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Functional and morphological damage of endothelium in rabbit ear artery following irradiation with cobalt^{60}

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1 The relaxant actions of acetylcholine and A23187 were examined in the rabbit central ear artery at different intervals following exposure to different doses of radiation with a cobalt^{60} unit. The artery was irradiated with a dose of 10 Gy, 20 Gy and 45 Gy. Radiation caused dose- and time-dependent impairment of the endothelium-dependent relaxations. The impaired endothelium-dependent relaxations occurred as early as 1 week postirradiation and persisted throughout the experimental period (10 weeks).

2 The endothelium-independent response to sodium nitroprusside was well preserved up to 6 weeks after irradiation. The contractile response to noradrenaline was unaltered by irradiation throughout the experimental period, but in contrast to control vessels, an increase in the sensitivity to noradrenaline in the presence of the nitric oxide synthase (NOS) inhibitor N^G-nitro-L-arginine was not observed in the irradiated vessels.

3 The impaired endothelium-dependent relaxations in the irradiated vessels were not improved by pretreatment with the NOS substrate L-arginine, the cyclo-oxygenase inhibitor indomethacin or the free radical scavengers superoxide dismutase and catalase.

4 Scanning electron microscopy indicated morphologically intact endothelial cells within the first 4 weeks after irradiation.

5 Western blot analysis showed a significant decrease in the expression of endothelial NOS (eNOS) in the irradiated vessels.

6 These data indicate that endothelial cell function is specifically impaired in the irradiated vessels before morphological endothelial cell damage can be detected. This impairment may be related to diminished eNOS expression.

Keywords: Irradiation; EDRF; contraction; relaxation; nitric oxide synthase; endothelium; artery

Introduction

Surgical procedures performed on irradiated tissues have an increased incidence of postoperative complications such as infections and impaired wound healing (Robinson, 1975; Rudolph, 1982). Impaired healing of radiation wound is usually attributed to alterations in fibroblasts, myofibroblasts and dermal vessels (Rudolph *et al*., 1981; Young & Hopewell, 1982; Luce, 1984). However, progressive vascular damage may be the most profound change seen after radiation injury (Ariyan & Krizek, 1990). Histological studies of vessels within irradiated fields have not only demonstrated thickening of the walls of these vessels, increased deposition of collagen, and fragmentation of the elastic lamina (Sams, 1965; Watson, 1979), but studies by scanning electron microscopy have demonstrated dehiscence of the endothelial (Guelinckx *et al*., 1984). As 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 various degrees of morphological damage (Kwock *et al*., 1982, Lee *et al*., 1983).

Endothelial cells mediate relaxation of vascular smooth muscle cells by releasing non-prostanoid endothelium-derived relaxing factors (EDRFs) in response to various vasodilating substances (Furchgott, 1983). This has provoked extensive research on their possible role in the regulation of the vascular tone in physiological and pathophysiological situations (Furchgott & Vanhoutte, 1989; Ignarro, 1990; Vane *et al*., 1990). Several lines of evidence have suggested that endothelial cell function is affected by radiation (Rubin *et al*., 1984, 1985; Fajardo, 1989). It has been found that a single dose of 45 Gy X-radiation blunts endothelium-dependent relaxant responses to acetylcholine (ACh) and substance P in rabbit ear artery (Maynard *et al*., 1992). However, the question as to whether the radiation-induced impairment of endothelium-dependent relaxations occurs with or without morphological damage of endothelial cells remains unresolved.

In the present study, we investigated changes in endothelium-dependent relaxations and alterations in the morphology of endothelial cells in the rabbit ear artery at different time intervals following exposure to different doses of radiation with a cobalt^{60} unit, that is commonly used in clinical radiotherapy for head and neck malignancies (Ackerman & Regato, 1970). Additionally, in order to determine whether vascular smooth muscle cells are functionally damaged by exposure to radiation, the contractile response to noradrenaline and the endothelium-independent relaxant response to sodium nitroprusside (SNP) were also evaluated.

Methods

Irradiation procedure

Male New Zealand White rabbits weighing 2 to 3 kg were anaesthetized with sodium pentobarbitone (25 mg kg^{-1}, i.v.).

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This dose of pentobarbitone provided general anaesthesia without depression of respiration. The ear of the rabbit was fixed to a flat plate with tape in order to keep the ear horizontally. A cobalt^{60} unit (Toshiba PCR-120-C_3, Tokyo, Japan) producing gamma rays at 1.25 meV and a SSD (source-skin-distance) of 55 cm was used. Although typical doses for cobalt^{60} used in clinical radiotherapy for head and neck malignancies are equal to less than 20 Gy as a single exposure, we chose a dose range of 10 to 45 Gy in our studies to bracket the range of the anticipated doses. Both ears of the animals were locally irradiated with three different doses of radiation (10, 20 and 45 Gy). The field size was 10×10 cm over the centre of the ear. A single 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 doses delivered to the ear central artery would be 90% and higher of the dose calculated at skin level.

