
c	Elongation factor 2[c]	21.5/6.3	95.2/6.4	207[c]	4[c]
d	Elongation factor 1-β	31.4/4.8	24.7/4.5	58	4
e	β-actin	30.4/5.2	42.1/5.3	61	7
f	β-actin	34.7/5.5	42.1/5.3	183	14

[a]Experimental molecular weights and p*I*s of proteins were calculated by PDQuest software based on 2D gel images using the molecular weight marker and p*I* marker, respectively. [b]After the acquisition of PMF using a MALDI-TOF MS spectrometer as described in MATERIALS AND METHODS section, proteins were identified with the Mascot search engine. Values of score and peptides matched were determined according to the Mascot search engine on the Swiss-Prot web server. EF-2 was identified using a MALDI-TOF MS/MS spectrometer as described in MATERIALS AND METHODS section. The values of score and peptide matched represent those acquired by MS/MS ion search in the Mascot search engine on the Swiss-Prot web server.

spots were detected on each gel and all spots were matched between gels of control and irradiated cells. The abundance of spots was represented as parts per million of total spots integrated on each gel. When comparing gels of non-irradiated cells (control) and irradiated cells, spots that had changed >2-fold or less than one-half-fold in abundance were searched for as described in MATERIALS AND METHODS section. Among 1200 spots, the abundance of only three spots was changed within 24 h after irradiation. The abundance of spots 'a' and 'b' increased with time after 5 Gy irradiation (Fig. 1B and C), and that of spot 'c' was temporally increased 3 h after 0.1 Gy irradiation (Fig. 1D). To identify these proteins, tryptic peptides were analysed by MALDI-TOF MS and a database search was performed using the Mascot search engine. The results are summarized in Table 1. Spots 'a' and 'b' corresponded to PRDX 2 and latexin, respectively. Spot 'c' could not be identified by MS analysis and database search under these conditions, and identified by MS/MS analysis as mentioned subsequently.

The effects of ageing on the alteration of radiation-responsive proteins were examined. The normalized relative abundance of PRDX 2 increased with time after 5-Gy irradiation in cells from young rats (1 month), while the induction was not observed in cells from adult (9 months) and aged rats (24 months) (Fig. 2A). The induction of latexin due to 5-Gy irradiation was also inhibited by ageing (Fig. 2B). The relative abundance of protein spot 'c' was increased 3 h and reduced 24 h after 0.1 Gy irradiation in young rat cells. Temporal induction was not observed in adult and aged rat cells (Fig. 2C).

Analysis of Phosphoproteins Responsive to 0.1 Gy Irradiation—Figure 3A and B show the phosphoprotein profile of rat astrocytes. About 300 spots were detected, and the abundance of three spots was changed within 24 h after irradiation. As shown in Fig. 3B, spots 'd' and 'e' were de-phosphorylated, and spot 'f' was phosphorylated due to 0.1-Gy irradiation. The identification of these phosphoproteins was summarized in Table 1. Spots 'd', 'e' and 'f' were identified as EF-1β, β-actin and β-actin, respectively. The effects of ageing on the alterations of phosphoproteins were examined, demonstrating that ageing did not decrease the capacity for phosphorylation and de-phosphorylation significantly (Fig. 4A–C).

Identification of 0.1 Gy-Responsive Proteins—In order to identify protein spot 'c', tryptic peptides extracted from spot 'c' were analysed by MALDI-TOF MS/MS. Figure 5A shows the mass spectra of peptides from spot 'c' and the Fig. 5B–D show the MS/MS spectra of the *m/z* 1494.82, 2184.25 and 2220.21 parent ions, respectively. Four peaks surrounding circles in Fig. 5A represent the selected parent ions subjected to collision-induced dissociation. Fragment ion masses obtained by MS/MS analysis were sent to the Mascot search engine as queries. The Mascot search score was 207 and four peptides were matched, concluding that spot 'c' was identified as EF-2. A part of the amino acid sequence of EF-2 is shown in Fig. 6 and bold characters represent the amino acid residues of spot 'c' detected by MS analysis. The theoretical molecular mass of the peptide from alanine-162 to lysine-336 was calculated by Compute pI/Mw Tool in ExPASy Proteomics Server (http://au.expasy.org/tools/pi_tool.html), with a result of 19.6 kDa, and almost identical to that of spot 'c' (about 21.5 kDa as shown in Table 1). These results suggest that spot 'c' was a fragment of EF-2 in the region roughly from alanine-162 to lysine-336.

