

Radio-sensitivities and angiogenic signaling pathways of irradiated normal endothelial cells derived from diverse human organs

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The purpose of the present investigation was to study the effects of ionizing radiation on endothelial cells derived from diverse normal tissues. We first compared the effects of radiation on clonogenic survival and tube formation of endothelial cells, and then investigated the molecular signaling pathways involved in endothelial cell survival and angiogenesis. Among the different endothelial cells studied, human hepatic sinusoidal endothelial cells (HHSECs) were the most radio-resistant and human dermal microvascular endothelial cells were the most radio-sensitive. The radio-resistance of HHSECs was related to adenosine monophosphate-activated protein kinase and p38 mitogen-activated protein kinase-mediated expression of MMP-2 and VEGFR-2, whereas the increased radio-sensitivity of HDMECs was related to extracellular signal-regulated kinase-mediated generation of angiostatin. These observations demonstrate that there are distinct differences in the radiation responses of normal endothelial cells obtained from diverse organs, which may provide important clues for protection of normal tissue from radiation exposure.

Keywords: ionizing radiation; normal endothelial cells; angiogenesis

INTRODUCTION

Exposure to ionizing radiation resulting from medical and industrial use of radiation or from nuclear accidents causes undesired damage in the human body. In particular, normal tissue damage associated with treating cancer with radiotherapy is of great concern. In fact, the damage to normal tissue is a limiting factor in achieving complete control of cancer by radiotherapy. It is known that radiation-induced tissue damage is closely related to blood vessel damage [1–3]. Microvessels are sensitive to ionizing radiation, which leads to capillary rupture, thrombosis and telangiectasia [4]. In medium-sized blood vessels, ionizing radiation causes neointimal proliferation, thrombosis, fibrinoid necrosis and acute arteritis [4]. On the other hand, it has been suggested that large blood vessels are less affected by ionizing radiation than other blood vessels [4]. The major components of blood vessels are endothelial cells, and thus

radiation-induced vascular damage is closely related to the radio-sensitivity of endothelial cells. The purpose of the present study was to compare the radio-sensitivity of endothelial cells derived from diverse normal tissues of human and reveal the molecular signaling involved in the radiation-induced endothelial damage.

Matrix metalloproteinases (MMPs), which degrade the structure of the extracellular matrix (ECM), and cleave diverse growth factors and cell surface receptors, have been demonstrated to promote angiogenesis and tumor progression [5–7]. Among MMPs, MMP-2 and -9 have been shown to be the most closely linked to the angiogenic process [8]. Furthermore, MMPs are up-regulated by various hormones, cytokines and growth factors, including vascular endothelial growth factor (VEGF), which plays a key role in vascular remodeling in response to diverse angiogenic stimuli [9]. In contrast to MMPs, angiostatin and endostatin generated via MMP-mediated proteolytic

cleavage of plasminogen and collagen XVIII, respectively, contribute substantially to anti-angiogenic responses [10]. Along with angiogenic factors, angiostatin and endostatin may play a role in regulating the balance between angiogenesis and angiostasis known as the opposite state of angiogenesis [11–13].

In the present study, we found that human hepatic sinusoidal endothelial cells (HHSECs) are the most radio-resistant and human dermal microvascular endothelial cells (HDMECs) are the most radio-sensitive among the different endothelial cells studied. Ionizing radiation promoted capillary-like tube formation by HHSECs via adenosine monophosphate (AMP)-activated protein kinase (AMPK) and p38 mitogen-activated protein kinase (MAPK)-mediated expression of MMP-2 and VEGF receptor-2 (VEGFR-2), but significantly suppressed capillary-like tube formation by HDMECs via extracellular signal-regulated kinase (ERK)-mediated generation of angiostatin.

MATERIALS AND METHODS

Reagents

Matrigel was obtained from BD Biosciences (Bedford, MA, USA). Antibodies against p38 MAPK, c-Jun N-terminal kinase 1/2 (JNK1/2), ERK1/2, AKT, AMPK and mTOR (mammalian target of rapamycin) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti- β -actin, anti-rabbit IgG and anti-mouse IgG were purchased from Sigma (St. Louis, MO, USA). Antibodies against phospho-p38 MAPK (Thr180/Tyr182), phospho-JNK1/2 (Thr183/Tyr185), phospho-ERK1/2 (Thr202/Tyr204), phospho-AKT (Ser473), phospho-AMPK (Thr172) and phospho-mTOR (Ser2448) were obtained from Cell Signaling Technology (Beverly, MA, USA). Inhibitors specific to JNK (SP600125), MEK/ERK (PD98059), PI3K/AKT (LY204002), p38 MAPK (SB203580) and AMPK (Compound C) were purchased from Calbiochem (San Diego, CA, USA).

Cell culture

HHSECs, human brain microvascular endothelial cells (HBMECs), human ovarian microvascular endothelial cells (HOMECS), human pulmonary endothelial cells (HPMECs), human umbilical vein endothelial cells (HUVECs) and HDMECs were obtained from ScienCell Research Laboratories (San Diego, CA, USA). Cells were plated on gelatin-coated 60-mm dishes and cultured in complete Endothelial cell culture medium (ECM) (ScienCell Research Laboratories) supplemented with 5% fetal bovine serum, 1% antibiotics and 1% endothelial cell growth supplement in a humidified 5% CO₂ incubator at 37°C. Cells from passages 2–5 were used for experiments.