After irradiation, rabbits were housed in FRP running-water flushing units (Clea, Tokyo, Japan) at a constant temperature of $23 \pm 3^{\circ}$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.

Organ bath experiments

On the day of the experiment, the animals were anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, i.v.), and the 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 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 (Sanei-Sokki 45196, Tokyo, Japan) and a carrier amplifier (Sanei-Sokki 1236). The output of the force transducer was registered on a pen recorder (TOA Electronics ERP-241A, Tokyo, Japan) through a polygraph recorder (Sanei-Sokki 142-8).

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. Then concentration-response curves for noradrenaline-induced contractions were constructed by adding cumulative concentrations of noradrenaline to the bath. The rings were rinsed thoroughly with the normal PSS, and allowed to recover to the baseline. Following the cumulative concentration-response curves, the rings were precontracted with 1 μM noradrenaline. When the noradrenaline-induced contraction reached a plateau level, the relaxant response to ACh, A23187 or SNP was examined in a cumulative manner. When the effects of L-arginine, indomethacin, superoxide dismutase (SOD) and catalase on the relaxant response to ACh in irradiated vessels were examined, each agent was added to the bath 15 min before the administration of noradrenaline. At the completion of the experiment the rings were carefully blotted dry and weighed.

Histological evaluation

For light microscopy, lengths of arteries were isolated from normal and irradiated ears at the time of ring preparations. They were fixed in 10% formalin, embedded in paraffin, and sectioned for haematoxylin and eosin (HE) staining.

For scanning electron microscopy, the ear artery was perfused *via* the proximal side with heparin-treated saline (1,000 units heparin in 500 ml physiological saline solution), and 2% 0.1 M phosphate buffered (pH 7.4) glutaraldehyde solution. The specimens were split longitudinally and immersed in 2% buffered glutaraldehyde solution for more than 4 h. The specimens were washed with 0.1 M phosphate buffer and electron stained with 2% titanic acid for 3 h at room temperature. Then the specimens were washed with distilled water several times and postfixed in 1% OsO_4 for 2 h. After repeated washes with the buffer, the specimens were dehydrated in graded alcohol solutions, dried in CO_2 at the critical point, mounted on stubs, coated with platinum-palladium by use of a vacuum anion coater, and examined by scanning electron microscopy.

Western blot analysis

Equal amounts of whole homogenates of control and irradiated ear arteries were run on a 8% polyacrylamide sodium dodecyl sulphate gel and electroblotted onto polyvinylidine defluoride filter (PVDF) membrane. To reduce non-specific binding, the PVDF was blocked for 60 min at room temperature in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM NaH_2PO_4, 1.5 mM KH_2PO_4) containing 1% bovine serum albumin. Thereafter, the PVDF was washed for 10 min three times in PBS-Tween buffer (TPBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM NaH_2PO_4, 1.5 mM KH_2PO_4, 0.1% Tween 20, 1% bovine serum albumin), and incubated overnight at 4°C with mouse monoclonal antibody raised against a polypeptide 1030 - 1209 of human endothelial nitric oxide synthase (eNOS; Transduction Laboratories, Lexington, KY, U.S.A) at 1 : 2500 dilution in TPBS. After extensive washing with TTBS, the PVDF was incubated with alkaline phosphatase-conjugated goat anti-mouse antibody (Wako Pure Chemical, Osaka, Japan) diluted at 1 : 3000 in TPBS at room temperature for 60 min. Then, the PVDF was washed for 10 min twice in TPBS. The blots were visualized by use of the enhanced chemiluminescence method according to the manufacturer's instructions (Bio-Rad, Hercules, CA, U.S.A.), exposed to X-ray film for 2–30 min and analysed by using the free software NIH image produced by Wayne Rasband (National Institute of Health, Bethesda, MD, U.S.A.).

Drugs

The drugs used were the following: (−) noradrenaline bitartrate and acetylcholine (ACh) chloride (Wako), A23187 (Calbiochem, San Diego, CA, U.S.A.), SNP, L-arginine, idomethacin, superoxide dismutase (SOD; from human erythrocytes; 2,000–4,000 u mg^{-1} protein), catalase (from bovine liver; 45,000 u mg^{-1} protein) and N^G-nitro-L-arginine (Sigma, St Louis, MO, U.S.A.). All drugs were dissolved in distilled water, except A23187, indomethacin and N^G-nitro-L-arginine. A23187 was dissolved in dimethyl sulphoxide and diluted in ethanol. Indomethacin was prepared in 50 mM Tris. N^G-nitro-L-arginine was prepared in 0.3 N HCl. Further dilutions to the desired concentrations were made with the normal PSS.