Alteration of Proteins and mRNA of EF-2 due to 0.1 Gy Irradiation—We examined whether the expression level of protein and mRNA of native EF-2 were altered by 0.1 Gy irradiation. Figure 7A shows immunoblot analysis using anti-EF-2 antibody in young and aged rat astrocytes each time after 0.1 Gy irradiation. The relative expressions of native EF-2 (97 kDa) were not altered significantly due to 0.1 Gy irradiation in both young and aged rat astrocytes (Fig. 7B). The bands of EF-2 fragment (21.5 kDa) corresponding to spot 'c' were not observed by immunoblotting using anti-EF-2 antibody. Figure 8 shows the relative expressions of EF-2 mRNA in young and aged rat astrocytes measured by quantitative real-time RT–PCR. There was no significant change of EF-2 mRNA due to 0.1 Gy irradiation in astrocytes from both young and aged rats. EF-2 mRNA in aged astrocytes tended to decrease compared with that in young cells. These results demonstrate that the expression of protein and mRNA of native EF-2 was not changed by 0.1 Gy irradiation, suggesting that the activity of protein synthesis, in which EF-2 plays an important role, is not inhibited by the increase of the EF-2 fragment due to 0.1 Gy irradiation.

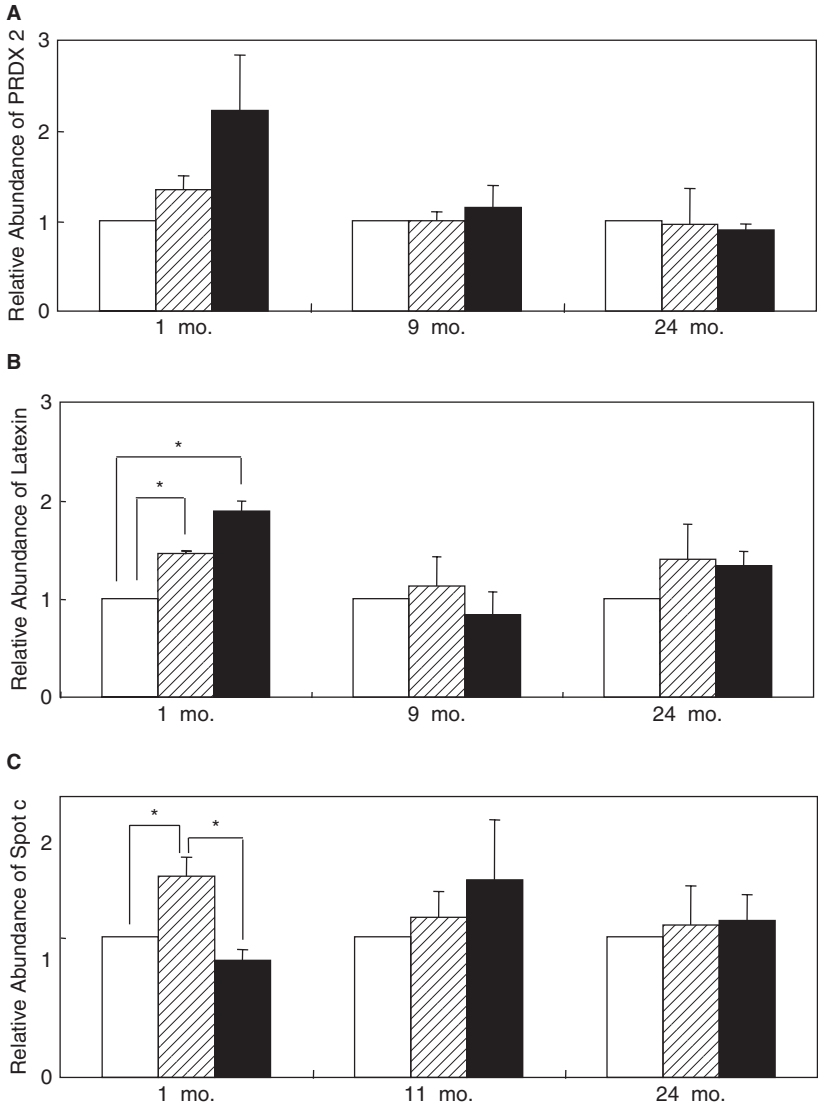

Fig. 2. **Alterations of (A) PRDX 2, (B) latexin and (C) elongation factor 2 in astrocytes from variously aged rats.** Alterations due to 5 Gy irradiation are shown in (A) and (B), and that due to 0.1 Gy irradiation is shown in (C). Open, hatched and closed bars represent the relative abundance of spots normalized against each internal standard in control, 3 h and 24 h after irradiation, respectively. Protein spots of ATP synthase beta chain mitochondrial precursor, proteasome