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Mitogen-Activated Protein Kinase, ERK1/2, is Essential for the Induction of Vascular Endothelial Growth Factor by Ionizing Radiation Mediated by Activator Protein-1 in Human Glioblastoma Cells

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Vascular Endothelial Growth Factor (VEGF)/Vascular Permeability Factor plays an important role in angiogenesis and cell proliferation of cancer cells. Glioblastoma cells are most malignant and show resistance to radiation therapy inducing VEGF to cause angiogenesis and brain edema. In the present study, the regulatory mechanism of the expression of VEGF by ionizing radiation was studied in three human glioblastoma cells. Induction of VEGF mRNA by ionizing radiation was dependent on dose and incubation time. Activator protein-1 (AP-1) was activated by 10 Gy of ionizing radiation in 1 h in T98G glioblastoma cells on an electrophoretic mobility shift assay. We constructed chimeric genes containing various regions of the VEGF promoter gene and the coding region for chloramphenicol acetyltransferase (CAT) and transiently transfected them to T98G cells. CAT assay with the VEGF promoter gene containing an AP-1 site demonstrated that the promoter activity of the VEGF gene was enhanced by ionizing radiation. Immunological analysis of the activity of mitogen-activated protein kinase, ERK1/2, showed that this activity is up-regulated by ionizing radiation.

These results suggest that ERK1/2 pathway is involved in the up-regulation of VEGF expression ionizing radiation mediated by AP-1, which may lead to further neovascularization and proliferation of glioblastoma cells resistant to radiation therapy.

Keywords: Ionizing radiation, VEGF, ERK1/2, AP-1, glioblastoma cells

INTRODUCTION

Resistance of malignant cells to ionizing radiation and chemotherapy is a major problem during the treatment of cancers. Glioblastoma is one of the most malignant forms of neoplasm, and often

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shows resistance to radiation therapy and chemotherapy.[1] This resistance is due to direct factors, such as a low intrinsic radiation sensitivity, a high recovery capacity, an increased number of clonogens, and a high hypoxic fraction.[2]

It has been reported that ionizing radiation induces the expression of particular genes, such as tumor necrosis factor-α (TNF-α), c-*fos*/c-*jun*, c-*myc*, gadd 45, and platelet-derived growth factor.[3] Furthermore, ionizing radiation activates transcription factors, such as nuclear factor-kappa B (NF-κB).[4–6] Although the precise mechanisms responsible for the specific gene expression are still unclear, transcriptional modulation to known to play a major role in the repair of DNA damage, proliferation and other cellular functions.[5]

Vascular endothelial growth factor (VEGF) is a growth factor and plays a key role in angiogenesis and vascular permeability. It is produced in many cells such as vascular endothelial cells, vascular smooth muscle cells and some tumor cells. VEGF was purified from bovine pituitary folliculostellate cells.[7,8] The human gene for VEGF and its promoter region was cloned and characterized by Tischer *et al.*[9]

VEGF mRNA is highly expressed in tumor cells within human brain tumor tissue.[10,11] Especially VEGF mRNA is up-regulated in glioblastoma cells and it is supposed that the VEGF mRNA levels are closely related to tumor enlargement and tumor angiogenesis.[12] Takano *et al.* reported that there is a correlation between the clinical grade of glioblastomas and the secretion of VEGF in them, suggesting that VEGF represents a useful marker and measurable element of glioblastoma angiogenesis.[13] It is also suggested that VEGF functions as a survival factor for newly formed vessels during developmental neovascularization.[14]

Analysis of the VEGF promoter region reveals several potential binding sites for transcription factors such as activator protein (AP)-1, AP-2 and specificity protein-1 (SP-1).[9] NF-κB activity for the VEGF expression was found in response to hypoxia.[15] Ryuto *et al.* reported that VEGF is induced by TNF-α mediated by SP-1 in human glioma cells.[16] These reports suggested that the expression of VEGF and the malignancy of glioblastoma cells are related. It has been reported that ionizing radiation induces VEGF in human cancer cell lines.[17,18] In the present study, we found that ionizing radiation stimualtes a mitogen-activated protein kinase (MAPK), ERK1/2, activity which cause an up-regulation of the expression of VEGF mRNA mediated by AP-1.

