Exogenously-added copper/zinc superoxide dismutase rescues damage of endothelial cells from lethal irradiation

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The vascular endothelium is important for the early and late effects observed in lethally irradiated tissue and organs. We examined the effects of exogenously added superoxide dismutase on cell survival and angiogenesis in lethally irradiated human primary umbilical vein endothelial cells. Cell survival was significantly improved in superoxide dismutase-treated cells; the addition of superoxide dismutase to cells after irradiation was also effective for increased survival, as it was before irradiation. Moreover, treatment of cells with superoxide dismutase enhanced the phosphorylation of mitogen-activated protein/extracellular signal-regulated kinase/extracellular signal regulated kinases 1 and 2 in human primary umbilical vein endothelial cells. The addition of superoxide dismutase to cells after irradiation attenuated the reduction of angiogenesis by irradiation, and inhibition of the mitogen-activated protein/extracellular signal-regulated kinase/extracellular signal regulated kinases signaling pathway abrogated the rescue effect of superoxide dismutase. Our results suggest that superoxide dismutase rescues human primary umbilical vein endothelial cells from endothelial dysfunction caused by irradiation via a pathway requiring activation of mitogen-activated protein/extracellular signal-regulated kinase/extracellular signal regulated kinases 1 and 2.

Key Words: SOD, irradiation, angiogenesis, survival

Damage to blood vessels is one of the most common effects of therapeutic irradiation or accidental exposure of normal tissue. Accumulating evidence suggests that the role of vascular endothelial dysfunction is crucial for the pathogenesis of early and also delayed irradiation toxicity in organs and tissues, although there is still debate as to whether vascular endothelial cells have primary involvement in gastrointestinal syndrome caused by high-dose irradiation.2-4 When endothelial cells are exposed to irradiation, cell death, including apoptosis, and the production of pro-inflammatory molecules occur, leading to early structural changes and increased permeability of blood vessels. In addition to this early response, irradiation also causes slow progressive changes, including prothrombic and procoagulant effects, and hypertrophy of the vascular wall, resulting in altered blood flow.5,6 The above experimental data are supported by experiences of radiation therapy and accidental irradiation exposure. Recent studies have shown that the cross-talk between endothelial cells and vascular smooth muscle cells can initiate molecular mechanisms involved in irradiation-induced vascular damage.7,8 Moreover, studies have reported that many agents, such as lovastatin, sphingosine-1-phosphate, pentoxifylline, as well as inhibitors of angiotsin-converting enzyme, are experimentally effective for the treatment of radiation injury in multiple organs or tissues including endothelial cells.7,8 However, an effective treatment of irradiation-damaged endothelial cells has not yet been established.

Superoxide dismutases (SODs) are enzymes that catalyze the conversion of superoxide ($O_2^-$) to hydrogen peroxide (H2O2) and act as key cellular defense systems against oxidant stress. Eukaryotic cells contain three types of SODs. Manganese superoxide dismutase (MnSOD) is localized to the matrix of mitochondria, and its expression is subjected to intense modulation by various stimuli including irradiation.9 Copper/Zinc SOD (Cu/ZnSOD) is located in cytosol and is constitutively expressed. Therefore, Cu/ZnSOD is considered to be a house-keeping enzyme. The third SOD, extracellular SOD (ECSOD), is found in extracellular space and is thought to be important for removing membrane-related oxidase-generated O2-9. In spite of these apparent advantages, however, technical reasons have limited the use of SOD as a drug like other proteins, even in the laboratory, and rapid renal clearance, slow extravasation due to the molecular radius, and charge density are factors affecting the pharmacodynamics and pharmacokinetics of enzymes used as drugs.10 Experimental attempts at modifying SOD proteins to improve these properties have been made, including the delivery of SOD by liposomes in animals.11,12 Recently, a synthetic compound termed Eukarion (EUK) containing SOD, catalase and peroxidase activities has been shown to mitigate apoptosis of endothelial cells.13 In addition, clinical trials of SOD have been carried out, including for bladder inflammation due to irradiation, and for successful renal transplantation.14 However, assessment of SOD for clinical use has been slow, since the mechanisms of SOD in mitigation or treatment activity are not yet fully understood in irradiated tissues and organs.