activator complex subunit 2, and hypoxanthine-guanine phosphoribosyltransferase were used as internal standards in the normalization of PRDX 2, latexin and elongation factor 2, respectively. Data are the mean ± SE of values from three independent experiments for each age. $^{*}P < 0.05$ *vs* control cells from each aged rat. 1 month, 9 months and 24 months; astrocyrtes from 1-, 9- and 24-month-old rats, respectively.

DISCUSSION

When astrocytes were exposed to 5 Gy irradiation, the radiation-induced loss of S-phase cells and cell cycle arrest was shown 8 and/or 18 h after irradiation and cell growth was inhibited significantly by irradiation, while 0.1 Gy irradiation affected neither the cell cycle distribution nor cell growth in astrocytes (*29*, *43*). We examined the radiation effects on protein expressions in variously aged rat astrocytes using proteome analysis (Fig. 1, Table 1). Five Gy irradiation induced the expression of PRDX 2 (spot 'a') and latexin (spot 'b') in young cells, and their induction was suppressed by ageing (Fig. 2). PRDX is ubiquitously found in all living organisms and plays a role in antioxidant defence systems, which eliminate peroxides generated during cellular metabolism (*44*). The expression of PRDXs was changed due to oxidative stress (*45*, *46*) and irradiation (*18*, *20*, *23*) in various biological systems by proteomics studies. Considering that radiation generates ROS intracellularly, that is, oxidative stress, the induction of PRDX 2 due to 5 Gy irradiation should correspond to the antioxidant defensive response; therefore, our results indicate that the antioxidant defensive response to 5 Gy irradiation was suppressed by ageing in rat astrocytes.