Irradiation

Cells were exposed to γ -rays with a ¹³⁷Cs irradiation source (Model 68; J.L. Shepherd and Associates, Glenwood, CA, USA) at a dose rate of 2–3 Gy/min.

Clonogenic cell survival assay

Appropriate numbers of endothelial cells were plated onto gelatin-coated 60-mm dishes with ECM, incubated overnight, irradiated with various doses of γ -ray and then cultured for 14 days in a 5% CO₂ incubator at 37°C. The formed colonies were fixed with 95% methanol and stained with 0.5% crystal violet, and the colonies containing more than 50 cells were counted. The averages of triplicate dishes were obtained for each sample, the results were normalized with respect to the plating efficiencies of the corresponding non-irradiated cells, and the surviving fractions were calculated.

RNA preparation and reverse

transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from endothelial cells using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and 1 μ g of total RNA was reverse transcribed into cDNA. The following targets were amplified from cDNA by PCR using the indicated primers: *MMP-2*, 5'-GAC AGT GGA TGA TGC CTT TGC-3' (forward primer) and 5'-GAG CAC CAG AGG AAG CCA TC-3' (reverse primer); *MMP-9*, 5'-AAC TAC TCG GAA GAC TTG CC-3' (forward primer) and 5'-GTG GTG CAG GCA GAG TAG G-3' (reverse primer); *TIMP-1*, 5'-TGG CTT CTG CAC TGA TGG TG-3' (forward primer) and 5'-TGG TCA TCT TGA TCT CAT AAC G-3' (reverse primer); *TIMP-2*, 5'-GTC AGT GAG AAG GAA GTG GAC-3' (forward primer) and 5'-TCT GTG ACC CAG TCC ATC CAG-3' (reverse primer); *VEGFR-2*, 5'-CTG ATG GTA ACA GAA TTT CCT G-3' (forward primer) and 5'-TTG TGT GAT TGG ACT CAA GGG-3' (reverse primer); *plasminogen*, 5'-CTG GAT GAC TAT GTG AAT AC-3' (forward primer) and 5'-TTG TCT GGA TTC CTG CAG TAG-3' (reverse primer). Amplified products were separated on 1% (w/v) ethidium bromide-stained agarose gels, and expression levels were measured by luminescent image analysis.

Western blot analysis

Endothelial cells plated on gelatin-coated dishes were treated with lysis buffer [40 mmol/l Tris-Cl (pH 8.0), 120 mmol/l NaCl, and 0.1% NP40] supplemented with protease inhibitors, then centrifuged for 15 min at 12 000 \times g. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline and subsequently incubated

for 1 h with primary antibodies at room temperature. Blots were developed with a peroxidase-conjugated secondary antibody, and immunoreactive proteins were visualized using enhanced chemiluminescence reagents (Amersham Biosciences, Piscataway, NJ, USA), as recommended by the manufacturer. β -actin was used as a loading control.

Gelatin zymography

Production of MMPs in endothelial cells was analyzed by gelatin zymography. Subconfluent cells (~70% confluence) were washed, replenished with serum-free ECM and exposed to 4-Gy irradiation in a single exposure. At 24 h after irradiation, 20 μ l of serum-free conditioned medium was mixed with sodium dodecyl sulfate (SDS) sample buffer without heating or reduction and applied to 10% polyacrylamide gels copolymerized with 0.1 mg/ml gelatin. After electrophoresis, gels were washed in 2.5% (v/v) Triton X-100 for 1 h at room temperature to remove the SDS, rinsed twice with water, and then incubated in developing buffer [50 mM Tris-HCl, pH 7.5, 0.2 M NaCl, 5 mmol/l CaCl₂, 0.1% Triton X-100] for 16 h at 37°C. Subsequently, gels were fixed and stained with 30% methanol and 10% acetic acid containing 0.5% Coomassie Blue R250. Gels were destained with 5% methanol and 8% acetic acid.

Measurement of angiostatin generation

Subconfluent cells (~70% confluence) were washed, replenished with serum-free ECM and exposed to 4-Gy irradiation in a single exposure. At 24 h after irradiation, angiostatin levels in conditioned media were measured with a commercially available ELISA kit (RayBiotech, GA, USA).

Capillary-like tube formation assay

Four-well culture plates were coated with cold Matrigel and placed in a 5% CO₂ incubator at 37°C to allow the Matrigel to polymerize. Cells were then seeded on plates (1×10^5 per well) in complete ECM, and exposed to 4 Gy of ionizing radiation. After incubating cells for 6 h, the area covered by the newly formed tubes was determined using the Image J program (NIH, Bethesda, MD, USA). In brief, the number of pixels in the tube area was quantified for two images per well at 40 \times magnification [14]. An image file was opened in the Image J program, and then subjected to the 'Polygon Selection Tool' to designate the area of the tube network. The designated area was measured using the 'Measure' option of the Image J program.

Statistical analysis

All data presented are representative of at least three separate experiments. Comparisons between groups were analyzed using Student's t-test (SPSS Statistics version 17.0,

Chicago, IL, USA). *P*-values < 0.05 (indicated by * on figures) were considered to be significant.