The central core of Cu/ZnSOD polypeptide is homologous to ECSOD, which is secreted into the extracellular environment. Therefore, ECSOD is referred to as extracellular Cu/ZnSOD.14 Cu/ZnSOD is quite resistant to physical or chemical denaturation. We examined the effects of exogenously-added Cu/ZnSOD on cell growth and angiogenic activity using human umbilical vein endothelial cells (HUVECs). The mitogen-activated protein kinase (MAPK) pathway is a key component in the transduction of signaling leading to cell survival or death; activation of the mitogen-activated protein/extracellular signal-regulated kinase/extracellular signal regulated kinases 1 and 2 (MEK/ERK1/2) pathway is closely linked to irradiation-induced apoptosis in endothelial cells.15,16 We also determined whether the activation

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of MEK/ERK1/2 was involved in the mechanism of the Cu/ZnSOD-mediated effect on irradiated endothelial cells.

Materials and Methods

Cells and cell culture. HUVECs (Cambrex BioScience Walkersville, Inc., Walkersville, MD) were cultured in the manufacturer’s growth medium (EGM®-2 Bullet Kit®, Cambrex) in a humidified atmosphere of 5% CO₂ and 95% air at 37°C. Twenty-four hours before the experiments, HUVECs were subcultured into new plates with fresh EGM-2 medium without vascular endothelial growth factor (VEGF), fibroblast growth factor-β, or hydrocortisone. HUVECs were irradiated by Cesium-137 (Cs) gammator (model M Gammator, Irradiation Machinery, Parsippany, NJ) at a dose rate of 8.0 Gy/min on a rotating platform.

Reagents. Superoxide dismutase from bovine erythrocytes, Cu/Zn type (Cu/Zn SOD), was obtained from Wako Pure Chem. Ind. (Osaka, Japan). Specific activity was 2100 units/mg protein. MEK inhibitor PD98059, p38 MAPK inhibitor SB203580, and PI3K inhibitor LY294002 were from Calbiochem-Novabiochem Corp. (San Diego, CA).

Surviving fraction. For the determination of survival after irradiation, the cell monolayer was irradiated in 25-cm² culture flasks in the exponential growth phase, before cells reached 70% confluence. One culture flask per dose was irradiated with doses ranging from 1 to 6 Gy. Immediately after irradiation, the cells were rinsed, trypsinized and seeded in culture dishes at low density, with 5 replications per dose. After incubation for 13 days, the colonies were fixed and stained with Giemsa. Colonies of more than 50 cells were scored as survivors.

Matrigel tube formation assay. Matrigel™ (BD Matrigel Matrix, BD Biosciences Discovery Labware, Bedford, MA) was thawed at 4°C, and 50 µL of growth factor-reduced Matrigel was layered onto each well of a 96-well plate. Matrigel was allowed to solidify at 37°C for 30 min. HUVECs were irradiated with γ-ray, treated with trypsin/EDTA after irradiation, and resuspended in EGM-2 medium at 1 × 10⁵ cells/ml. Fifty micro-liters of the cell suspension (5000 cells of HUVECs) was seeded onto presolidified Matrigel of each well, and 50 µL of 2-fold concentrated SOD was added. After incubation for 16 h, the formation of capillary-like structures was examined microscopically. Tube-like formation was scored for each well by counting the number of lumen structures within four randomly selected view fields of the microscope (40×), and photos were taken. Tube formation in the presence of SOD and/or irradiation was scored and compared to tube formation in media alone; the tube-forming capacity of HUVECs media alone was assumed to be 100%.