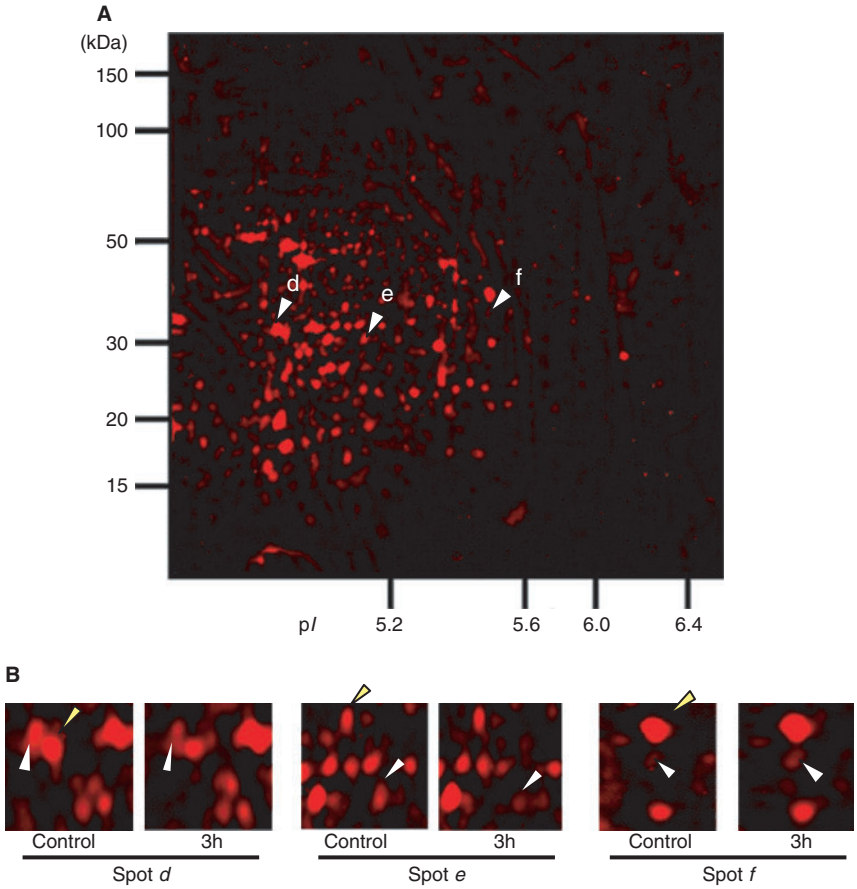

Fig. 3. **Phosphoprotein profile of rat astrocytes.** 2D gel was stained with Pro-Q Diamond phosphoprotein gel stain. (**A**) Spots d, e, and f indicated with white arrowheads represent 0.1-Gy-responsive phosphoproteins. (**B**) Regions around spots 'd', 'e' and 'f' are enlarged to facilitate the identification of each spot indicated by white and yellow arrowheads. The panels of control and 3 h represent the gels of control and 0.1-Gy irradiated cells at 3 h post-irradiation, respectively. Spots indicated with yellow arrowheads were identified as 14-3-3 protein epsilon (panel of spot 'd'), β-actin (panel of spot 'e') and tubulin-α(panel of spot 'f'), and were used as internal standards in the normalization of spots 'd', 'e' and 'f', respectively.

Latexin discovered in the rat brain (*47*) is known to be an inhibitor of carboxypeptidase A1 (CPA1), CPA2 and mast-cell CPA (MCCPA) (*48*). Latexin was up-regulated by nerve growth factor in PC12D and PC12-22a cells, and its up-regulation was mediated by several signalling molecules such as AP-1, NF-κB and protein kinase C (*49*), which were also activated due to ionizing irradiation; therefore, it seemed that 5 Gy radiation activated these signalling molecules, followed by the induction of latexin.

Low-dose irradiation is known to induce a radiation adaptive response in various biological systems. Previously we reported that young rat astrocytes exposed to 0.1-Gy pre-irradiation achieved resistance against 2 Gy additional irradiation in cell growth (*29*). The radiation adaptive response was reduced with ageing and not observed in 24-month-old rat cells. Furthermore, when the interval between 0.1 Gy pre- and 2 Gy additional irradiation was 3 h, there was a radiation adaptive response, while it did not occur with a 24-h-interval (*43*). This suggests that any proteins responsible for the adaptive response should be induced 3 h after 0.1-Gy irradiation, resulting in the acquisition of radio-resistance in astrocytes from young rats. Thus, in order to explore the proteins responsible for the radiation adaptive response in this system, we examined the effects of 0.1 Gy irradiation on protein expression and phosphorylation in young rat astrocytes using proteomics. As a result, 0.1 Gy irradiation altered the expression of the EF-2 fragment (spot 'c'), phospho-β-actin (spots 'e' and 'f'), and phospho-EF-1β (spot 'd') (Figs. 1D, 3B and Table 1). The EF-2 fragment increased 3 h and reduced 24 h after 0.1 Gy irradiation, and ageing decreased the up-regulation of the EF-2 fragment as well as the induction of the radiation adaptive response (Fig. 2C). Alteration of the EF-2 fragment was not observed in 5 Gy irradiated cells, demonstrating that the response of the EF-2 fragment was specific to low-dose irradiation. On the other hand, the alterations of phospho-β-actin and phospho-EF-1β were not significantly affected by ageing (Fig. 4A–C); therefore, the alteration of EF-2 fragment corresponded to the occurrence of radiation adaptive response.