MATERIALS AND METHODS

Materials

Human glioblastoma cells (T98G, A172 and KG1C) and human GAPDH cDNA were purchased from the Health Sciences Research Resources Bank (Tokyo, Japan). The cells were maintained in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal calf serum at 37°C in 5% CO_2 under 100% humidity. VEGF121 cDNA[19] was donated by H.A. Weich. The sequences of the oligonucleotides for AP-1 and SP-1 are: AP-1, 5′-AGCTTGTCTGACTCAT-GTCTGACTCAT-3′ and 3′-ACAGACTGAGTA-CAGACTGAGTACTAG-5′; SP-1, 5′-AATTACC-GGG CGGGCGGGCTACCGGGCGGGCT-3′ and 3′-TGGCCCGCCCGCCCGATGGCCCGCCCGA-TGCA-5′.

Exposure to Ionizing Radiation

For each experiment the cells were transferred to fresh medium with adjustment to a cell density of 1×10^6 cells/ml. The cells were preincubated in this fresh medium for 2 h at 37°C in T-75 flasks (Corning, Costar, NY, USA) prior to exposure to ionizing radiation. Cells were irradiated with a ^{137}Cs source emitting a fixed dose rate of 1.0-Gy per min at room temperature (Toshiba Exs-300,

Tokyo, Japan). Mock-irradiated control cells (0 Gy) were treated identically.

Northern Blots

The cloned cDNA was isolated as described by Godwin *et al.*[20] A VEGF probe (721 bp corresponding to nucleotides 141-861 of human VEGF) was generated from human umbilical vein endothelial cells (HUVEC) total RNA by the reverse-transcriptase polymerase chain reaction as described.[19] Isolation of cytoplasmic RNA and Northern blotting were essentially as described by Sambrook *et al.*[21] Cytoplasmic RNAs isolated from T98G cells were subjected to electrophoresis in 1% agarose gels containing 0.6 M formaldehyde, subsequently transferred to nylon membranes, and then hybridized with ^{32}P-labeled VEGF probe. Autoradiographed membranes were analyzed using a Fujix Bio-Analyzer BAS-2000 (Fuji Photo Film, Tokyo, Japan). After stripping, the membranes were rehybridized with ^{32}P-labeled glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe. The relative radioactivity was expressed as a ratio of photostimulated luminescence (PSL) corrected by the intensity of GAPDH.

Stability of VEGF mRNA

The stability of VEGF mRNA was estimated basically according to a method described previously.[16] T98G cells were treated with 10 Gy of ionizing radiation and then incubated with 1 μg/ml of actinomycin D, and total RNAs were extracted 0, 2, 4, and 6 h later. Total RNAs were analyzed by hybridizaton with a ^{32}P-labeled cDNA probe.

Electrophoretic Mobility Shift Assay

The electrophoretic mobility shift assay for AP-1 was performed as described by Sen and Baltimore[22] with a slight modification. Briefly, cell-nuclear extracts were incubated with ^{32}P-oligonucleotides specific for AP-1 or SP-1. The binding reaction proceeded in a 20-μl reaction mixture containing 10 μg of extract, 4 μl of a binding buffer (10 mM Tris, pH 7.5, 40 mM NaCl, 1 mM EDTA, 1 mM 2-mercaptoethanol, 4% glycerol), 2 μg of poly(dI-dC) as a non-specific competitor DNA, 2 μg of bovine serum albumin and labeled oligonucleotide (3000–6000 cpm). After a 30 min-binding reaction at room temperature, samples were loaded on a 6% non-denaturing polyacrylamide gel and subjected to electrophoresis in 50 mM Tris, 45 mM borate, and 0.5 mM EDTA, pH 8.0. As a specificity control, a 100-fold excess of unlabeled probe was applied. The sequence of the binding site for the AP-1 probe was prepared according to the nucleotide sequences of the VEGF promoter region containing 5′-···TGACTCA···-3′. The DNA-binding activity of the extracts was quantified by estimating the amount of the ^{32}P-labeled AP-1-DNA complex excised from the dried gels and was expressed as PSL. Similarly, the sequence of the binding site for the SP-1 probe prepared according to the nucleotide sequences of the VEGF promoter region contained 5′-···GGGAGGG···-3′. The DNA-binding activity of the extracts was quantified by estimating the amount of the ^{32}P-labeled SP-1-DNA complex excised from the dried gels and was expressed as PSL.

