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Normalization by edaravone, a free radical scavenger, of irradiation-reduced endothelial nitric oxide synthase expression

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Abstract

We investigated the therapeutic effect of edaravone, a free radical scavenger, on alterations in endothelium-dependent relaxation and endothelial nitric oxide synthase (eNOS) expression in the rabbit ear central artery at 2 weeks after exposure to a dose of 45 Gy radiation with a cobalt^{60} unit. For treatment with edaravone, edaravone was given daily to the animals from the day before irradiation at an intrapenetreal dose of 10 mg/kg twice a day. The endothelium-dependent relaxant response to acetylcholine was markedly impaired in irradiated vessels. Edaravone treatment improved the response to the level observed in nonirradiated control vessels. Using immunohistochemical and Western blot techniques, we showed that protein expression of eNOS in irradiated vessels was reduced to about 50% of control and that edaravone treatment returned it nearly to intact levels. Gene expression of eNOS, analyzed by reverse transcription-competitive polymerase chain reaction, was found to be reduced from the control level by 47% following irradiation. The reduced level of eNOS mRNA in irradiated vessels was almost completely normalized by edaravone treatment. These results suggest that edaravone has a protective effect on the reduced expression of eNOS and its associated endothelial cell dysfunction in the vessels following irradiation. We thus assume that oxygen-free radicals may be closely related to the irradiation-induced derangement of the eNOS gene regulation.

Keywords: Irradiation; Endothelium-dependent relaxation; endothelial nitric oxide synthase (eNOS); Free radical scavenger; Artery

1. Introduction

Endothelium-derived nitric oxide (NO), produced by endothelial NO synthase (eNOS), plays a central role in the modulation of vascular tone and blood pressure (Fleming and Busse, 1999). It also decreases platelet aggregation and adhesion (Radomiski et al., 1987, 1990), limits vascular smooth muscle proliferation (Garg and Hasid, 1989), inhibits neointima formation (von der Leyen et al., 1995), prevents monocyte chemotaxis (Bath et al., 1991) and inhibits leukocyte adhesion to the endothelium (Kubes et al., 1991). Therefore, the functional consequence of the decline in eNOS activity would contribute to the pathogenesis of the vascular complications of diseases.

There can be little doubt that the vascular system is very sensitive to radiation. This holds true not only for morphological criteria but also for the functional changes in the vascular system (Reinhold et al., 1991). Since vascular endothelial cells appear to absorb a much higher radiation dose than any other target or nontarget tissue in the body, numerous studies have focused on the influence of radiation exposure on endothelial cells and revealed their biochemical and functional alterations following irradiation (Rubin et al., 1984, 1985, 1991; Fajaedo, 1989). In reference with the influence of irradiation on eNOS-dependent endothelial cell function, it has been reported that a single dose of 45 Gy X-irradiation blunts endothelium-dependent relaxant responses to acetylcholine and substance P in rabbit ear artery (Maynard et al., 1992). In a previous publication, we also showed that radiation with a cobalt^{60} unit caused dose- and time-dependent impairment of endothelium-dependent relaxations to acetylcholine and A23187 in rabbit ear artery (Qi et al., 1998). Interestingly, we found that the impaired

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endothelium-dependent relaxations within 4 weeks after irradiation were not accompanied by morphological damage of endothelial cells but were associated with the decrease in eNOS protein expression. Furthermore, our recent work demonstrated a lack of eNOS-mediated relaxations in cervical arteries from the neck region of patients who had radiation therapy (Sugihara et al., 1999). In irradiated human cervical arteries, no expression of eNOS protein was detectable despite of morphologically intact endothelial cells.