EF-2 plays a role in the peptide elongation step of protein synthesis (*50*). The appearance of the EF-2

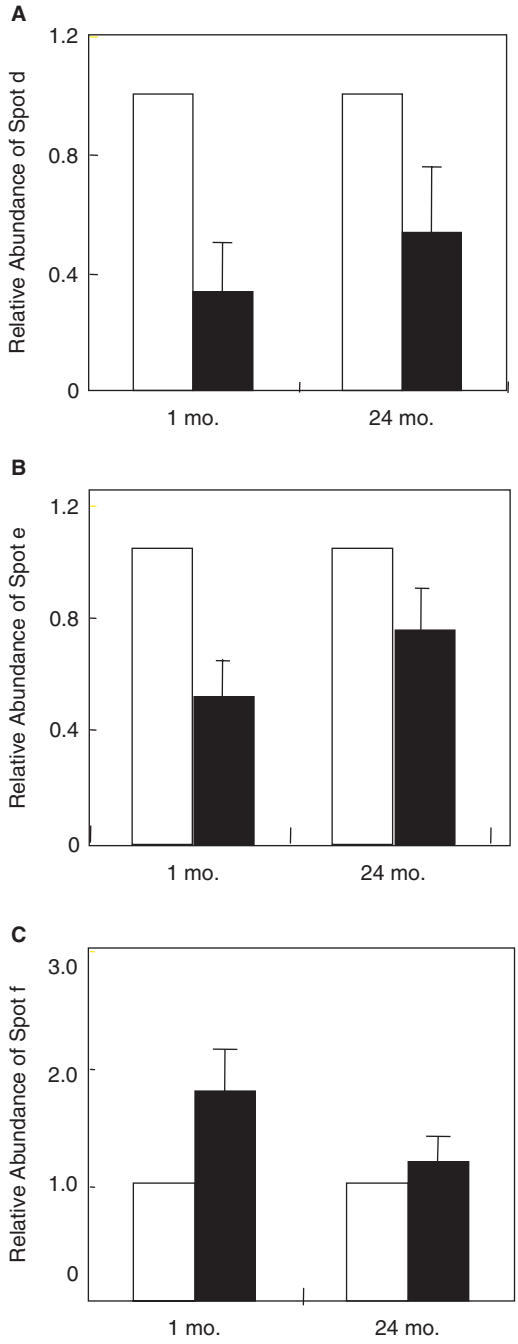

Fig. 4. **Alteration of the relative abundance of phosphoprotein spots due to 0.1 Gy irradiation in young and aged rat astrocytes.** Open and closed bars represent the relative abundance of spots normalized against each internal standard in control and 0.1 Gy irradiated cells 3 h after irradiation, respectively. Each internal standard is described in the legend of Fig. 3. Data are the mean ± SE of values from three independent experiments for each age. (**A**) spot 'd', elongation factor 1β (**B**) spot 'e', β-actin (**C**) spot f, β-actin.

fragment is considered to be due to the degradation of native EF-2, leading to the inhibition of protein synthesis. Therefore, we examined the effects of low-dose irradiation on the expression of native EF-2 protein and EF-2 mRNA by immunoblot and quantitative real-time RT–PCR, respectively; however, neither the expression of native EF-2 protein (97 kDa) nor mRNA was decreased by 0.1 Gy irradiation in astrocytes from young and aged rats. These suggest that temporal up-regulation of the EF-2 fragment due to 0.1-Gy irradiation did not affect the abundance of native EF-2, namely the function of protein synthesis, probably because the abundance of the EF-2 fragment was much less than that of native EF-2 in cells.

EF-2 fragmentation was reported to occur under severe oxidative stress or with ageing (*51*, *52*). We also reported previously that 100 μM H_2O_2 treatment induced EF-2 fragmentation in astrocytes and that the molecular mass of the fragment was about 38.6 kDa (*13*). Since the molecular mass of the EF-2 fragment in the present study was about 21.5 kDa, the mechanism of EF-2 fragmentation caused by 0.1-Gy irradiation was possibly different from that under severe oxidative stress such as 100 μM H_2O_2 treatment.