RESULTS

Identification of radio-resistant and radio-sensitive endothelial cells

We first performed clonogenic cell survival assay to compare the radio-sensitivities of HHSECs, HBMECs, HOMECS, HPMECs, HUVECs and HDMECs. As shown in Fig. 1, HHSECs were the most radio-resistant and HDMECs were the most radio-sensitive among the different endothelial cell lines studied. We then investigated the effect of ionizing radiation on the angiogenic potential of radio-resistant HHSECs and radio-sensitive HDMECs by determining the ability of the cells to form capillary-like tubular structures on Matrigel-coated plates. Although the ability of unirradiated control HDMECs to form capillary-like tubular structures was much greater than that of unirradiated control HHSECs, 4-Gy irradiation increased tube formation by radiation-resistant HHSECs but effectively suppressed tube formation by radio-sensitive HDMECs (Fig. 2A and B). These data suggest a direct or indirect relationship between the degree of radio-sensitivity as determined with a clonogenic assay method and the changes in angiogenic process.

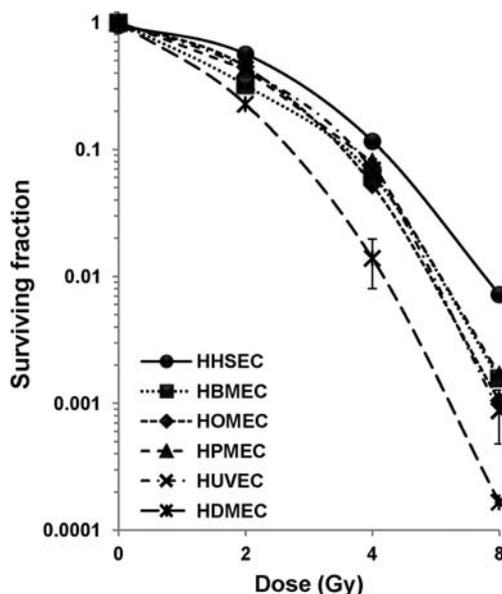


Fig. 1. Effect of ionizing radiation on the clonogenic survival of the normal endothelial cells from diverse human organs. HHSEC, HBMEC, HOMECS, HPMEC, HUVEC and HDMEC were irradiated with increasing doses of ionizing radiation, cultured for 14 days, stained with 0.5% crystal violet, and the numbers of colonies were counted. The results from three independent experiments are expressed as means \pm SEM.

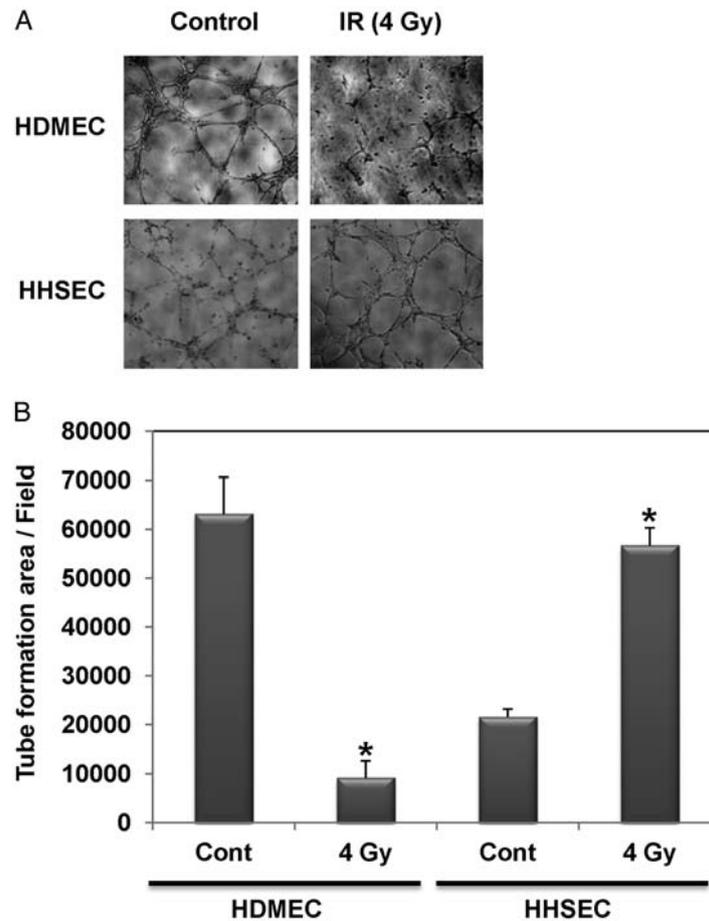


Fig. 2. Effect of ionizing radiation on the capillary-like tube formation by HDMEC and HHSEC. HDMEC and HHSEC were incubated on Matrigel for 6 h after irradiation with 4 Gy. (A) Photographs of the formed tubes. (B) Quantification of tube formation of HDMEC and HHSEC. Results from three independent experiments are expressed as means \pm SEM (*, $P < 0.05$). Cont = control.