Mitogen-Activated Protein Kinase Assay

Detection for the phosphorylation of MAPKs such as the extracellular signal-regulated kinases 1 and 2 (ERK1/2, p44/p42), c-Jun NH_2-terminal kinase (JNK, p54/p46), and $p38^{MAPK}$ was performed by immunoblotting. The cells were rinsed twice with ice-cold phosphate buffered saline (137 mM NaCl, 8.1 mM Na_2HPO_4, 2.68 mM KCl, 147 mM KH_2PO_4, PH 7.4), and lysed in buffer containing 20 mM HEPES (pH 7.4), 50 mM β-glycerophosphate, 1% Triton X-100, 10% glycerol, 2 mM

EGTA, 1 mM DTT, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 2 μM leupeptin, 2 μM aprotinin, 2 μM pepstatin A, and 1 mM PMSF. Soluble extracts were prepared by centrifugation at 10,000× *g* for 10 min at 4°C. Proteins (25 μg) were separated on 10% polyacrylamide gels using SDS-PAGE and transferred to Hybond-ECL nitrocellulose membranes. Membranes were incubated for 1 h with primary antibodies (rabbit polyclonal phospho-specifc ERK1/2, JNK and $p38^{MAPK}$ antibodies, New England Biolabs, Inc., Berverly, MA, USA). After incubation with secondary antibodies (HRP-conjugated goat anti-rabbit antibody) for 1 h, phosphorylated form of proteins were detected by ECL chemiluminescence.

CAT Assay at Human VEGF Promoter

VEGF promoter and its 5′-deleted constructs were used for the transfection T98G cells. Synthetic oligonucleotides (20-mer) were prepared on the basis of the published DNA sequence of the VEGF promoter region[9] as described.[16] The reporter plasmids for expression in T98G cells were obtained as follows. The NdeI-BamHI VEGF promoter fragment was cloned into the *Hind* III linkers and designated V1. VEGF promoter deletions from −624 to +430 were constructed.[16] The promoter fragment was obtained from a subclone, and from this, fragments from −268, −129, −94, or −49 to +430 were isolated and designated V2–5, respectively. Site directed mutagenesis of AP-1 was constructed using a LAPCR *in vitro* Mutagenesis Kit (Takara Co. Ltd., Tokyo, Japan). The sequence of the AP-1 binding site between −490 and −484 bp was changed from 5′-TGAGTGA-3′ to 5′-TGAGTTG-3′ and designated V6. T98G cells were transfected with a mixture of VEGF-CAT construct (10 μg) using Lipofectin (Life Technologies, Rockville, MD, USA). Each CAT assay was performed using identical amounts of protein as described previously.[23] The CAT activities of the transient transfection assay were normalized to β-galactosidase activity. The activity of β-galactosidase was estimated according to the method described by Rosenthal.[24]

Statistical Analysis

The data are given as the mean ± SD. Differences were calculated with Student's two-tailed *t*-test.

RESULTS

Expression of VEGF mRNA

The expression of VEGF mRNA by ionizing radiation was determined using three human glioblastoma cell lines, T98G, A172, and KGIC, which were independently established from patients with glioblastoma. Figure 1 shows the results of Northern blot analysis for VEGF mRNA. When the relative intensity of VEGF mRNA in T98G cells is expressed as 100% that in A127 cells was 132 ± 2.5%, and KG1C cells 109 ± 1.8% (mean of three independent analyses); 10 Gy of ionizing radiation induced the expression of VEGF mRNA. After 6 h, there was an increase in the levels of VEGF mRNA by 1.7 ± 0.3-fold in T98G cells, 1.5 ± 0.4-fold in A172 cells, and 1.6 ± 0.2-fold in KG1C cells. The following experiment was performed using T98G cells to elucidate the mechanisms by which VEGF mRNA is induced by ionizing radiation.

Figure 2 shows the time and dose dependent effect of ionizing radiation on the levels of VEGF mRNA estimated by Northern blot analysis. The time course study (Figure 2(A)) showed that the expression of VEGF mRNA increased within 1 h of the treatment with 10 Gy of radiation, showed a peak in 6 h and the induction continued till 12 h. The induction of VEGF by ionizing, radiation was dose-dependent (Figure 2(B)).

The Stability of VEGF mRNA on Ionizing Radiation

To know if the up-regulation of VEGF mRNA caused by ionizing radiation is due to stabilization