The functional region of EF-2 has been proposed by Kohno *et al*. (*53*). The amino-terminal region of EF-2 encompassing the first 160 amino acids is considered to contain domains essential for GTP-binding and GTPase activities, whereas the carboxyl-terminal half is considered to contain the domain involved in the interaction of EF-2 with the ribosome and toxins. Since the EF-2 fragment in the present study was located in the region roughly from alanine-162 to lysine-336, it contained neither domains essential for GTP binding nor interaction with ribosome, and corresponded to a region of unknown function. Among the factors involved in protein synthesis on the ribosome, eukaryotic initiation factor-2 (eIF-2) was known to be phosphorylated under environmental stress such as ER stress or nutritional stress, and to act as a transcriptional regulator activating transcription factor ATF4 (*54*, *55*). EF-2 fragment might act as a transcriptional regulator of the adaptive response similarly to eIF-2.

To our knowledge, this is the first report to explore the protein responsible for the radiation adaptive response using 2D PAGE. In order to identify the EF-2 fragment as the protein responsible for the radiation adaptive response, further investigation is needed, in relation to the generality of the alteration of EF-2 fragment caused by low-dose irradiation and the function of EF-2 fragment in radiation adaptive response. However, it is expected that the present results will clarify the molecular mechanism of adaptive response and prevent suppression of the adaptive response due to ageing.

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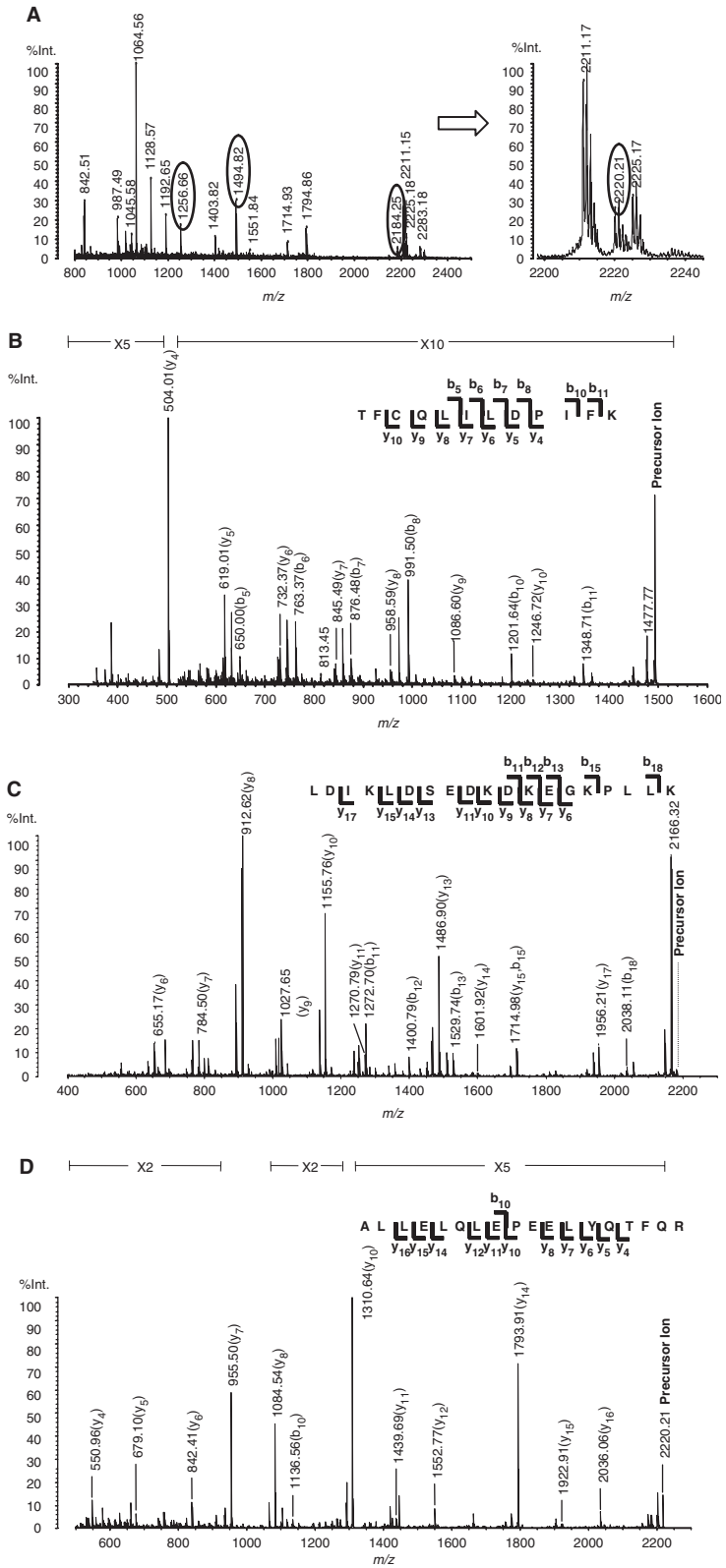