Differential modulation of the expression of angiogenic and angiostatic factors in radio-resistant and radio-sensitive cell lines by ionizing radiation

We next examined the effects of ionizing radiation on the expression levels of pro- and anti-angiogenic factors in HDMECs and HHSECs using RT-PCR. As shown in Fig. 3A, MMP-2 mRNA levels in HHSECs after 4-Gy irradiation increased for 48 h. Although 4-Gy irradiation also slightly increased MMP-2 mRNA levels in HDMECs, the extent of the increase in MMP-2 expression was significantly lower in HDMECs than in HHSECs (Fig. 3A). The 4-Gy irradiation did not alter MMP-9 mRNA expression levels in HDMECs or HHSECs (Fig. 3A). Tissue inhibitor of metalloproteinases (TIMP) 1 and 2 mRNA levels were also unaltered in HDMECs and HHSECs following exposure to 4-Gy irradiation (Fig. 3A). Vascular endothelial growth factor receptor 2 (VEGFR-2) mRNA expression levels were

significantly increased in HHSECs by 4-Gy irradiation (Fig. 3A). Although 4-Gy irradiation also slightly increased VEGFR-2 mRNA levels in HDMECs, the extent of the increase in VEGFR-2 expression was substantially lower in HDMECs than in HHSECs (Fig. 3A). Although the basal mRNA expression level of plasminogen, the precursor of angiostatin, was much lower in HDMECs than in HHSECs before irradiation, the plasminogen level in HDMECs was markedly increased by 4-Gy irradiation, whereas that in HHSECs gradually decreased after irradiation (Fig. 3A). We then investigated the effect of ionizing radiation on the activity of MMP-2 and -9 in both HDMECs and HHSECs using zymography. Although the enzymatic activity of MMP-2 increased in a time-dependent manner after 4-Gy irradiation in both HDMECs and HHSECs, the increase was much greater in HHSECs than in HDMECs (Fig. 3B). The enzyme activity of MMP-9 was negligible before and after irradiation in both HDMECs and HHSECs (Fig. 3B). Proteolytic

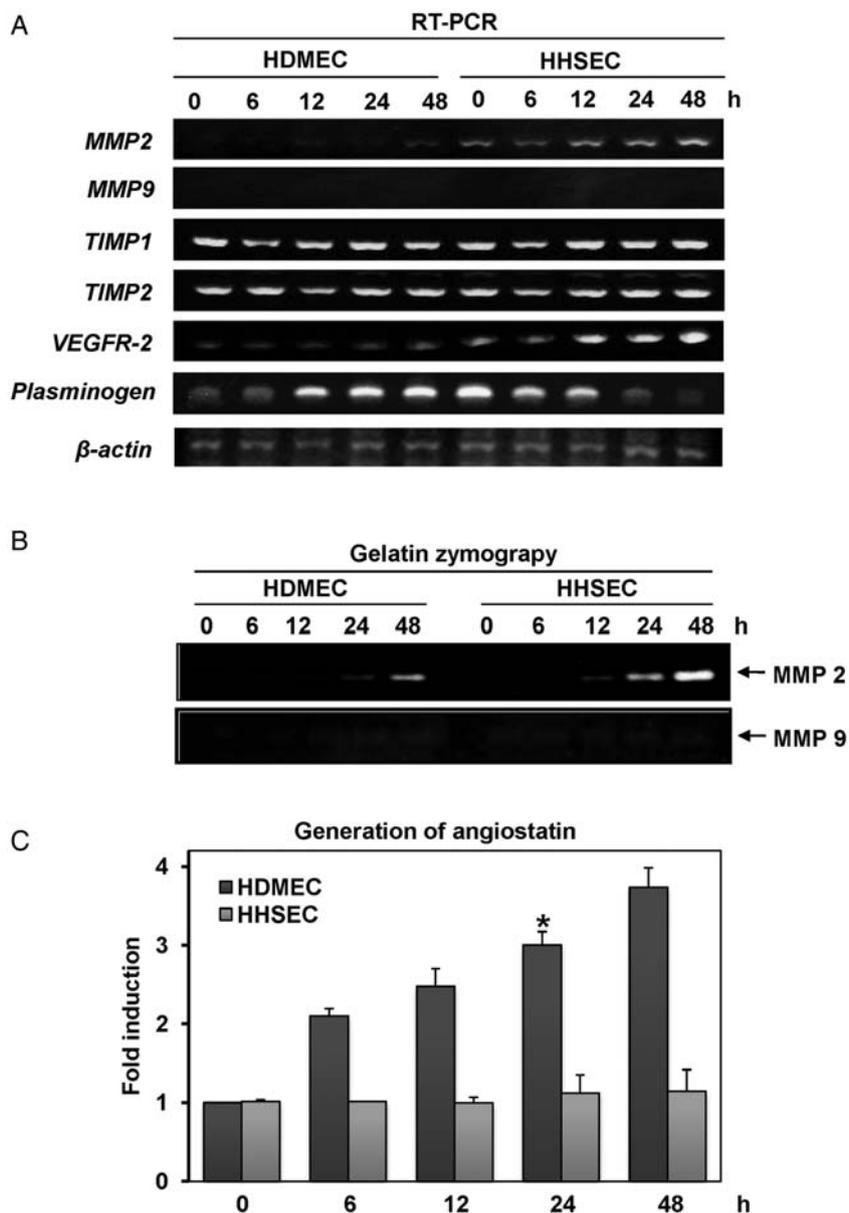


Fig. 3. Ionizing radiation induces different angiogenic factors in HDMEC and HHSEC. (A) HDMEC and HHSEC were irradiated with 4 Gy, and RT-PCR was used to assess the mRNA expression of *MMP-2*, *MMP-9*, *TIMP1*, *TIMP2*, *VEGFR-2*, *plasminogen* and *β-actin*. Representative results of three repeated experiment are shown. (B) HDMEC and HHSEC were irradiated with 4 Gy for the indicated times, and zymography was used to analyze the activities of MMP-2 and MMP-9. The experiments were repeated in triplicate and the data are representative of a typical experiment. (C) HDMEC and HHSEC were irradiated with 4 Gy, and ELISA was used to analyze the generation of angiostatin. The results from three independent experiments are expressed as means \pm SEM (*, $P < 0.05$).

cleavage of plasminogen by several enzymes, including MMP-2 and -9, has been previously reported to generate angiostatin, which has a potent anti-angiogenic effect [10]. Therefore, we measured angiostatin generation in irradiated HDMECs and HHSECs. As shown in Fig. 3C, the levels of angiostatin in the culture media of HDMECs significantly

increased after 4-Gy irradiation, whereas angiostatin levels in HHSECs remained unchanged after 4-Gy irradiation. These results indicate that increased plasminogen expression in HDMECs, and up-regulation of MMP-2 and VEGFR-2 in HHSECs may have crucial roles in anti- and pro-angiogenic responses, respectively, to ionizing radiation.