Western blot analysis. HUVECs were lysed in ice-cold buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 3 mM Na₂S, 0.1% (w/v) SOD, 1% (v/v) NP-40 and 0.5% sodium deoxycholate. The protein concentration of each sample was measured. Ninety micro-grams of protein from the total extract was separated in 10% SDS polyacrylamide gel. After wet blotting to PVDF and blocking of non-specific binding (5% dry milk in TBS/Tween 20; 1 h at room temperature), filters were incubated for overnight at 4°C with the corresponding primary antibody diluted to 1:1000 in 5% dry milk in TBS/0.1% Tween 20. After washing and incubating with secondary peroxidase-coupled anti-rabbit or anti-mouse antibody (1:5000), proteins were visualized by autoradiography using ECL (Amersham Biosciences, Boston, MA).

Statistical analysis. All values were presented as mean ± SD or SE as indicated. Data were analyzed using the Student’s t test (Ekuseru-Tokei® 2003 software, Social Survey Research Information Co., Ltd., Tokyo, Japan). Differences were considered significant at p<0.05.

Results

Improved survival of irradiated HUVECs by Cu/ZnSOD. To determine the effect of SOD in irradiated HUVECs, clonogenic survival analysis was performed. Cells were treated with 50 units/ml of SOD for 1 h and then irradiated at a dose of 0, 1, 2, 4, or 6 Gy. Cells were plated and cultured for 13 days. The clonogenic survival results after irradiation with γ-rays are shown in Fig. 1. Plating efficiency was 0.027 ± 0.005 in untreated control cells and 0.027 ± 0.004 SOD-treated cells, showing no statistically significant difference. Pretreatment with SOD significantly increased the survival of these cells irradiated with 1 Gy (p = 0.0109, Table 1). HUVECs cultured with SOD before irradiation also had significantly increased survival fractions as compared to cells without SOD at higher doses of irradiation (Table 1). Upon irradiation with 6 Gy, survival was 17 times higher in SOD-treated cells compared to control cells (p = 0.0061).

In parallel, we also studied the effects of SOD treatment after irradiation on survival fractions in these cells. Cells were irradiated and 50 units/ml of SOD was added to cell cultures 30 min later. The addition of SOD after irradiation also increased radiation resistance significantly at each of the doses used. However, pre-irradiation SOD-treatment of cells was more effective for improving survival fractions compared to post-irradiation at a dose of 4 or 6 Gy. Addition of SOD to cell culture did not affect the pH level of culture supernatants in the present study (data not shown).

Effects of Cu/Zn SOD on tube forming capacity in irradiated HUVECs. Since treatment with SOD even after irradiation improved the survival rate of these HUVECs, we determined whether SOD affects angiogenesis in irradiated cells. Endothelial
cells plated on Matrigel undergo attachment, migration, and differentiation events to form interconnected networks of endothelial cell-lined tubes, recapitulating the angiogenic process. Thus, tube formation is an important mechanism in angiogenesis. We first examined the effects of SOD on the ability of endothelial cells to form tubular structures. Cells were cultured with SOD for 16 h (Fig. 2A). The endothelial cells plated on Matrigel formed abundant networks of branching and organized to lumen-like structures with multicentric anastomoses. For quantification of tube formation, the numbers of endotubes were counted in the microscopic fields (Fig. 2B). When these HUVECs were plated onto Matrigel covered plates in the presence of SOD, tube formation was significantly increased. Treatment of cells with as low as 1 unit/ml SOD resulted in about 40% increase in capability of tube formation (compared to control: \( p = 0.0164 \)), with tube formation reaching a maximum in cells treated with 5 units/ml of SOD (compared to control: \( p = 0.0456 \)). The capability of tube formation was reduced thereafter, and no significant effects on tube formation capability were observed in cells treated with 50 or 100 units/ml of SOD.