Fig. 5. **MALDI-TOF MS and MALDI-TOF MS/MS spectra of 0.1 Gy-responsive protein, spot 'c'.** (**A**) MALDI-TOF MS spectra of spot 'c' and the region of *m/z* 2200–2240 mass/charge are enlarged to facilitate the identification of *m/z* 2220.21 peak. (**B–D**) Peptide sequence analysis by MALDI-QIT-TOF MS/MS. The parent ions at *m/z* 1494.82 (B), 2184.25 (C), and 2220.21 (D) were subjected to sequence analyses by collision-induced MALDI-QIT-TOF MS/MS. The sequences of peptides at *m/z* were assigned to TFCQLILDPIFK, LDIKLDSEDKDKEGK PLLK and ALLELQLEPEELYQTFQR in EF-2, respectively. Experimental details are described in MATERIALS AND METHODS section.

101 LIDSPGHVDF SSEVTAALRV TDGALVVVDC VSGVCVQTET VLRQAIAERI
151 KPVLMMNKMD R**ALLELQLEP EELYQTFQR**I VENVNVIIST YGEGESGPMG
201 NIMIDPVLGT VGFGSGLHGW AFTLKQFAEM YVAKFAAKGE GQLGPAERAK
251 KVEDMMKKLW GDRYFDPANG KFSKSANSPD GKKLPR**TFCQ LILDPIFKVF**
301 **DAIMNFRK**EE TAKLIEK**LDI KLDSEDKDKE GKPLLK**AVMR RWLPAGDALL
351 QMITIHLPSP VTAQKYRCEL LYEGPPDDEA AMGIKSCDPK GPLMMYISKM

Fig. 6. **Part of the amino acid sequences of MALDI-TOF MS after trypsinization of 0.1 Gy responsive EF-2.** Bold characters represent peptides detected by protein spot.