Statistical analysis

All values are expressed as means ± s.e.mean. Comparisons of variables obtained by various treatments with basal values were made by one way analysis of variance with a repeated measurements design, and if any significant difference was found the Scheffés multiple comparison test was applied. Student's *t* test was used to make comparisons between control and irradiated groups. Nonparametric data were analysed by means of Mann-Whitney *U*-test. P values <0.05 was considered statistically significant.

Results

Contractile response to noradrenaline

The cumulative addition of noradrenaline (10 nM – 100 μM) produced a concentration-dependent contraction in all preparations tested. The vessels at 1 week after being irradiated with one of three different doses of radiation (10, 20 and 45 Gy) showed no significant difference in either the maximum contraction or sensitivity to noradrenaline when compared with the control vessels (Figure 1). Similarly, there was no significant difference in contractile response to noradrenaline between control and irradiated vessels at 1, 4, 6 and 10 weeks following exposure to a single dose of 45 Gy (Figure 2).

Incubation with the NOS inhibitor N^G-nitro-L-arginine (100 μM) resulted in a significant leftward shift of the concentration-response curve for noradrenaline of control preparations ($pD_2 = 7.20 \pm 0.04$ versus 6.51 ± 0.10, $n=6$, $P<0.05$) without affecting the maximum contraction (2.0 ± 0.2 versus 2.2 ± 0.2 g mg^{-1} wet weight). By contrast, there was no significant shift in the vessels at 1 week after irradiation with a dose of 45 Gy ($pD_2 = 6.71 \pm 0.06$ versus 6.81 ± 0.10, $n=6$).

Endothelium-dependent relaxant responses

In control preparations precontracted with 1 μM noradrenaline, ACh produced concentration-dependent relaxations. Such relaxant responses were not observed in tissues without endothelium. Inhibition of nitric oxide synthase with 100 μM N^G-nitro-L-arginine virtually abolished ACh-induced relaxations. A dose of 10 Gy had no visible effect on ACh-induced relaxations at 1 week after irradiation (Figure 3). However, with higher doses (20 and 45 Gy), mild to severe impairments of the relaxant response to ACh were observed (Figure 3). After 45 Gy irradiation, the relaxant response to ACh was progressively attenuated with elapsed time (Figure 4). Thus, the response was unaffected at 1 h after irradiation, but was markedly reduced at 1, 4, 6 and 10 weeks after irradiation without significant variation in the results obtained at these time points.

The calcium ionophore A23187 produced concentration- and endothelium-dependent relaxations. The relaxant response to A23187 was significantly attenuated in the vessels at 1 week after 45 Gy irradiation (Figure 5). Figure 5 also shows the postirradiation time-related alterations in the relaxant response to A23187: the relaxant response to A23187 was greatly reduced at 1, 4, 6 weeks following irradiation, and was nearly completely eliminated after 10 weeks.

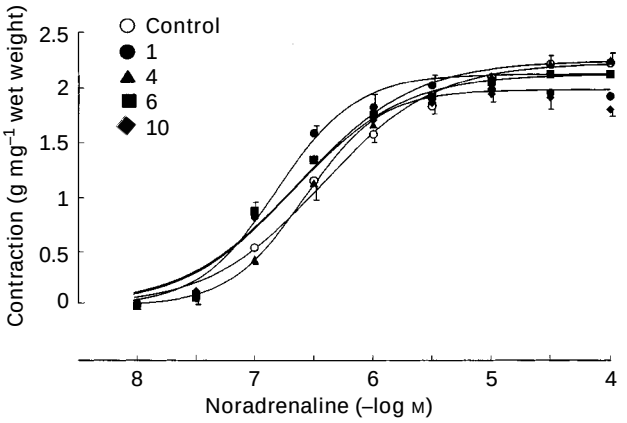

Figure 2 Concentration-response curves for noradrenaline-induced contractions in control and irradiated vessels at 1, 4, 6 and 10 weeks after exposure to a dose of 45 Gy radiation. The contractions induced by noradrenaline are expressed as g developed tension mg^{-1} tissue wet weight. Points are means and vertical lines s.e.mean of six experiments. There was no significant difference in the values of maximum contraction and pD_2 between each group.