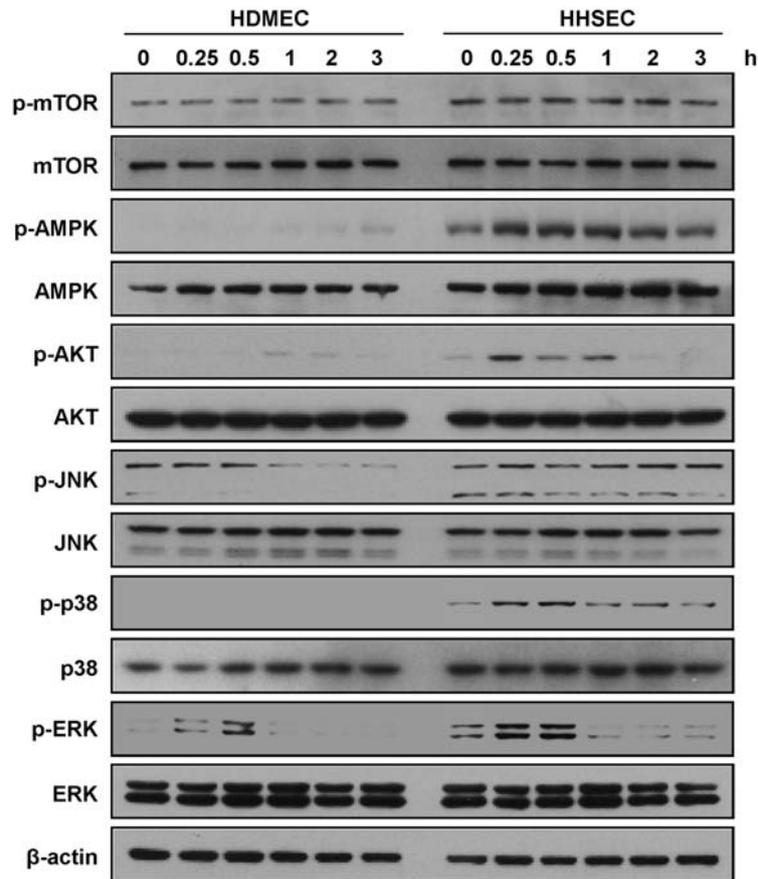


Fig. 4. Ionizing radiation activates different signaling pathways in HDMEC and HHSEC. HDMEC and HHSEC were irradiated with 4 Gy, and cell lysates were subjected to western blot analysis for the indicated proteins at the indicated times. The data represent the results of a typical experiment conducted three times with similar results.

Effects of ionizing radiation on the activity of signaling pathways involved in angiogenesis by HDMECs and HHSECs

mTOR, AMPK, AKT and MAPKs have been shown to play important roles in pro- and anti-angiogenic responses to diverse stimuli [15, 16]. We thus determined the potential involvement of these proteins in the angiogenic responses of HDMECs and HHSECs to ionizing radiation. As shown in Fig. 4, the level of phosphorylated mTOR remained unchanged for 3 h after 4-Gy irradiation in both HDMECs and HHSECs. The levels of phosphorylated forms of AMPK, AKT and p38 MAPK increased after 4-Gy irradiation in HHSECs, but not in HDMECs. Phosphorylated JNK levels in HDMECs decreased after irradiation, but were unaltered in HHSECs (Fig. 4). The basal phosphorylation status of ERK was much higher in HHSECs than in HDMECs before irradiation, but in both cells, the levels of phosphorylated ERK increased for 30 min and then decreased within 1 h after 4-Gy

irradiation. There was no change in total protein levels of mTOR, AMPK, AKT, JNK, p38 MAPK or ERK. These results indicate that ionizing radiation differentially activates angiogenic signaling pathways in HDMECs and HHSECs.

The effect of radiation-induced ERK activation on the generation of angiostatin in HDMEC

To examine the potential involvement of ERK in the anti-angiogenic response of HDMECs to ionizing radiation, we pretreated HDMECs with PD98059, a MEK/ERK inhibitor prior to 4-Gy irradiation. Pretreatment with PD98059 efficiently attenuated the irradiation-induced suppression of tube formation by HDMECs (Fig. 5A and B). Moreover, inhibition of ERK activity with PD98059 effectively blocked the irradiation-induced increase in angiostatin generation (Fig. 5C). These results strongly suggested that the ERK pathway plays an active role in angiostatin generation, leading to suppression of radiation-induced angiogenesis.