It has been postulated that irradiation-induced alterations could result from the generation of reactive oxygen species during radiation exposure which is considered as the most important indirect mechanism of radiation injury (Dubner et al., 1995). Thus, a possible explanation for the impairment of eNOS expression after irradiation may be the enhanced production of oxygen-derived free radicals in the irradiated vessels. Edaravone, a newly developed free radical scavengers for clinical use, has potent-free radical quenching action by trapping a variety of free radical species, especially hydroxyl radicals (Watanabe et al., 1994), and appears to be very effective in preventing cerebral damage in patients with cerebral infarction possibly due to the result of suppression of delayed neuronal death by free radical scavenging (Houkin et al., 1998). In the present study, we examined the effect of edaravone treatment on endothelium-dependent relaxation and eNOS expression in rabbit ear artery following exposure to radiation with a cobalt^{60} unit in order to search the involvement of free radicals in the irradiation-induced endothelial impairment.

2. Materials and methods

2.1. Study design

Male New Zealand white rabbits ($n = 10$ for each of 4 groups) weighing 2 to 3 kg were anesthetized with sodium pentobarbital (25 mg/kg, i.v.). This dose of pentobarbital provided general anesthesia without depression of respiration. The ear of the rabbit was locally irradiated with a dose of 45 Gy according to the method described previously (Qi et al., 1998). Briefly, the ear of the animal was fixed to a flat plate with tape in order to keep the ear horizontally. A cobalt^{60} unit (PCR-120-C_3, Toshiba, Tokyo, Japan) producing gamma rays at 0.87–0.93 Gy/mm and a source-skin-distance of 50 cm was used. The field size was 5×10 cm over the center of the ear. A double layer of wet gauze was placed over the skin to provide dose buildup on the surface of the ear central artery. In that way, the dose delivered to the ear central artery would be 90% and higher of the dose calculated at skin level. Some rabbits were given 10 mg/kg of edaravone i.p. twice a day. Edaravone (Mitsubishi Pharma, Osaka, Japan), 150 mg, was dissolved in 2.5 ml of 1 N NaOH, the pH was adjusted to 7.0 with 1 N HCl, and the total volume was prepared to 50 ml by adding distilled water. Edaravone treatment was begun from the day before irradiation and was continued up to the day before the animals were killed. Rabbits who did not receive radiation served as control subjects, and half of the control animals were treated with edaravone.

All rabbits were housed in fiberglass reinforced plastics (FRP) running-water flushing units (Clea, Tokyo, Japan) at a constant temperature of 23 ± 3 °C, constant humidity ($50 \pm 10\%$) with a daily 12 h light–dark cycle. All animals received care that was in compliance with the institutional guidelines and the experimental procedure was approved by the Hokkaido University School of Medicine Animal Care and Use Committee.

2.2. Organ bath experiments

Two weeks after irradiation, the animals were anesthetized with sodium pentobarbital (60 mg/kg, i.v.) and central ear arteries were dissected from the ears. The arteries were cleaned of adherent fat and surrounding tissues and cut into rings of 4-mm in length. Care was taken to ensure that the endothelial layer was not damaged during tissue preparation. Each ring was suspended by a pair of stainless steel hooks under a resting tension of 2 g in a water-jacketed bath filled with 25 ml of normal physiological salt solution (PSS). The composition of the normal PSS was (in mM): NaCl 118.2, KCl 4.7, $MgCl_2$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25.0 and glucose 10.0. The solution in the bath was gassed with 95% O_2 and 5% CO_2, and its temperature was maintained at 37 °C. Force generation was monitored with an isometric transducer (45196, Sanei-Sokki, Tokyo, Japan) and a carrier amplifier (1236, Sanei-Sokki). The output of the force transducer was registered on a pen recorder (ERP-241A, TOA Electronics, Tokyo, Japan) through a polygraph recorder (142-8, Sanei-Sokki).

Following the equilibration period of at least 60 min, the rings were exposed several times to high K^+ (40 mM) solution until reproducible contractile responses were obtained. High K^+ solution was prepared by replacement of NaCl with equimolar KCl in order to avoid a change in tonicity of the solution. After being rinsed thoroughly with the normal PSS and allowed to recover to the baseline, the rings were precontracted with 3 μM phenylephrine. When the phenylephrine-induced contraction reached a plateau level, the relaxant response to acetylcholine was examined in a cumulative manner. The degree of relaxations was expressed as a percentage of the height of contraction induced by phenylephrine.