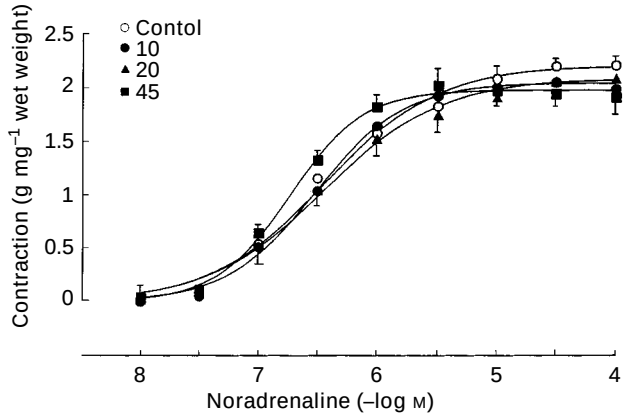

Figure 1 Concentration-response curves for noradrenaline-induced contractions in control and irradiated vessels at 1 week after exposure to 10 (●), 20 (▲) and 45 Gy (■) radiation. The contractions induced by noradrenaline are expressed as g developed tension mg^{-1} tissue wet weight. Points are means and vertical lines s.e.mean of six experiments. There was no significant difference in the values of maximum contraction and pD_2 ($-\log EC_{50}$) between each group.

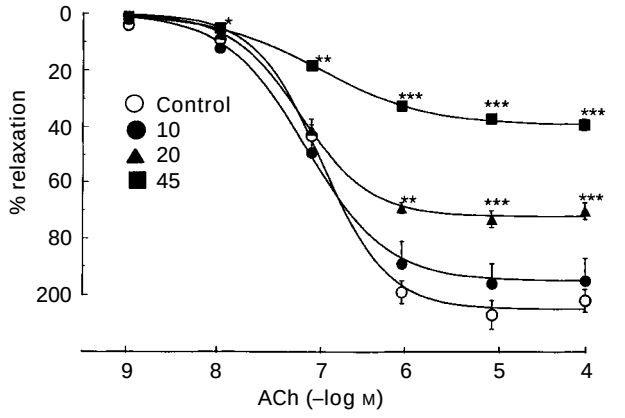

Figure 3 Effects of different doses of radiation on the relaxant response to ACh. The relaxant responses obtained in control preparations were compared with those in the preparations at 1 week after irradiation with doses of 10 Gy, 20 Gy and 45 Gy. The responses are expressed as % relaxation of noradrenaline-induced contraction. Points are means and vertical lines s.e.mean of six experiments. *$P<0.05$, **$P<0.01$ and ***$P<0.001$ vs the respective control values.

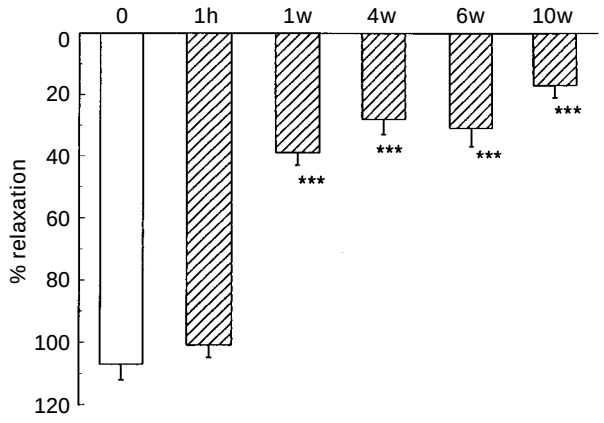

Figure 4 Time-dependent effect of 45 Gy irradiation on the relaxant response to ACh. The maximum relaxations produced by ACh were determined in preparations taken from control animals and from animals at various time points after 45 Gy irradiation. The responses are expressed as % relaxation of noradrenaline induced contraction. Columns are means ± s.e.mean of six experiments. ***$P<0.001$ vs the control value.

As shown in Figure 6, incubation with L-arginine (1 mM) failed to improve the impaired maximum relaxant response to ACh in vessels at 1 week after 45 Gy irradiation. Addition of the cyclo-oxygenase inhibitor indomethacin (10 μM) did not cause a significant improvement in the ACh response in the irradiated vessels. Further, pretreatment with the free radical scavengers SOD (60 u ml^{-1}) and catalase (600 u ml^{-1}) did not lead to a recovery from the diminution of the relaxant response after irradiation.

Relaxant response to SNP

The endothelium-independent vasodilator SNP produced concentration-dependent relaxations in precontracted preparations taken from control animals. As shown in Figure 7, there was no significant variation in this response in the vessels at 1, 4 and 6 weeks following 45 Gy irradiation. However, the vessels at 10 weeks after irradiation exhibited a significantly diminished response.

Macroscopic and microscopic findings

In rabbits irradiated with 10 or 20 Gy, no morphological alterations were macroscopically observed in the irradiated sites of the ear at 1 and 4 weeks after radiation; hair growth and appearance of the skin were normal. Furthermore, the dissected irradiated vessels did not show any thickening or other changes under the dissection microscope. In animals which received radiation of 45 Gy, morphological alterations detected macroscopically in the irradiated sites were hair loss, which became evident progressively from 1 week after irradiation, and reactive responses of the skin, such as reddening, thickening, desquamation and necrotic ulcer formation which became manifest in the later postirradiation period, typically at 10 weeks after irradiation. Severe thickening of the irradiated vessels was seen under the dissection microscope; this developed later than 6 weeks after irradiation.