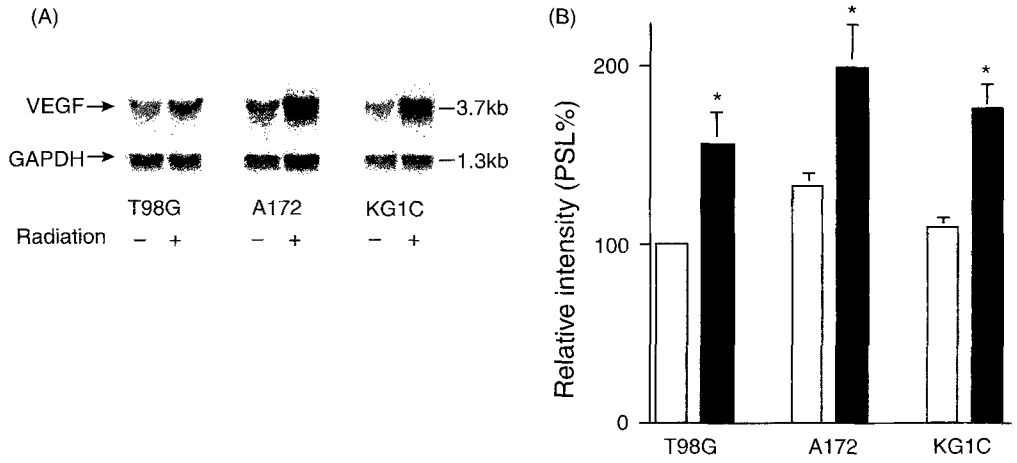

FIGURE 1 Induction of VEGF mRNA by ionizing radiation. The effect of ionizing radiation on the expression of VEGF mRNA was examined. Three human glioblastoma cells were treated with 10 Gy of radiation and RNAs were prepared after 6 h. The expression of VEGF mRNA was estimated from Northern blots (A). Values were normalized with the GAPDH mRNA level and are expressed as relative intensity (PSL%) when the steady state levels of VEGF in T98G cells were 100% (B). The data are the mean ± SD of 3 independent analyses. *$p < 0.01$ vs each control.

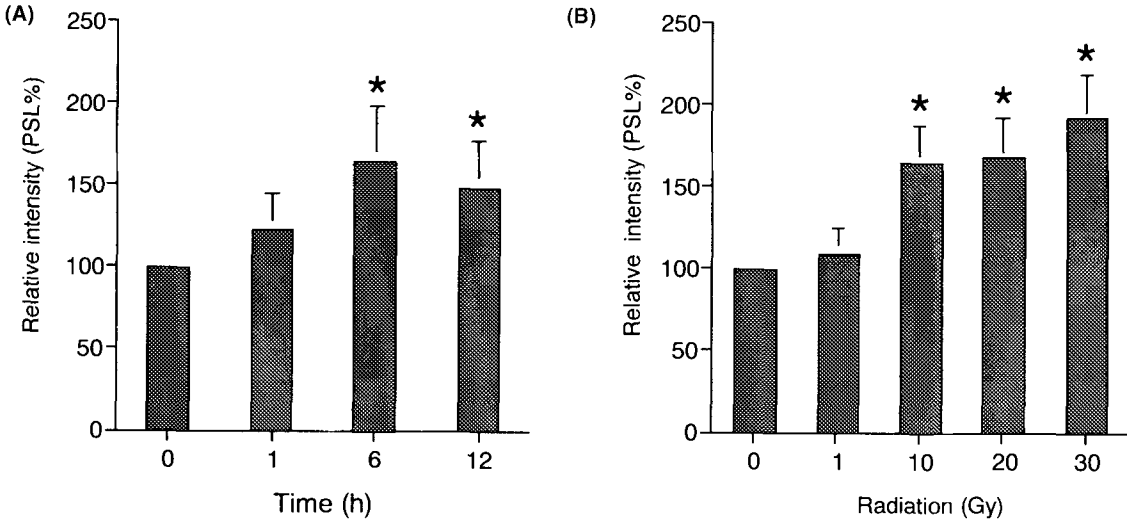

FIGURE 2 Time and dose dependent effect of ionizing radiation on the levels of VEGF mRNA. The effect of incubation time (A) after 10 Gy of ionizing radiation and dose-dependent effect of ionizing radiation (B) after 6 h were estimated in T98G cells by Northern blot analysis. Values were normalized with the GAPDH mRNA level and are expressed as relative intensity (PSL%) when the levels of VEGF in control T98G cells were 100%. The data are the mean ± SD of 3 independent analyses. *$p < 0.01$.

of the mRNA in T98G cells, the effect of ionizing radiation on the stability of VEGF mRNA was studied. T98G cells were pre-treated with 1 μg/ml actinomycin D for 1 h and then treated with 10 Gy of ionizing radiation. In control cells, the half-life of VEGF mRNA was approximately 3 h and ionizing radiation had no effect on it (Figure 3).