2.3. Immunofluorescence staining

For immunohistochemical determination of eNOS, we used antihuman eNOS rabbit polyclonal antibody (Affinity BioReagents, Golden, CT, USA). Arteries were fixed in 10% formalin overnight and then processed routinely for paraffin embedding. The preparations were cut in 5-μm

sections transversely, deparaffinized and treated for 5 min three times with citrate buffer (10 mM citric acid, pH 6.0) in a microwave oven (750 W) before immunostaining. After overnight incubation at 4 °C, with the primary antibody at a dilution of 1:100, the sections were rinsed in phosphate buffer solution and then exposed to the fluorescence secondary antibody, Cy3-conjugated Affini-Pure antirabbit immunoglobulin G (IgG) (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 2 h according to the manufacturer's instructions. The samples processed without the primary antibody served as negative controls. The coverslips were mounted with Immunon (Thermo Shandon, Pittsburgh, PA, USA). Immunofluorescent images were observed under Laser Scanning Confocal Imaging System (MRC-1024, Bio-Rad, Hemel Hempstead, UK).

2.4. Western blot analysis

Ear central arteries were exposed and placed in ice-cold Dulbecco's phosphate-buffered saline (136.9 mM NaCl, 2.7 mM KCl, 8.1 mM NaH_2PO_4, 1.5 mM KH_2PO_4). Fat and connective tissues were trimmed from arteries. Arteries were minced finely with scissors and homogenized in 2 volumes of lysis buffer (1% sodium dodecylsulfate (SDS), 1 mM sodium orthovanadate, 10 mM Tris-HCl, pH 7.4) by means of a polytron. The homogenate was centrifuged at 6000 × *g* for 15 min, and the supernatant was retained. The protein concentration of the supernatant was determined by the method of Lowry et al. (1951), with bovine serum albumin used as standard. The supernatant was stored at − 80 °C until used.

Samples (10 μg) were subjected to an 8% polyacrylamide SDS gel and electroblotted onto a polyvinilidine difluoride filter (PVDF) membrane. The PVDF was washed in Tris-buffered saline (TBS: 500 mM NaCl, 20 mM Tris-HCl, pH 7.5) and was blocked for 90 min at room temperature in TBS containing 1% bovine serum albumin. Thereafter, the PVDF was washed twice in TBS-Tween buffer (500 mM NaCl, 20 mM Tris-HCl, 0.05% Tween 20, pH 7.5) and incubated overnight at 4 °C with antihuman eNOS rabbit polyclonal antibody (Affinity BioReagents) at 1:1000 dilution in TBS-Tween buffer. The PVDF was washed twice in TBS-Tween buffer, and then was incubated with horseradish peroxidase-conjugated antirabbit antibody (Bio-Rad Laboratories, Hercules, CA, USA.) diluted at 1:6000 in TBS-Tween buffer at room temperature for 60 min. After washing with TBS-Tween buffer twice, the blots were visualized with the enhanced chemiluminescence detection system (Amersham, Little Chalfont, Buckinghamshire, UK), exposed to X-ray film, and analyzed by the free software NIH image produced by Wayne Rasband (National Institutes of Health, Bethesda, MD, USA). The intensity of total protein bands per lane was evaluated by densitometry. Negligible loading/transfer variation was observed between samples.

2.5. RNA extraction and reverse transcription-competitive polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from ear arteries by the guanidinium thiocyanate-phenol-chloroform method used routinely in our laboratory (Matsuda et al., 1999). RNA purity was determined by the ratio of optical density (OD) measured at 260 and 280 nm (OD_{260}/OD_{280}), and RNA quantity was estimated at OD_{260}. The concentration of total RNA was adjusted to 1 μg/ml with RNase-free distilled water.