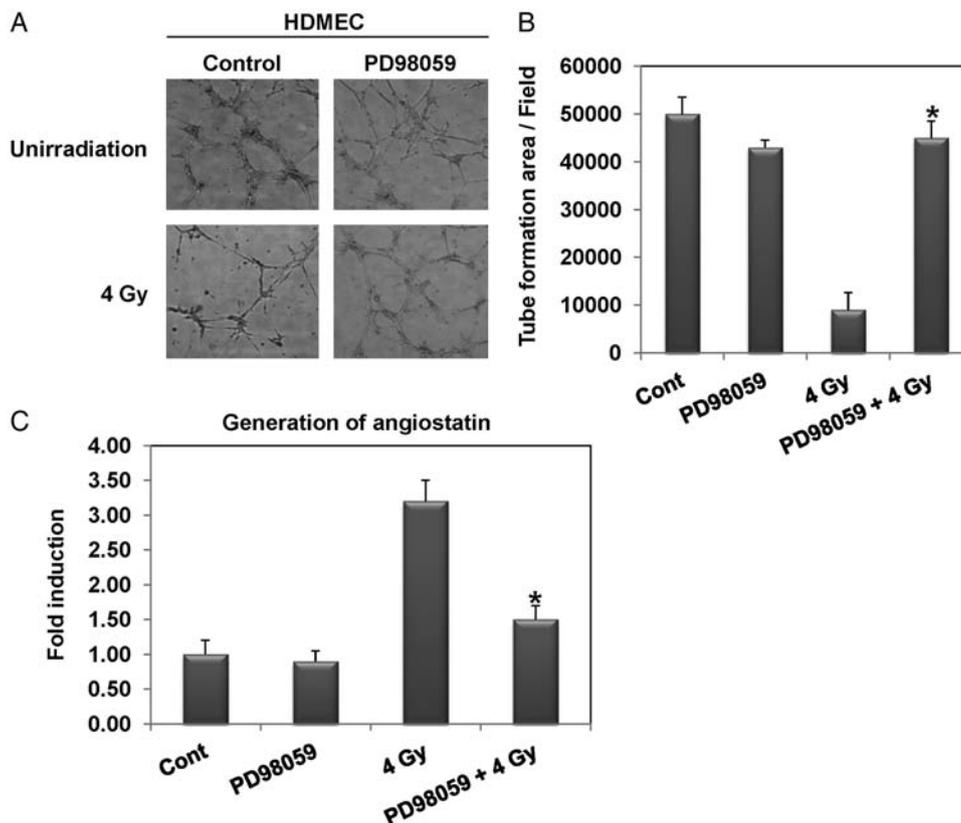


Fig. 5. The activation of ERK is necessary for the angiostatin-mediated suppression of tube formation by HDMEC. (A) HDMECs were incubated on Matrigel for 6 h after irradiation with 4 Gy in the presence or absence of 5 μ M PD98059. (B) Quantification of tube formation by HDMEC. The results from three independent experiments are expressed as means \pm SEM (*, $P < 0.05$). (C) HDMECs were irradiated with 4 Gy for 24 h in the presence or absence of 5 μ M PD98059, and ELISA was used to analyze the generation of angiostatin. The results from three independent experiments are expressed as means \pm SEM (*, $P < 0.05$). Cont = control.

The role of AMPK and p38 MAPK in the radiation-induced increase in HHSEC angiogenic capacity

To investigate the potential involvement of AMPK, AKT, ERK and p38 MAPK in pro-angiogenic response of HHSECs to ionizing radiation, we pretreated HHSECs with Compound C, LY204002, PD98059 and SB203580, specific inhibitors of AMPK, PI3K/AKT, MEK/ERK and p38 MAPK, respectively, prior to 4-Gy irradiation. Pretreatment with either Compound C or SB203580 efficiently attenuated the radiation-induced increase in capillary-like tube formation and expression of both MMP-2 and VEGFR-2 in HHSECs, whereas pretreatment with LY204002 or PD98059 had no effect on these events in HHSECs (Fig. 6A, B and C). These results strongly implicate that both AMPK and p38 MAPK pathways are involved in promoting the radiation-induced angiogenic behavior of HHSECs via up-regulation of MMP-2 and VEGFR-2.

DISCUSSION

The radiation-induced morphological and pathophysiological changes in blood vessels in normal tissues have been extensively investigated [4, 17, 18]. The effect of radiation on the endothelial cells *in vitro* has also been reported [19–22]. However, little is known about the radiobiological characteristics of endothelial cells of normal tissues due mainly to the technical difficulty of isolating endothelial cells from normal tissues. In the present study, we compared the radio-sensitivity of endothelial cells obtained from diverse normal tissues and investigated radiation-induced changes in the molecular signaling pathways involved in the angiogenic activities of these cells. We found that HHSECs and HDMECs are the most radio-resistant and radio-sensitive, respectively, compared with other endothelial cells tested. Moreover, we showed that ionizing radiation promotes capillary-like tube formation by