To determine whether SOD could affect tube formation with radiation, HUVECs were irradiated at a dose of 20 Gy and then plated onto Matrigel with or without SOD. The numbers of endotubes were counted after 16 h. Exposure to irradiation with 20 Gy resulted in almost 75% reduction of the capability of tube formation compared to that of control. On the other hand, SOD attenuated the reduction of the tube-forming capability by irradiation. Treatment of HUVECs with 5 units/ml of SOD increased the capability by more than 2-fold compared to that of cells only irradiated (\( p = 0.0198 \)). However, there was no significant difference in the capability of tube formation among the cell groups receiving 5, 50, and 100 units/ml of SOD, whereas cells treated with 50 or 100 units/ml of SOD also had a significantly higher capability than cells exposed to irradiation alone (irradiation alone vs 50 units/ml: \( p = 0.0139 \), irradiation alone vs 100 units/ml: \( p = 0.0399 \)).

### Role of MEK pathway for rescue effects of SOD on angiogenesis in irradiated HUVECs

To examine whether the MEK signal pathway is involved in the rescue effects of SOD on tube formation, PD98059, an inhibitor of the MEK pathway, was used. PD98059 is a cell-permeable inhibitor of MEK that inhibits the activation of MAPK and the subsequent phosphorylation of MAPK kinase substrates. Cells were cultured with 10 \( \mu \)M of PD98059 for 1 h and then irradiated with 20 Gy. After irradiation, cells were plated onto Matrigel including 5 units/ml of SOD (Table 2). Treatment with PD98059 reduced the effect of SOD on angiogenesis by 44.1 \( \pm \) 6.7% in HUVECs (\( p = 0.0197 \)) cultured with SOD alone (PD98059 vs SOD alone: \( p = 0.0079 \), PD98059 vs SOD + irradiation: \( p = 0.0004 \), PD98059 + irradiation vs SOD + irradiation: \( p = 0.0197 \)).

### Table 1. \( p \) values from comparison of surviving fractions among the three cell groups

| Dose (Gy) | Control vs Before-SOD | Control vs After-SOD | Before- vs After-SOD |
|----------|-----------------------|----------------------|---------------------|
| 1        | 0.0109                | 0.0017               | 0.1027 (ns)         |
| 2        | 0.0013                | 0.0005               | 0.1810 (ns)         |
| 4        | 0.0003                | 0.0007               | 0.0031              |
| 6        | 0.0061                | 0.0019               | 0.0237              |

Statistical analysis was performed for results presented in Fig. 1 and Table 1 as described in "Materials and Methods". ns: not significant.

### Table 2. Effect of MEK/ERK pathway inhibition on angiogenesis by SOD in irradiated HUVECs

|          | Untreated control | Irradiation | PD98059 | Irradiation + SOD | PD + Irradiation + SOD |
|----------|-------------------|-------------|---------|-------------------|------------------------|
| (%)      | 100.0 \( \pm \) 25.8** | 38.7 \( \pm \) 5.6*** | 95.7 \( \pm \) 9.7** | 65.6 \( \pm \) 10.4**** | 44.1 \( \pm \) 6.7**** |

Cells were cultured with 10 \( \mu \)M of PD98059 for 1 h and then irradiated with 20 Gy. After irradiation, cells were plated onto Matrigel including 5 units/ml of SOD. Experiments were triplicated and results were expressed as percentage tube-forming capacity of untreated control cells.

\( * p = 0.0079 \), **\( * p = 0.0084 \), ***\( * p = 0.0197 \).
with 5 units/ml of SOD after irradiation, whereas treatment with PD98059 alone failed to affect the capacity significantly as compared to untreated control. We also used highly specific inhibitors of p38MAPK (SB203580) and PI3K (LY294002). SB203580 is a highly selective inhibitor of p38 MAPK that acts by competitively inhibiting ATP binding.\(^{(20)}\) LY294002 specifically inhibits PI3K activity via competitive inhibition of the ATP-binding site located on the p85α subunit of PI3K.\(^{(21)}\) However, these inhibitors did not affect the tube-forming capacity in irradiated HUVECs (data not shown).