RT-PCR was performed by using primers derived from the human eNOS sequence. The oligonucleotide sequence pair used for gene amplification in this study generated PCR products of expected size that has been sequenced to verify eNOS identity: sense primer, 5′-GCTGGAGTGGTTG-CAGCC-3′ and antisense primer, 5′-CTCCAGGATGTTG-TAGCGGTGA-3′ (175 bp). cDNA was reverse transcribed from 1 μg total RNA according to the manufacturer's instructions. The PCR conditions were 25 cycles of denaturation at 85 °C for 30 s, annealing at 64 °C for 30 s and extension at 72 °C for 30 s. After amplification, the resulting PCR products visualized on 2% agarose gels stained with ethidium bromide. The products were quantified using an Image Analyzer (Bio-Rad Laboratories). To standardize the amount of the target molecule, the amount of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) mRNA, a ubiquitously expressed housekeeping gene was determined using the primer pair (sense, 5′-TGATGACATCAAGAAG-GAGGTGAAG-3′, and antisense, 5′-TCCTTGGAGGC-CATGTGGACCAT-3′; 252 bp).

2.6. Statistical analysis

All data are expressed as means ± S.E.M. Statistical analysis was performed by analysis of variance (ANOVA)

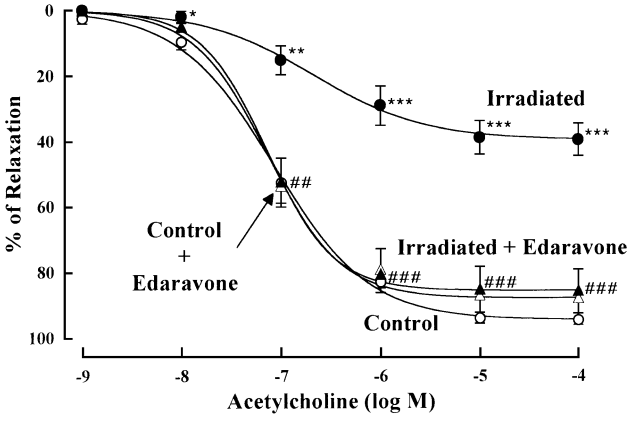

Fig. 1. Concentration–response curves for acetylcholine-induced relaxations in ear arteries from control (○), irradiated (●), edaravone-treated control (△) and edaravone-treated irradiated (▲) rabbits. The responses are expressed as percent relaxation of phenylephrine-induced contraction. Points are means ± S.E. of six to seven experiments. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ vs. the respective control values. $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$ vs. the respective values obtained in irradiated vessels.