Light macroscopy revealed that nonirradiated vessels dissected from the ear showed no intimal proliferation, well organized smooth muscle cells and normal connective tissue in adventitia (Figure 8a). There were no microscopic changes in the vascular wall immediately after a 45 Gy irradiation (data not shown), and no obvious intimal proliferation was observed

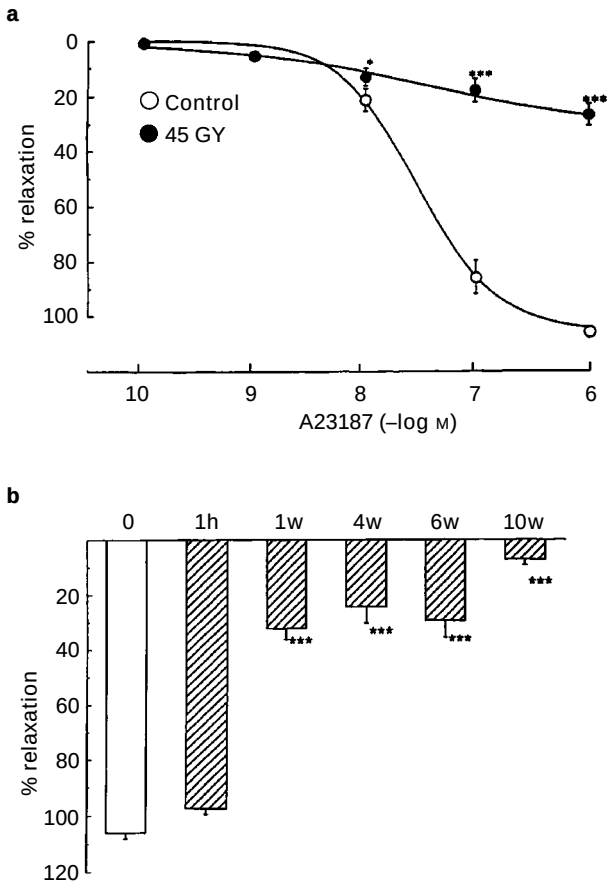

Figure 5 Effect of 45 Gy irradiation on the relaxant response to A23187. (a) The relaxant responses obtained in control preparations were compared with those in the preparations at 1 week after 45 Gy irradiation. (b) The maximum relaxations produced by A23187 in the preparations at various time points after 45 Gy irradiation are shown. The responses are expressed as % relaxation of noradrenaline-induced contraction. Columns are means ± s.e.mean of six experiments. *$P<0.05$, and ***$P<0.001$ vs the respective control value.

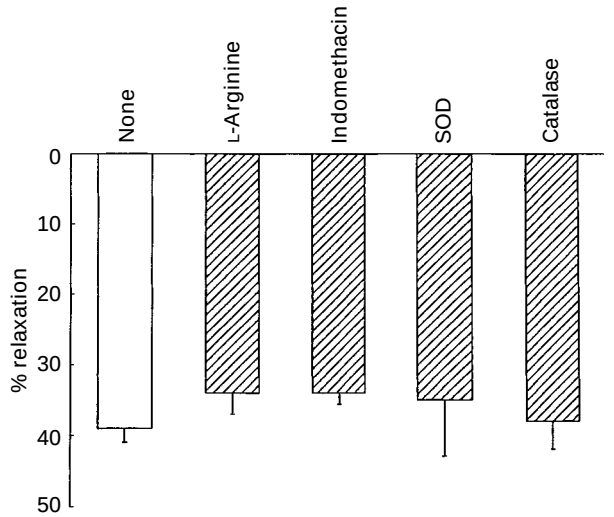

Figure 6 Effects of L-arginine (1 mM), indomethacin (10 μM), SOD (60 u ml^{-1}) and catalase (600 u ml^{-1}) on the diminished relaxant response to ACh at 1 week after 45 Gy irradiation. The maximum responses to ACh are expressed as % relaxation of noradrenaline-induced contraction. Columns are means ± s.e.mean of four to six experiments.