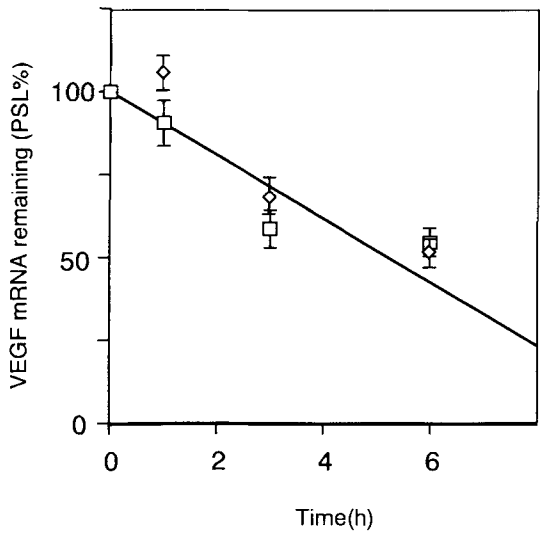

FIGURE 3 Stability of VEGF mRNA with or without the treatment of ionizing radiation. T98G cells were treated with or without 10 Gy of ionizing radiation and incubated for up to 6 h. RNAs prepared at the indicated times were hybridized with ^{32}P-labeled VEGF and GAPDH cDNA probes. Decay rates of VEGF mRNA were determined when mRNA levels at the indicated time points were normalized with the GAPDH mRNA level and are expressed as relative intensity (PSL%) when the levels of VEGF at 0 time were 100%. The data are the mean of duplicate analyses. (□) 10 Gy of ionizing radiation; (◇) control.

An Electrophoretic Mobility Shift Assay

There are oxidative stress response elements such as AP-1 and SP-1 on the 5′-flanking region of VEGF mRNA.[16] Figure 4 shows the results of an electrophoretic mobility shift assay. There was an increase in the AP-1-DNA binding activity within 1 h of the radiation of T98G cells with 10 Gy (Figure 4(A), lane 5). The AP-1-DNA binding activity was abolished by the treatment of the cells with competitor (lane 6), anti-c-Jun antibody (lane 7) and anti-c-Fos antibody (lane 8). There was no apparent increase in the SP-1-DNA binding activity within 1 h after the irradiation (Figure 4(B)). These results suggest a heterodimer of c-Fos/c-Jun binds to AP-1 site in T98G cells following ionizing radiation and that the AP-1-DNA binding activity activated by the radiation participates in the induction of VEGF mRNA.

The CAT Activity of VEGF Promoter

We constructed chimeric genes containing various regions of the VEGF gene promoter. T98G cells were transiently transfected with pSV00CAT containing the VEGF promoter construct. CAT activity stimulated by 10 Gy of irradiation was found in the VEGF promoter containing the AP-1 binding site (Figure 5, lane V1). Deletion (lane V2) and mutagenesis of the AP-1 site (lane V6) abolished the CAT activity stimulated by ionizing radiation. There are at least five SP-1 sites on the VEGF promoter. The CAT activity of the promoter region containing SP-1 sites but not an AP-1 site was not stimulated by ionizing radiation (lanes V2–V4). The CAT activity of the promoter region deleting AP-1 and SP-1 sites was not stimulated by ionizing radiation (lane V5). This strongly suggests that ionizing radiation stimulates the transcription of the VEGF gene mediated by AP-1.

MAPKs Activity

It has been reported that MAPKs are involved in the activation of AP-1 by reactive oxygen species (ROS).[25] Next, we confirmed whether ERK1/2 signal pathway is involved in the induction of VEGF mediated by AP-1 by ionizing radiation. Figure 6(A) shows the effect of ionizing radiation on the activity of MAPKs. The ERK1/2 phosphorylation elevated in 5 min after the treatment with 10 Gy of radiation, and showed a peak at 20 min. A specific inhibition of ERK1/2 activity with a specific inhibitor of ERK1/2, PD98059 (2-(2′-amino-3′-methylphenyl)oxanaphthalene-4-one), inhibited the ERK1/2 activity (B) and attenuated ionizing radiation-stimulated AP-1 activity (C, lane 6) and ionizing radiation-induced VEGF mRNA expression (D). Other MAPKs such as JNK (p54/p46) and p38^{MAPK} were not stimulated by ionizing radiation.