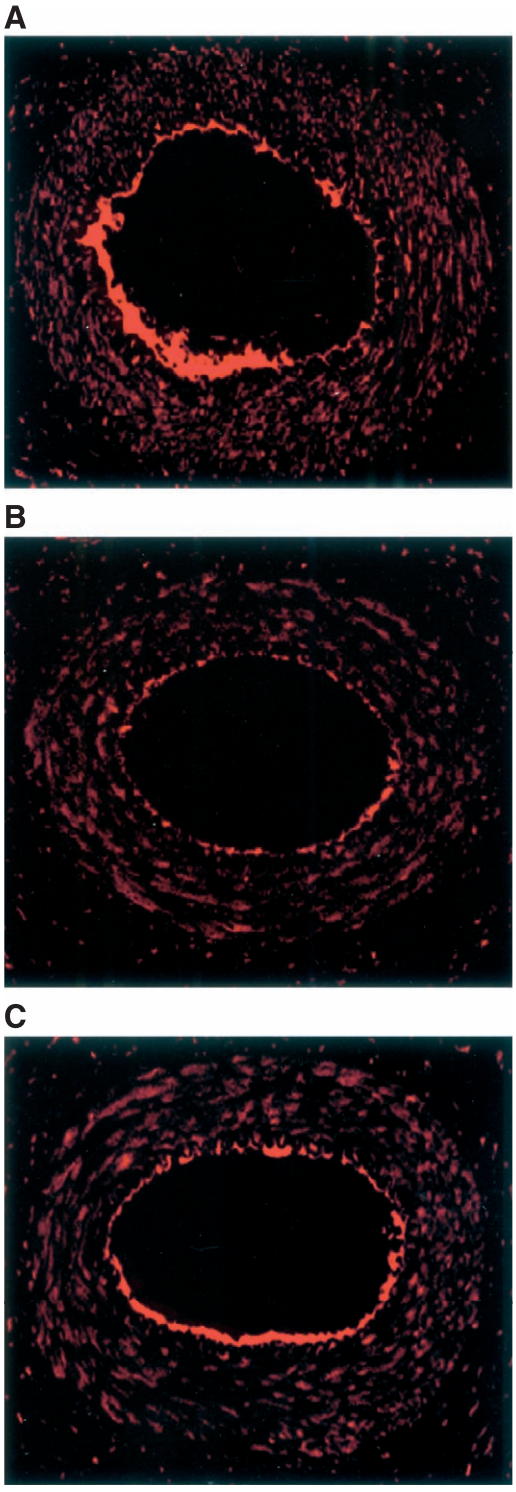

Fig. 2. Confocal images showing immunofluorescence labeling for eNOS protein in ear arteries from control (A), irradiated (B) and edaravone-treated irradiated (C) rabbits. Note that immunoreactivity (staining as red) was less in the irradiated vessel and this change was blocked by edaravone treatment. The same results were obtained with five other vessels in each group. Original magnification × 100.

with multiple comparisons by Fisher's protected least-significant difference *t*-test. Nonparametric data were analyzed by the Mann–Whitney's *U*-test or Wilcoxon signed-rank test. A $P<0.05$ was considered to be statistically significant.

3. Results

The contraction evoked by phenylephrine at a submaximal concentration (3 μM) showed no significant difference between control and irradiated ear arteries at 2 weeks following exposure to a single dose of 45 Gy (0.547 ± 0.067, $n=7$ versus 0.548 ± 0.080 g/mg wet weight, $n=6$). In arteries precontracted with 3 μM phenylephrine, however, the concentration-dependent relaxant response to acetylcholine was markedly impaired after irradiation (Fig. 1). Treatment of irradiated rabbits with edaravone nearly completely normalized the impaired acetylcholine relaxant response (Fig. 1). The relaxation elicited by acetylcholine remained unchanged when edaravone was given to control animals (Fig. 1).

Immunofluorescent studies showed less abundant eNOS protein expression in ear arteries from irradiated animals

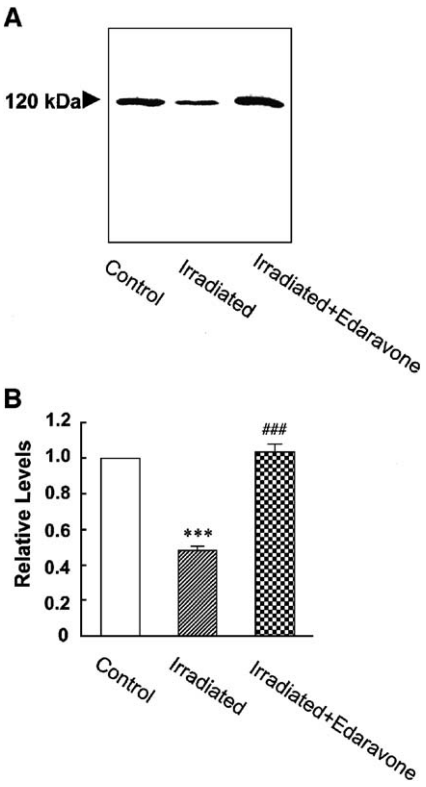

Fig. 3. Immunoblot analysis of eNOS protein in ear arteries from control, irradiated and edaravone-treated irradiated rabbits. (A) Representative Western blot showing a marked decrease in expression of 120 kDa band following irradiation and a complete reversal by edaravone treatment. (B) Bar graph comparing the immunostained band for eNOS protein in the three groups of vessels. Densitometric results are expressed as percent of the band obtained with control in each experiment. Bars are means ± S.E. of four experiments. ***$P<0.001$ vs. the control value. $^{###}P<0.001$ vs. the value obtained in irradiated vessels.

than those from controls (Fig. 2). The location of positive staining for eNOS was largely within vessels, especially the intimal surface. The results of quantitative analysis showed a 52% decrease in eNOS protein expression in irradiated vessels compared with controls ($P<0.001$). In irradiated rabbits treated with edaravone, arterial expression of eNOS protein was evidently increased nearly to that in controls (Fig. 2).