To further study the involvement of the MEK pathway in the tube-forming capacity of irradiated cells, western blot analysis was performed using an antibody against phosphorylation of ERK. HUVECs were irradiated with 20 Gy and harvested at different durations (Fig. 3A). A time-course study showed that the levels of phosphorylated ERK were increased by irradiation, reaching a plateau 4 h after irradiation and then gradually decreasing. Cells were also cultured with 5 or 50 units/ml of SOD for different durations (Fig. 3 B and C). We found that SOD markedly increased the levels of phosphorylated ERK at 2 h, and at 6 h they had returned close to baseline. However, significant activation of ERK was not observed 0.5 or 1 h after exposure or the addition of SOD to cells (data not shown).

In a parallel study, SOD was added to HUVECs either 1 h before or 30 min after irradiation with 20 Gy (Fig. 3D). Cells were then cultured for 4 h and harvested for western blot analysis.

Cells treated with SOD after irradiation had almost the same or increased level of the phosphorylated form of ERK compared to that of cells treated with SOD or irradiation alone. Moreover, treatment with PD98059 significantly inhibited phosphorylation of ERK by either irradiation, SOD, or the combinations with SOD and irradiation in these cells.

**Discussion**

Endothelial cells are one of the most critical targets for the pathologic effects of irradiation *in vivo*, and it is known that the vascular endothelium within tissues and organs including lung, brain, and skin is the actual target of radiation damage. Thus, vascular damage is a key mechanism in tissue and organ injuries caused by irradiation. In the present study, we found that exogenously-added Cu/ZnSOD effectively protected and also rescued HUVECs from lethal irradiation, and stimulated angiogenesis in these cells. Furthermore, this effect occurred through a pathway requiring activation of MEK. There are many reports of agents with radioprotective capability. The administration of agents to reduce either the incidence or severity of radiation-induced normal tissue injury is called mitigation or treatment.\(^{(7)}\) However, most of these radioprotective agents are effective only when they are added to cells or administered to experimental animals before irradiation, and the mechanistic basis of mitigation or treatment agents for the experimental successes has not been
established. It is noteworthy that, for the prevention of angiogenesis inhibition from irradiation, the addition of SOD to cells after irradiation is also effective. Thus, the present data suggest the possibility of Cu/ZnSOD as a therapeutic agent for the treatment of blood vessel injuries by irradiation, providing insight into how Cu/ZnSOD mediates angiogenesis in irradiated endothelial cells.

Endothelial cells constitutively produce reactive oxygen species (ROS) that are known to have a very high sensitivity to ROS. This production is augmented by exposure of cells to extra-cellular sources of ROS such as irradiation as well as hypoxia and ischemia. On the other hand, ROS play an important role in angiogenesis in endothelial cells. Many angiogenic responses are mediated through ROS in endothelial cells; angiogenic factors such as VEGF, a major angiogenesis factor, and also angiopoietin-1 (Ang1), are induced through an increase in ROS, and these factors stimulate proliferation and angiogenesis in these cells. Recent studies have shown that endothelial cells overexpressing either MnSOD or Cu/ZnSOD had a higher angiogenic potential dependent on the generation of H$_2$O$_2$. The activity of Cu/ZnSOD depends on copper for its enzymatic activity. Inhibition of Cu/ZnSOD by binding or chelating copper ion decreases angiogenesis, and diet-related depletion of copper has also been shown to inhibit angiogenesis. Thus, SODs, especially Cu/ZnSOD, are important for angiogenesis. In the present study, the addition of low concentrations of Cu/ZnSOD stimulated angiogenesis in control as well as in irradiated cells. Cu/ZnSOD added exogenously to cell culture is known to be incorporated into cells, as prior study has shown that exogenously-added Cu/ZnSOD penetrates the cellular membrane and increases total SOD activity. Further studies reported that exogenously-added Cu/ZnSOD induced neutrophil apoptosis via accumulation of H$_2$O$_2$, and effectively blocked monocytic differentiation of leukemic cells with platelet-activating factor (PAF) and stimulation of neonatal rat hepatocyte growth by tumor promoters. On the other hand, SOD activity is observed in the circulatory system of mammalian cells. ECSOD is a secreted enzyme that requires copper and zinc for enzymatic activity. The ECSOD gene is about 60% homologous with Cu/ZnSOD but possesses extensions at the N- and C-termini. Human ECSOD is related to dimeric Cu/ZnSOD, which is typically produced in vascular smooth muscle cells, and is secreted into the extracellular environment where it binds to the extracellular matrix and endothelial surface components. Thus, Cu/ZnSOD is structurally similar to ECSOD. In contrast, MnSOD is located in the matrix of mitochondria in eukaryotes, and mitochondrial localization is required for protection of cells by MnSOD from irradiation damage. There is no similarity in sequence or structure between Cu/ZnSOD and MnSOD. Exogenously-added Cu/ZnSOD may act in a way similar to ECSOD, whereas an alternative mechanism may involve the suppression of O$_2^-$ generating systems such as NADPH oxidase by extracellular Cu/ZnSOD.