DISCUSSION

Induction of VEGF is brought about by many stimuli. Among them, ROS such as superoxide,[26]

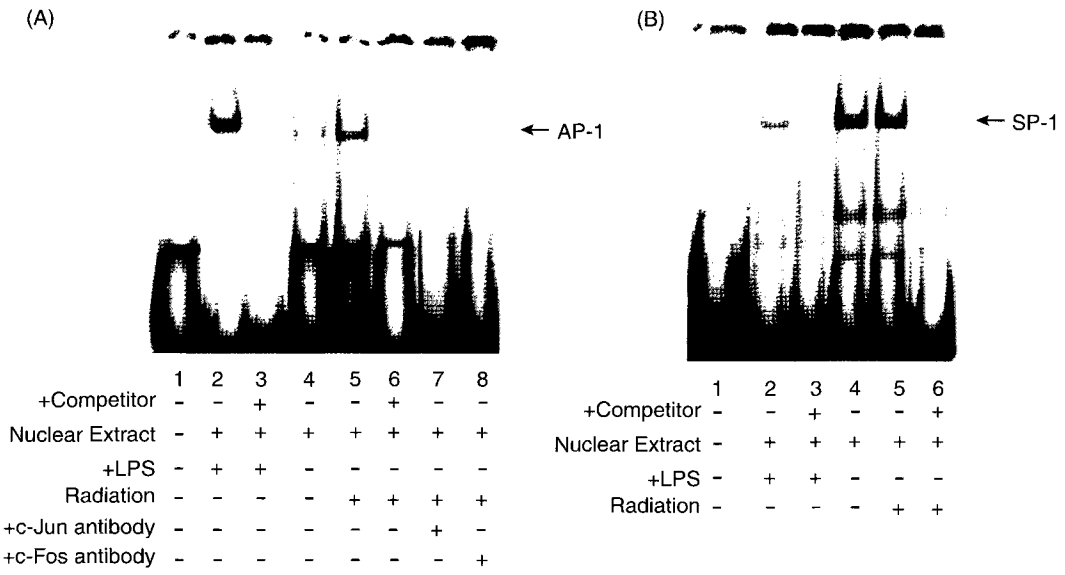

FIGURE 4 Electrophoretic mobility shift assay of AP-1 and SP-1. The effect of ionizing radiation on the AP-1 and SP-1-DNA binding activities was studied. T98G cells were treated with 10 Gy of ionizing radiation and cell-nuclear extracts drawn at the indicated times were incubated with an AP-1- or SP-1-specific ^{32}P-oligonucleotide for 30 min and then loaded on a 6% nondenaturing polyacrylamide gel. The DNA binding activities of the extracts were estimated by electrophoretic mobility shift assay for AP-1 (A) and SP-1 (B). (A), lane 1, free probe; lanes 2–8, cell-nuclear extracts from T98G cells; lanes 2 and 3, + 100 ng/ml of lipopolysaccharide (LPS) as a positive control; lanes 3 and 6, + competitor for AP-1; lane 4, 0 h; lanes 5–8, 1 h after the 10 Gy of ionizing radiation; lane 7, + anti-c-Jun antibody; lane 8, anti-c-Fos antibody. (B), lane 1, free probe; lanes 2–6, cell-nuclear extracts from T98G cells; lanes 2 and 3, + 100 ng/ml of LPS as a positive control; lanes 3 and 6, + competitor for SP-1; lane 4, 0 h; lanes 5 and 6, 1 h after the 10 Gy of ionizing radiation.

hydrogen peroxide[27] and nitric oxide,[28] are reported to have a role in the elevation of the VEGF levels. However, there has been no report on the effect of ionizing radiation on the VEGF expression. Since ionizing radiation produces ROS, it is speculated that VEGF is up-regulated during the treatment of glioblastoma cells with ionizing radiation. In the present study, induction of VEGF by ionizing radiation was found and the possible mechanism of it was elucidated.

It has been reported that the levels of oxidative stress-responsive transcription factors such as c-Fos/c-Jun and SP-1 are elevated in human glioblastoma cells in which the VEGF mRNA levels are closely correlated suggesting that the DNA-binding activity of these factors is involved in the VEGF expression.[16] Induction of VEGF mRNA by hypoxia is also AP-1-dependent in human glioma cells.[28] On the other hand, Kuroki *et al.* reported that ROS induce VEGF by enhancing mRNA stability but not the transcriptional activity.[25] Furthermore, Gorski *et al.* has reported that neutralizing anti-VEGF antibodies inhibits VEGF-induced angiogenesis by ionizing radiation in human cancer cells,[18] supporting the idea that induction of VEGF by ionizing radiation protects tumor blood vessels from radiation-mediated cytotoxicity and thereby induces radioresistance of tumor cells.