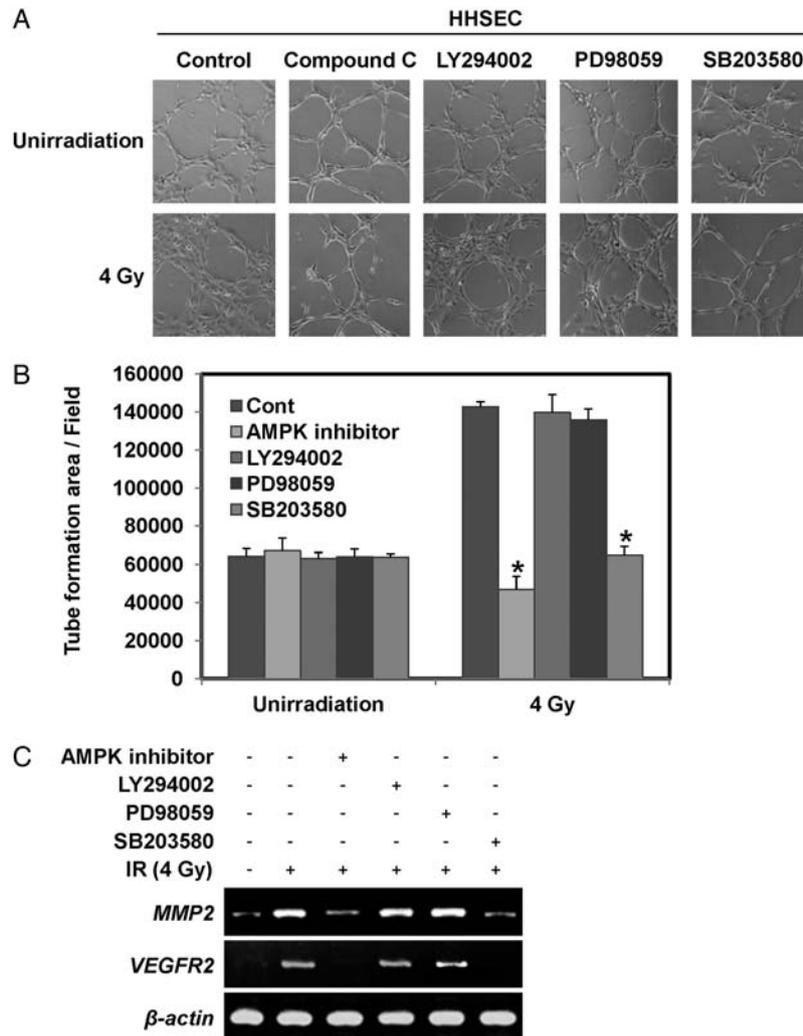


Fig. 6. AMPK and p38 MAPK activations are required for ionizing radiation-induced tube formation by HHSEC. (A) HHSECs were incubated on Matrigel for 6 h after irradiation with 4 Gy in the presence or absence of 5 μ M Compound C (AMPK inhibitor), 5 μ M LY294002 (PI3K/AKT inhibitor), 5 μ M PD98059 (MEK/ERK inhibitor) or 5 μ M SB203580 (p38 MAPK inhibitor). (B) Quantification of tube formation by HHSEC. The results from three independent experiments are expressed as means \pm SEM (*, $P < 0.05$). (C) HHSECs were irradiated with 4 Gy for 24 h in the presence or absence of 5 μ M Compound C (AMPK inhibitor), 5 μ M LY294002 (PI3K/AKT inhibitor), 5 μ M PD98059 (MEK/ERK inhibitor) or 5 μ M SB203580 (p38 MAPK inhibitor), and RT-PCR was used to assess the expression of *MMP-2*, *VEGFR-2* and β -actin. The data are representative of the results from a typical experiment conducted three times.

HHSECs but markedly suppresses that by HDMECs. We further found that AMPK and p38 MAPK-mediated up-regulation of MMP-2 and VEGFR-2 is involved in the promotion of tube formation by irradiated HHSECs, and that ERK activation is involved in the angiotensin-mediated suppression of tube formation by irradiated HDMECs.

Ionizing radiation inhibits angiogenesis of endothelial cells and also induces endothelial cell death [3]. Clonogenic survival assays revealed differences in the relative sensitivity of endothelial cells to radiation-induced cell

death, showing that HHSECs were the most radio-resistant and HDMECs were the most radio-sensitive of the endothelial cells studied (Fig. 1). Moreover, it has been previously shown that VEGF-mediated angiogenesis by HDMECs is effectively blocked by irradiation [23]. In agreement with these reports, tube formation by HDMECs was effectively suppressed by 4-Gy irradiation; in contrast, 4-Gy irradiation promoted tube formation by the radiation-resistant HHSECs (Fig. 2). This result is similar to a recent report that ionizing radiation caused the formation of capillary-like tubes by

HUVECs [24]. It has been reported that the process of angiogenesis in liver disease is associated with the development of fibrosis [25]. Given that ionizing radiation causes liver fibrosis [26], there is a possibility that ionizing radiation-induced angiogenesis by HHSECs may be involved in the radiation-induced liver damage. Additionally, ionizing radiation is known to induce the expression of diverse genes that play key roles in cell growth and immune responses, including interleukin-6 (IL-6), basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF), in various types of normal endothelial cell lines [27, 28]. Because these proteins are well known angiogenic factors and are thought to play important roles in modulating the radiation response via paracrine and/or autocrine mechanisms, it would be reasonable to assume that irradiated HHSECs may participate in angiogenic processes by secreting these and other angiogenic factors.

MMPs participate in both angiogenesis and vasculogenesis by increasing the expression and secretion of angiogenic growth factors such as VEGF [5–7]. Here, we found that ionizing radiation increased the expression and secretion of MMP-2 to a significantly greater degree in HHSECs than in HDMECs (Fig. 3). VEGFR-2 mRNA expression levels were also increased in irradiated HHSECs, but were unchanged in irradiated HDMECs (Fig. 3).