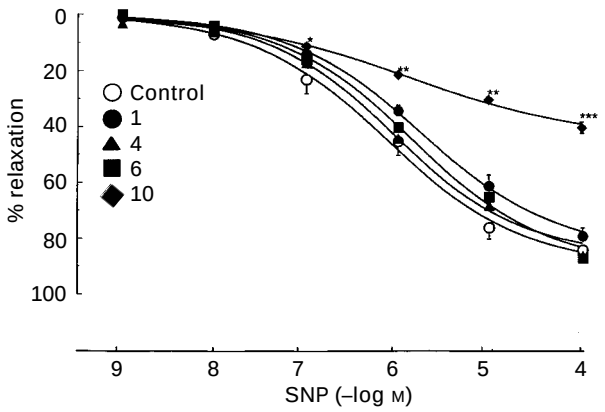

Figure 7 Effect of 45 Gy irradiation on the relaxant response to SNP. The relaxant responses obtained in control preparations were compared with those in the preparations at 1, 4, 6 and 10 weeks after 45 Gy irradiation. The responses are expressed as % relaxation of noradrenaline-induced contraction. Points are means and vertical lines s.e.mean of six experiments. $*P<0.05$, $**P<0.01$ and $***P<0.001$ vs the respective control values.

through the 10 weeks postirradiation period (Figure 8b, and c). However, the smooth muscle cells in the tunica media were disorganized in their arrangement to some extent from 1 week after irradiation (Figure 8b). Perivascular fibrosis in the adventitia was identified from 6 weeks postirradiation, and, at 10 weeks, there was marked infiltration of the adventitia with neutrophils, especially around the vasa vasorum, and there was an increase in the number of large fibroblasts with abundant basophilic cytoplasm in the adventitia (Figure 8c).

Scanning electron microscopy indicated that the endothelial cells of the arteries at 1 and 4 weeks after 45 Gy irradiation appeared normal (Figure 9a, b). Endothelial cells were flat, well defined, rhomboid in shape, with their long axis lying parallel with that of the vessel and with tight junctions with neighbouring cells. The luminal surface of the cells was covered with microvillous projections. At 6 and 10 weeks afer irradiation, the cells were shrunken like long and thin cords (Figure 9c). The junctions of the endothelial cells were widened, the basement membrane was exposed and detachment of the endothelial cells was also frequent. Platelets, degenerated protein deposits, red blood cells, fibrin deposits were found on the bare basement membrane. The internal elastic membrane revealed frequent defects through which the underlying fibres were exposed.

Western blot analysis

Figure 10 shows a representative Western blot for eNOS, a mouse monoclonal antibody raised against the human eNOS was used. The antibody identified one single protein band with a molecular mass of about 140 kDa, which was thought to be eNOS. We found markedly lower levels of eNOS in the vessels at 1 and 4 weeks following 45 Gy irradiation. Quantification of immunoblots of the band for eNOS with four different vessels in each group revealed a significant decrease to $69\pm7\%$ ($P<0.05$) and $70\pm3\%$ ($P<0.01$) of control in the vessels at 1 and 4 weeks after irradiation, respectively.

Discussion

The present study clearly demonstrates that the endothelium-dependent relaxant responses to ACh and A23187 were progressively attenuated in the rabbit central ear artery after exposure to radiation with a cobalt^{60} unit. The effect of radiation on the endothelium-dependent relaxations was dependent on the dose delivered. When the animals were irradiated with a dose of 45 Gy, the impairment of the endothelium-dependent relaxations occurred as early as 1 week postirradiation. This observation is consistent with the results of other investigators (Maynard *et al*., 1992) who have shown that the relaxant responses to ACh and substance P are reduced in the rabbit central ear artery at 1 week after a single dose of 45 Gy X-irradiation. The endothelium-dependent