Immunoblot analysis using the antiserum raised against human eNOS showed a single band with a molecular mass of approximately 120 kDa, which was referred to as eNOS, in rabbit ear arteries (Fig. 3A). The band obtained from the irradiated vessel was evidently weaker than that from the control vessel. Densitometric quantification of the signal revealed that eNOS protein level following irradiation was $48 \pm 2\%$ ($n=4$, $P<0.001$) of the control level (Fig. 3B). The decreased expression level of eNOS was completely recovered when irradiated rabbits were treated with edaravone ($104 \pm 4\%$, $n=4$). Edaravone treatment resulted in a small but significant increase in expression of eNOS in control animals. Thus, an $18 \pm 6\%$ ($n=5$) increase was found when edaravone was given to control animals.

Gene expression of eNOS in ear arteries from control, irradiated and edaravone-treated irradiated rabbits was analyzed by the RT-PCR method (Fig. 4). GAPDH mRNA was used as an internal standard and for adjustment of sample-to-sample variations. The transcript band for eNOS was evidently faint in the irradiated vessel when compared with that in control. Densitometric analysis showed that irradiation significantly ($P<0.05$) reduced the eNOS mRNA expression level to 47% of control. Edaravone treatment resulted in complete prevention of the irradiation-induced reduction in eNOS transcripts. Edaravone alone had no effect on arterial expression of eNOS gene in control animals: when the data were presented as the ratio of eNOS mRNA to GAPDH mRNA, the values were $96 \pm 1\%$ and $97 \pm 1\%$ ($n=4$) in control animals without and with edaravone treatment, respectively.

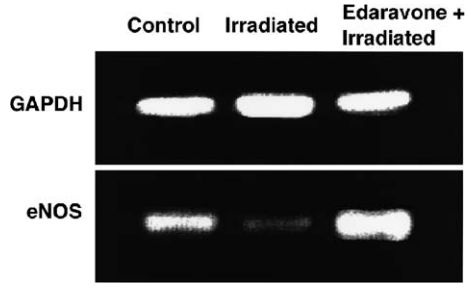

Fig. 4. RT-PCR analysis showing gene expression of eNOS in ear arteries from control, irradiated and edaravone-treated irradiated rabbits. While expression of eNOS mRNA was less abundant following irradiation, the reduced mRNA signal was reversed by edaravone treatment. Note that there is no apparent difference in GAPDH mRNA used as an internal control among groups. The same results were obtained with two other vessels in each group.

4. Discussion

In our previous work (Qi et al., 1998), the endothelium-dependent relaxations were found to be markedly impaired in the rabbit ear artery following exposure to radiation with a cobalt^{60} unit. As N^{G}-nitro-L-arginine, an inhibitor of NOS, virtually eliminated the endothelium-dependent relaxations in normal nonirradiated rabbit ear artery, the relaxant response of this artery is most likely to be completely dependent on eNOS. When the animals were irradiated with a dose of 45 Gy, a significant reduction in arterial expression of eNOS, in addition to the impaired endothelium-dependent relaxations, was observed at 1 and 4 weeks after irradiation. However, the morphological damage of endothelial cells of the arteries was identified from 6 weeks postirradiation. In the current study, we used the ear arteries at 2 weeks following 45 Gy irradiation. Thus, it would be expected that our irradiated arterial preparations exhibit the impairment of endothelium-dependent relaxations and the reduction in eNOS expression without any morphological alteration in endothelial cells. Furthermore, it should be noted that the dose of 45 Gy employed in this study is equal to the total preoperative dose of radiation required for patients with a neck malignant tumor (Sugihara et al., 1999).