Angiostatin is a proteolytic fragment of plasminogen (comprising kringles 1–4) that functions as a potent endogenous inhibitor of angiogenesis [10]. It acts by competitively inhibiting a pro-angiogenic factor that serves as a mitogen for endothelial cells [12], and also inhibits the proliferation and migration of endothelial cells [29]. In agreement with these previous reports, we found that ionizing radiation significantly increased the expression of plasminogen and the generation of angiostatin in HDMECs (Fig. 3), suggesting that irradiation-induced angiostatin generation is involved in suppressing the angiogenic properties of HDMECs via autocrine regulation. It has been previously demonstrated that angiostatin exerts a disruptive influence on endothelial cells by binding to ATP synthase and inhibiting its activity on the endothelial cell surface, thereby interrupting ATP synthesis on the cell membrane [30]. In addition, extracellular ATP generated by ATP synthase on endothelial cells has been strongly implicated in the proliferation of endothelial cells [31]. Therefore, as angiostatin is likely to exert an anti-angiogenic effect on HDMECs via binding to cell surface ATP synthase following irradiation, future studies designed to investigate the expression of ATP synthase in HDMECs and its potential participation in the ionizing radiation-induced suppression of their angiogenic properties are warranted. Angiostatin has been reported to potentiate the effects of irradiation on various types of cancer [32]. However, little is known about whether and how irradiation contributes to the generation of angiostatin

by endothelial cells. In the present study, we observed that irradiation-induced activation of ERK was crucial for the generation of angiostatin by HDMECs (Figs 4 and 5). Although direct involvement of ERK activation in the generation of angiostatin has not been reported, previous studies have indicated that the activation of ERK participates in the expression of interleukin-6 (IL-6), which is known to contribute to up-regulation of plasminogen [33]. Given that irradiation induces the expression of IL-6 in human endothelial cells [34], we speculate that the irradiation-induced activation of ERK in HDMECs up-regulates IL-6, thereby increasing the expression of plasminogen, which can be converted to angiostatin via proteolytic cleavage. Further studies will be needed to provide support for the potential involvement of IL-6 in the irradiation-induced generation of angiostatin in HDMECs. Recently, inhibition of JNK activity was reported to suppress the capillary-like tube formation by HDMECs [35]. Consistent with this report, our results indicated that ionizing radiation decreased the levels of phosphorylated JNK in HDMECs (Fig. 4). Therefore, it can be assumed that decreased JNK activity caused by ionizing radiation also contributes to the decreased angiogenic capacity of irradiated HDMECs.

AMPK in endothelial cells has been implicated in the regulation of fatty acid oxidation, small G protein activity and nitric oxide production in relation to inflammation and angiogenesis [15]. Furthermore, AMPK activation is reported to be involved in the angiogenic process [36]. Recently, it has been demonstrated that ionizing radiation also induces the activation of AMPK which appears to participate in a signaling pathway triggered by irradiation, leading to G2/M cell cycle arrest in human cancer cells [37]. Consistent with these observations, we found that ionizing radiation rapidly activated AMPK in HHSECs, and inhibition of AMPK with Compound C effectively blocked ionizing radiation-induced capillary-like tube formation and expressions of MMP-2 and VEGFR-2 in HHSECs. Collectively, these results indicate that AMPK is a crucial regulator of ionizing radiation-induced angiogenesis in HHSECs (Figs 4 and 6). Although p38 MAPK has often been implicated in endothelial cell death and anti-angiogenic signaling pathways [38], some studies have found that p38 MAPK plays a positive role in activation of angiogenic signaling pathways [39]. In our study, ionizing radiation activated p38 MAPK in HHSECs, and the activation of p38 MAPK was strongly associated with ionizing radiation-induced angiogenesis by HHSECs (Figs 4 and 6). Pretreatment with the p38 MAPK inhibitor SB203580, like pretreatment with Compound C, also effectively attenuated ionizing radiation-induced capillary-like tube formation and expression of MMP-2 and VEGFR-2 in HHSECs, indicating that p38 MAPK signaling has a key role in these events in HHSECs (Fig. 6). It has been previously reported that

AMPK is an upstream activator of p38 MAPK in metabolic, inflammatory and angiogenic processes [40–42]. In addition, both AMPK and p38 MAPK are known to activate nuclear factor κ B (NF κ B), which leads to transcriptional up-regulation of MMP-2 and VEGFR-2 [15, 43, 44]. Further studies will be needed to define the crosstalk between AMPK and p38 MAPK, and establish the potential role of AMPK and p38 MAPK in ionizing radiation-induced expression of MMP-2 and VEGFR-2 in HHSECs.

It is well known that AKT is a positive regulator of mTOR, which plays an important role in cell survival and proliferation [16]. Notably, although activation of AKT lasted for 1 h after irradiation in HHSECs, we did not observe the activation of mTOR, a downstream target of AKT in HHSECs (Fig. 4). Previous studies indicated that AMPK is a potent negative regulator of mTOR [15]. Consistent with these findings, we observed that AMPK was significantly activated after irradiation in HHSECs (Fig. 4). Therefore, it is reasonable to assume that activation of AMPK was responsible for suppressing AKT-mediated mTOR activation in irradiated HHSECs.

In conclusion, we demonstrated that HHSECs are the most radio-resistant and HDMECs are the most radiosensitive endothelial cells among the established endothelial cell lines tested. Irradiation promoted capillary-like tube formation by HHSECs via AMPK and p38 MAPK-mediated expression of MMP-2 and VEGFR-2, but suppressed capillary-like formation by HDMECs via ERK-mediated generation of angiostatin.

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