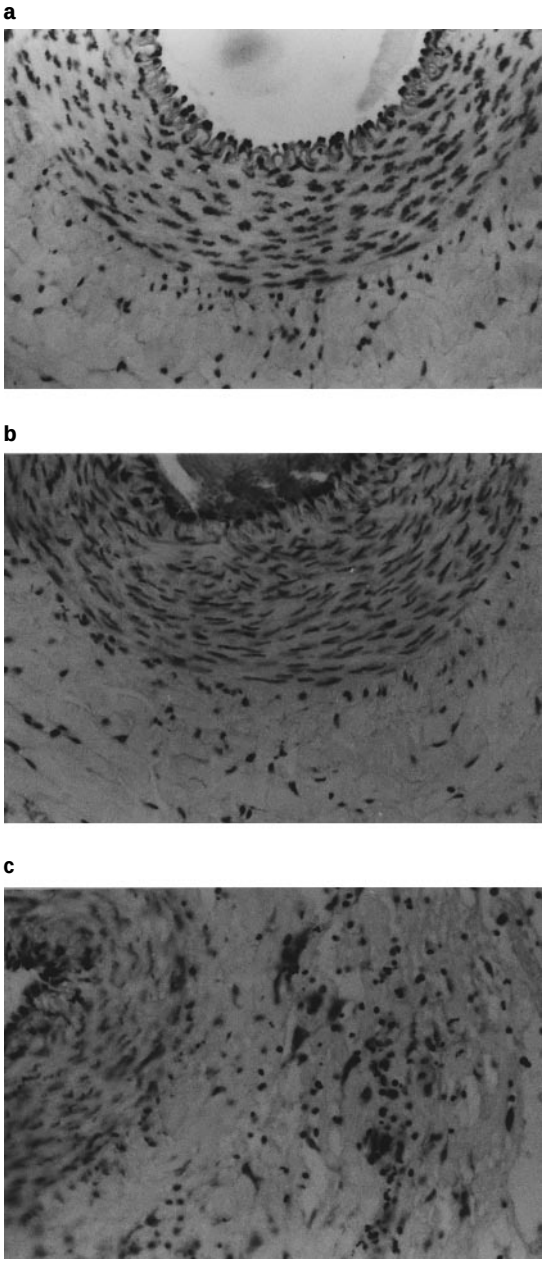

Figure 8 Light microscopic appearance of the arteries. (a) Nonirradiated vessels served as control. No intimal proliferation was observed. There was well organized smooth muscle cells and normal connective tissue in adventitia (HE; ×66). (b) No obvious intimal proliferation was noted at 1 week after 45 Gy irradiation. Smooth muscle cells in the tunica media were disorganized to some extent (HE; ×66). (c) Neutrophil infiltration was evident in the adventitia at 10 weeks after irradiation. Fibroblasts with large body and abundant basophilic cytoplasm were also identified (HE; ×66). The same results were obtained with three other vessels in each group.

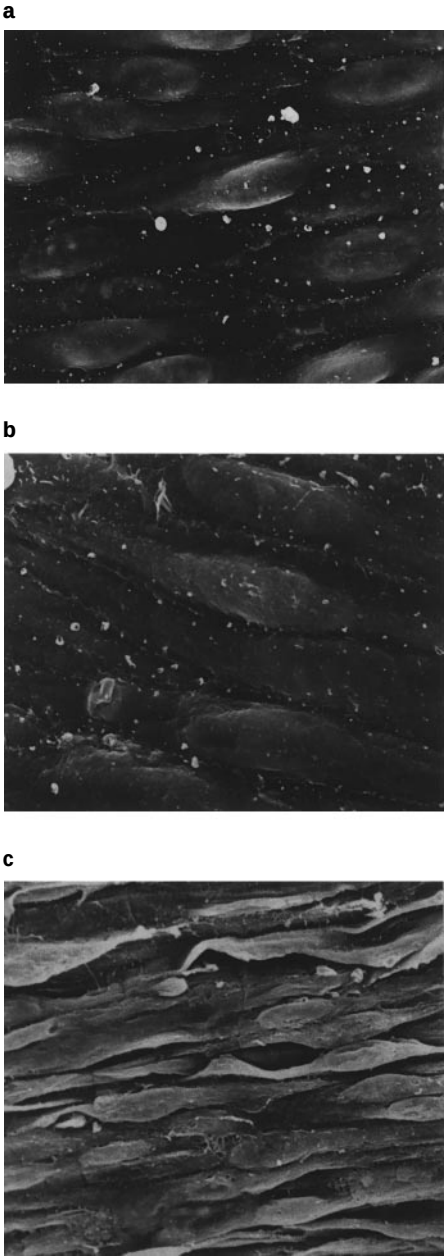

Figure 9 Scanning electron microscopy of the endothelial surfaces of the arteries taken from animals at 1 week (a, SEM; ×1500), 4 weeks (b, SEM; ×2500) and 10 weeks (c, SEM; ×1000) after irradiation with a single dose of 45 Gy. The same results were obtained with three other vessels in each group.

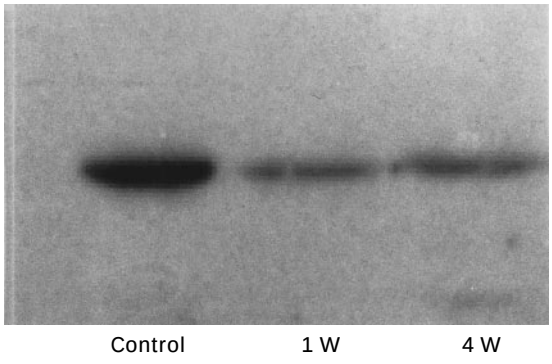

Figure 10 Representative autoradiograms of eNOS Western blot in the arteries taken from control animals and from animals at 1 and 4 weeks after irradiation with a single dose of 45 Gy.