It has been reported that basic fibroblast growth factor (bFGF) and vascular VEGF trigger angiogenic response through a pathway requiring activation of the MEK/ERK pathway, and inhibition of this pathway is known to lead to reduced angiogenesis. Recent studies have also revealed that the MEK/ERK pathway is closely linked to irradiation-induced apoptosis in endothelial cells; bFGF and VEGF have been known to protect these cells from radiation-induced cell death via the MEK/ERK pathway. Thus, activation of the MEK/ERK pathway is a key event for cell function and survival in irradiated endothelial cells. In the present study, treatment with SOD increased cell survival without affecting plating efficiency and stimulated angiogenesis with concomitant activation of ERK1/2 phosphorylation in irradiated HUVECs, and inhibition of the MEK/ERK pathway abrogated the effect of SOD in these cells. These results suggest that activation of the MEK/ERK pathway is closely related to the rescue effect of Cu/ZnSOD on damage caused by irradiation. Our study also shows that the rescue effects of SOD may act through a different mechanism from those of VEGF and bFGF, since these growth factors are known to increase plating efficiency of endothelial cells. Recently, it has become more evident that H$_2$O$_2$ serves an important regulatory role in cell growth/proliferation and signaling pathways. Furthermore, H$_2$O$_2$ has been shown to regulate pro-angiogenic responses in a variety of systems. The accumulation of H$_2$O$_2$ by SOD may trigger the activation of the ERK pathway, leading to the rescue effect in irradiated cells. Moreover, treatment of cells with SOD after lethal irradiation effectively rescued these cells and also increased the capability of tube formation. We also found that the level of the phosphorylated form of ERK1/2 was increased in cells treated with SOD even after irradiation. Recent studies have shown that irradiated cells can exhibit persistent and prolonged generation of ROS that can last from minutes to days. NADPH oxidases are the primary physiological ROS producers, and O$_2^-$ is the first member of ROS generated by NADPH oxidase. Detoxifying the prolonged generation of O$_2^-$ as well as shorter generation may contribute to the rescue effect of SOD in irradiated endothelial cells.

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**Abbreviations**

Ang1 angiogenesis factor, and also angiopoietin-1
Cu/ZnSOD Copper/Zinc SOD
ECSOD extracellular SOD
HUVECs human umbilical vein endothelial cells
H$_2$O$_2$ hydrogen peroxide
ERK1/2 extracellular signal regulated kinases 1 and 2
SODs Superoxide dismutases
MAPK mitogen-activated protein kinase
MEK/ERK1/2 mitogen-activated protein/extracellular signal-regulated
MnSOD Manganese superoxide dismutase
ROS reactive oxygen species
VEGF vascular endothelial growth factor

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