The expression level of eNOS protein was reduced by about 50% in the vessels at 2 weeks after irradiation, as determined by Western blot and immunohistochemical analyses. Our results using the RT-PCR method indicated that the irradiated vessels showed a decrease in eNOS mRNA to 47% of control. Thus, the reduction in expression of eNOS protein was paralleled by a similar decrease in its mRNA. This suggests that irradiation causes downregulation of eNOS expression at the transcriptional level.

The principal finding of this work is that the reduced levels of expression of eNOS mRNA and protein in the irradiated vessels was nearly completely normalized when the animals were treated with edaravone. Reactive oxygen species, particularly hydroxyl radical and superoxide anion, are known to be produced during irradiation by water radiolysis (Stark, 1991). Edaravone has been proven to show prominent free radical scavenging and antioxidant characteristics (Watanabe et al., 1994; Yamamoto et al., 1996). It seems reasonable, therefore, to infer that edaravone could protect against inhibition of eNOS expression by scavenging free radicals generated in irradiated tissues. Consistent with this explanation is the recent report showing that vitamin C treatment prevented irradiation-induced impairment of endothelium-dependent vasodilation in rats (On et al., 2001). We thus suggest that generation of free radical species may be a primary cause of the reduced expression of eNOS in irradiated vessels. However, the potential mechanism(s) of the action of free radical species in reducing eNOS expression is unclear from the present data. Furthermore, our idea cannot

readily be reconciled with the previous report showing that expression of eNOS in cultured endothelial cells is upregulated by H_2O_2 (Drummond et al., 2000). At the present time, we do not have a clear understanding of this paradoxical result, but the discrepancy may be due to fundamental differences between in vivo and in vitro experimental models. With regard to the striking difference of our experimental model from cultured endothelial cells, it is intriguing to note that irradiation does not affect expression of eNOS mRNA in bovine aortic endothelial cells (Hirakawa et al., 2002).

Because of the very unstable chemical nature of NO, endothelium-derived NO can be rapidly inactivated by oxygen-derived free radicals due to the formation of the complex between NO and $O_2{}^{*-}$, and thus, an enhanced production of oxygen-free radical species appears to be involved in the accelerated breakdown of NO (Gryglewski et al., 1986; Rubanyi and Vanhoutte, 1986; Mian and Martip, 1995). However, we previously showed that incubation with superoxide dismutase and catalase failed to improve the impaired endothelium-dependent relaxation in the vessels after irradiation (Qi et al., 1998), suggesting that the impairment of the response in irradiated vessels is not caused by reduced bioavailability of NO due to interactions between NO and $O_2{}^{*-}$.

The reduction in endothelial NO formation due to the reduced eNOS expression in irradiated vessels would decrease local blood flow and favor thrombus formation. This could result in the potential development of stenosis, which does indeed occur in arteries following irradiation (Reinhold et al., 1991). Furthermore, such an irradiation-induced impairment of endothelial cell function may be one of the factors that aggravate postoperative complications, including impaired wound healing, when surgical procedures are performed on irradiated tissues (Robinson, 1975; Rudolph, 1982). Therefore, the beneficial effect of edaravone to reverse eNOS expression shown in this study suggests that this radical scavenger may become a therapeutic agent for preventing a high risk of postoperative complications in irradiated tissues.

In conclusion, we demonstrated that in vivo treatment with edaravone, a free radical scavenger, normalized the reduced expression of eNOS mRNA and protein in the rabbit ear artery following irradiation. We assume that the generation of free radical species may play a key role in the irradiation-induced reduction in eNOS expression in the vessels, thereby resulting in the impaired endothelium-dependent relaxations in the irradiated vessels. On considering the importance of eNOS-mediated endothelial function in maintenance of the fluidity of the blood, modulation of vascular tone, and inflammatory and immunological processes (Lüscher and Vanhoutte, 1990), our results may lead to the possibility that edaravone could prove useful in preventing the development of vascular stenosis and poor surgical wound healing in irradiated tissues.

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