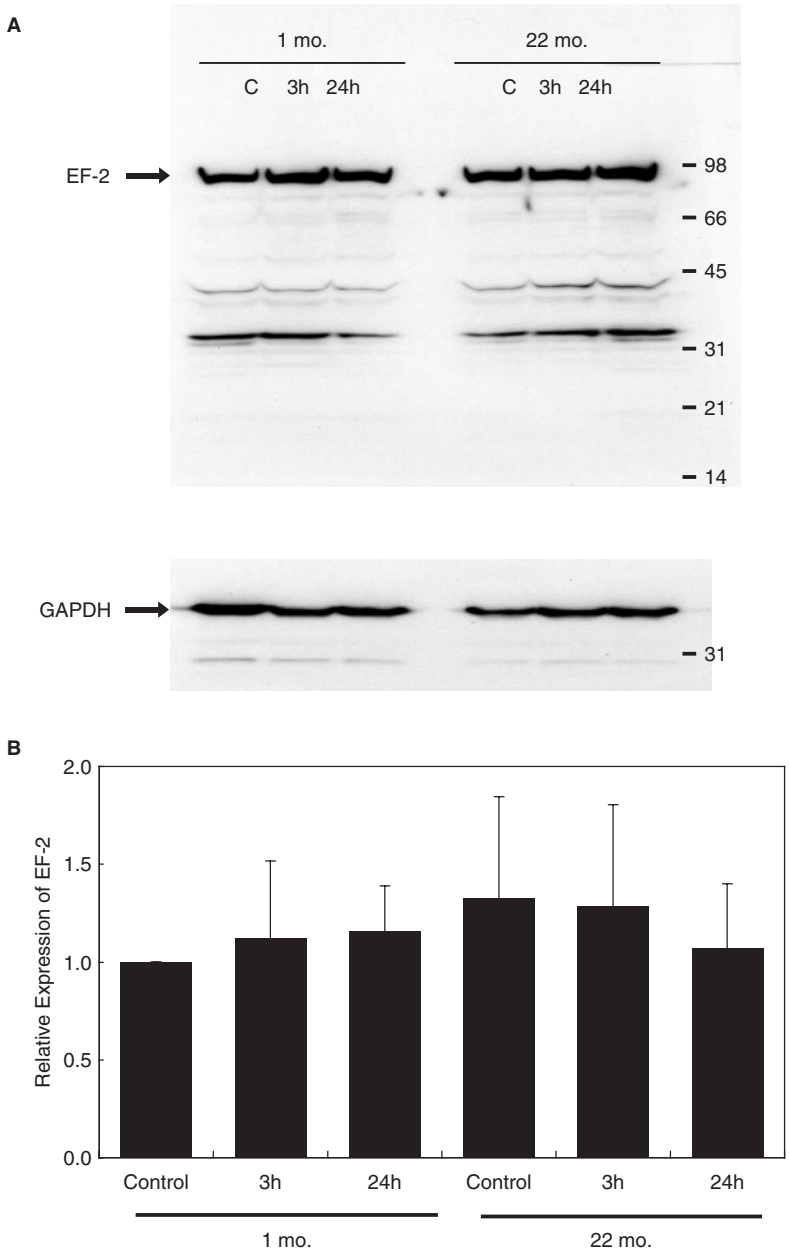

Fig. 7. **Effect of 0.1 Gy irradiation on EF-2 expression in astrocytes cultured from 1- and 22-month-old rats.** (**A**) Western blot analysis of EF-2 and GAPDH in astrocytes cultured from 1- and 22-month-old rats. (**B**) Effects of 0.1-Gy irradiation on the relative expression of EF-2. Relative intensity of EF-2 was determined from the intensity of GAPDH as an internal standard, and ordinate represents the ratio of relative intensity to control. Control: control, 3 h: 3 h after 0.1-Gy irradiation, 24 h: 24 h after 0.1-Gy irradiation. Three independent experiments were carried out, and values represent the means ± SE.

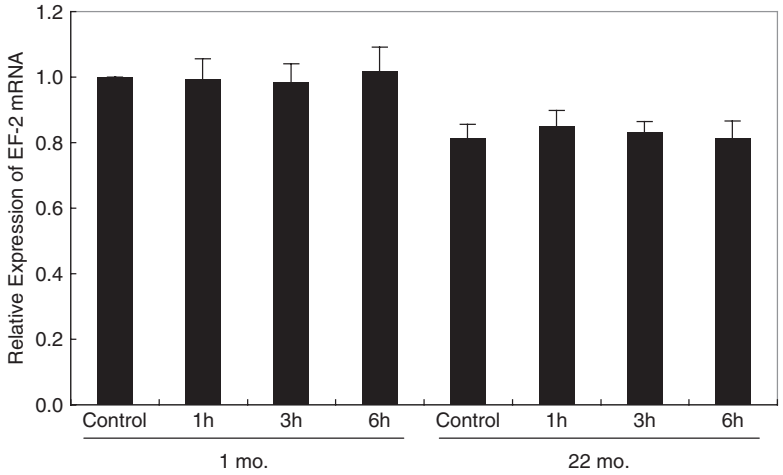

Fig. 8. **Effects of 0.1 Gy irradiation on expression of EF-2 mRNA in astrocytes cultured from 1- and 22-month-old rats.** EF-2 mRNA levels were measured by quantitative real-time RT–PCR normalized to GAPDH. 1 h, 3 h and 6 h represent each time after 0.1 Gy irradiation. Values are the mean ± SE of five independent experiments. Experimental details are described in MATERIALS AND METHODS section.

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