In the present study VEGF mRNA was elevated by ionizing radiation in human glioblastoma cells (Figures 1 and 2), which results are in good agreement with those reported by others.[17,18] Previously Damert *et al.* reported that stabilization of VEGF mRNA contributes to the up-regulation of this mRNA under hypoxia.[28] In this study, the VEGF mRNA stability was not changed by ionizing radiation in T98G cells (Figure 3). An electrophoretic mobility shift assay (Figure 4) as well as CAT activity for the VEGF

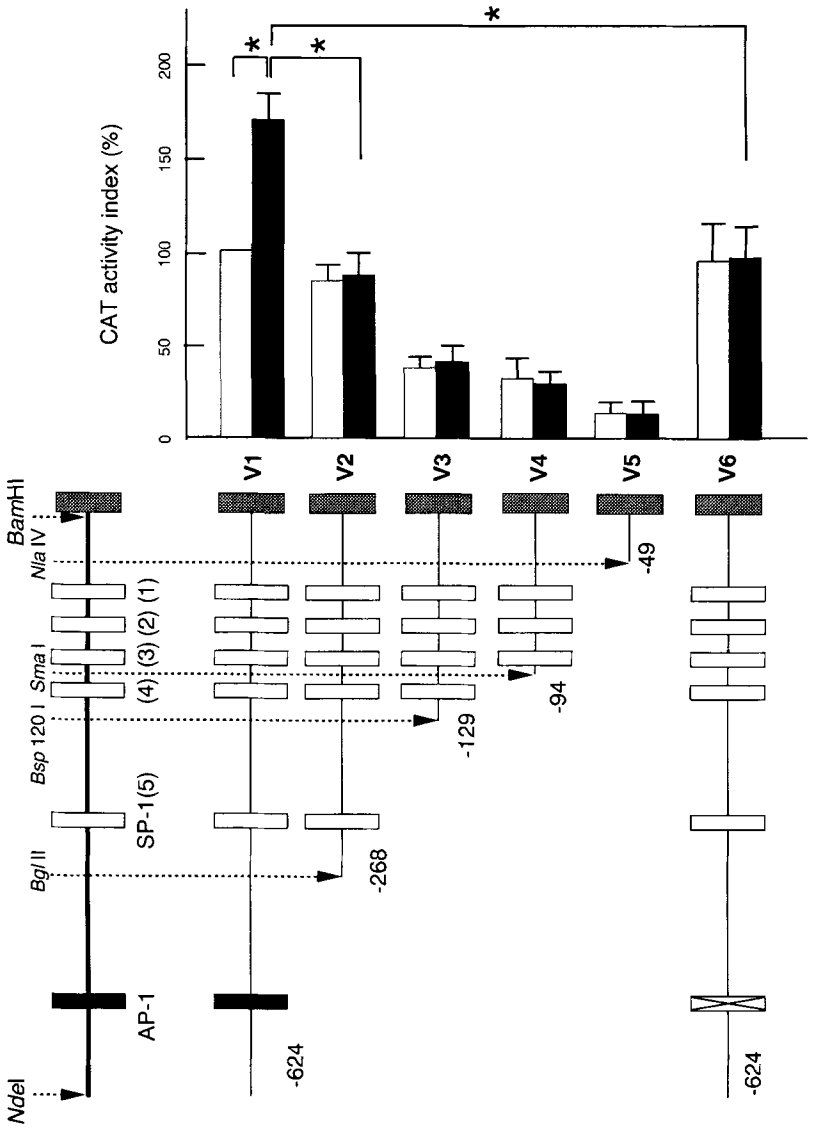

FIGURE 5 CAT activity of VEGF promoter. T98G cells transiently transfected with VEGF promoter-CAT gene fusion plasmid were treated with 10 Gy of ionizing radiation and incubated for 6 h. Putative consequences in the 5′-upstream region of human VEGF gene and restricting enzymes used are illustrated (upper left). Numbers indicate the distance in base pairs from the start of transcription. V1, a NdeI-BamHI fragment of promoter; V2, V1 lacking AP-1; V3, V1 lacking AP-1 and SP-1 (5); V4, V1 lacking AP-1, SP-1 (5) and SP-1 (4); V5, V1 lacking AP-1 and SP-1 (1–5); V6, V1 with a mutation at AP-1 site. The CAT activity was corrected for differences in transfection efficiency among the cells as estimated by β-galactosidase activity and then normalized to corrected activity of transient VEGF-CAT-transfected cells. The relative fold increase was determined when normalized by endogenous CAT activity of the transfected cells in the absence of ionizing radiation. Data are the mean ± SD (%) of 3 independent analyses. $^*p < 0.01$.

promoter (Figure 5) suggested that ionizing radiation up-regulates the VEGF expression mediated by AP-1. The ERK1/2 activity was activated by ionizing radiation (Figure 6). Quite recently, we found that intracellular ROS produced from oxidized low density lipoprotein stimulates the MAPK activity which further stimulates AP-1 activity.[29] The results obtained

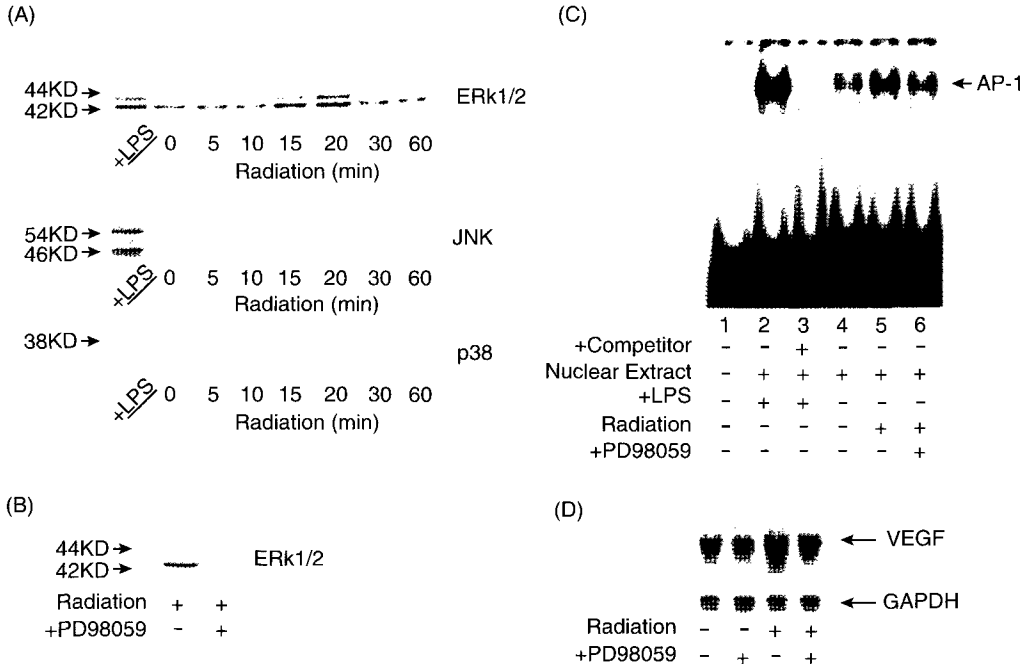

FIGURE 6 Effect of ionizing radiation on MAPKs phosphorylation in T98G cells. The phosphorylation of MAPKs in response to ionizing radiation was estimated. T98G cells were treated with 10 Gy of ionizing radiation and cell lysates were prepared at the indicated times, aliquots (25 μg) of the 1% Triton X-soluble fractions were separated by 10% SDS-PAGE and the fractionated proteins were transferred to nitrocellulose membranes. The membranes were probed with rabbit polyclonal phospho-specifc anti- ERK1/2, -JNK and -p38^{MAPK} antibodies (A). First lane, 100 ng/ml of LPS was added as a positive control. Effect of a specific inhibition of ERK1/2 with PD98059 was estimated on the ionizing radiation-stimulated ERK1/2 activity using SDS-PAGE (B); AP-1-DNA binding activity on electrophoretic mobility shift assay (C). Lane 1, free probe; lanes 2–6, cell-nuclear extracts; lanes 2 and 3, + LPS as a positive control; lane 3, + competitor; lanes 5 and 6, 1 h after the 10 Gy of ionizing radiation; lane 6 + PD98059. Ionizing radiation-induced VEGF mRNA expression on Northern blots (D).

in the present study suggest that ROS produced by ionizing radiation is involved in the induction of VEGF mediated by AP-1 activity stimulated by ERK1/2 activity.

Since up-regulation of VEGF expression is a major event leading to vascularization, the induction of VEGF by ionizing radiation may correlate to the capacity of glioblastoma cells to promote tumor neovascularization and enhance vascular permeability.[30]

In conclusion, it is suggested that high VEGF mRNA levels in glioblastomas, where it may be involved in promoting tumor angiogenesis and stroma generation, were further up-regulated by ionizing radiation, affecting endothelial cell mitogenesis and vascular permeability.

Acknowledgment

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