relaxation in normal, nonirradiated rabbit ear artery that was caused by ACh could be virtually eliminated by N^G-nitro-L-arginine, an inhibitor of NOS (Moore *et al*., 1990), suggesing that the EDRF in this artery is most likely to be nitric oxide. Thus, the impaired endothelium-dependent relaxation following irradiation may occur by a diminution of the production of nitric oxide or of its action on the vascular smooth muscle. In fact, it is unlikely that the impairment of the endothelium-dependent relaxation was at level of the vascular smooth muscle sensitivity to nitric oxide, as we found that the relaxant response to the nitric oxide donor SNP was unaffected in the vessels at 1, 4 and 6 weeks following 45 Gy irradiation, although it was significantly reduced at 10 weeks after irradiation. This observation also excludes the possibility that irradiation might have induced a general impairment of the relaxant capacity of the arterial smooth muscle. The present results therefore indicate that endothelial cell function is selectively impaired in the irradiated vessels.

In contrast to the impaired endothelium-dependent relaxations, the contractions induced by noradrenaline were unaltered even at 10 weeks following irradiation. Thus, there appeared to be no damage to the vascular smooth muscle contractile mechanism after irradiation. Incubation with N^G-nitro-L-arginine produced the expected increase in the sensitivity to noradrenaline in control, nonirradiated vessels, indicating inhibition of basal EDRF release. However, no significant leftward shift of the concentration-response curve for noradrenaline was observed in the irradiated vessels. This suggests a reduction in basal EDRF release in the irradiated vessels. These observations are also in keeping with our key finding that there was specifically a marked impairment in endothelium-dependent relaxant responses in the vessels after irradiation.

At 6 and 10 weeks after exposure to a dose of 45 Gy of $cobalt^{60}$ radiation, marked alterations in the morphology of the endothelial cells of the rabbit central ear artery were observed by using scanning electron microscopy. Cellular shrinking, widened cellular junctions and detachment of cells from the basement membrane were all noted at these times after irradiation. This morphological damage of the endothelial cells may account for the impaired endothelium-dependent relaxations observed at 6 and 10 weeks after irradiation. However, the impairment of the endothelium-dependent relaxant responses noted within the first 4 weeks after irradiation was apparently not accompanied by significant morphological changes. Thus, the damaging effect of irradiation on endothelial cell function at the early stage is not likely to be a result of direct radiation-induced changes to the morphology of endothelial cells.

Previous studies have shown that radiation leads to dose-dependent increases in the production of thromboxane A_2 (Schneidkraut *et al*., 1984; Ward *et al*., 1988). Although platelets primarily serve as a source of thromboxane A_2 (Hamberg *et al*., 1975), a great deal of evidence suggests that thromboxane A_2 can also be produced by a variety of blood vessels (Salzman *et al*., 1980; Maurer *et al*., 1980; Serneri *et al*., 1993). Since thromboxane A_2 is the most potent vasoconstrictor prostanoid end product (Moncada & Vane, 1979), its release might counteract the endothelium-dependent relaxations in the irradiated vessels. In the present study, the impaired endothelium-dependent relaxant response to ACh at 1 week after irradiation was not restored by treatment with the cyclo-oxygenase inhibitor indomethacin, indicating that the vasoconstrictor prostanoid does not participate in the impaired endothelial response.

Oxidative processes in endothelial cells can generate oxygen-derived free radicals (Rosen & Freeman, 1984).

Because of the very unstable chemical nature of nitric oxide, endothelium-derived nitric oxide can be easily inactivated by oxygen-derived free radicals after its release from endothelial cells (Rubanyi & Vanhoutte, 1986). In fact, increased production of oxygen-derived free radicals and decreased free radical scavenger systems have been suggested to play an important role in abnormal endothelium-dependent relaxations in some pathological conditions such as diabetes mellitus (Hattori *et al*., 1991; Tesfamariam & Cohen, 1992). Thus, a possible explanation for the impairment of the endothelium-dependent relaxations after irradiation is the enhanced production of oxygen-derived free radicals in the irradiated vessels. However, our study showed no improvement of ACh relaxation with the free radical scavengers SOD and catalase in the vessels at 1 week after irradiation, suggesting that excessive nitric oxide destruction by free radicals is unlikely to be the cause of the impaired relaxation in the irradiated vessels. Clearly, the present study does not exclude a role of free radicals in morphological damage of endothelial cells observed at the late time points following irradiation.

In endothelial cells, nitric oxide is synthesized in a process involving oxidation of L-arginine by Ca^{2+}-calmodulin-dependent constitutive NOS (Palmer *et al*., 1988; Moncada *et al*., 1991). Incubation with the NOS substrate, L-arginine, failed to improve the impaired endothelium-dependent relaxant response to ACh in the vessels at 1 week after irradiation, and it is therefore reasonable to conclude that the impaired response was not due to a lack of substrate *per se*. On the other hand, Western blot analysis showed a significant decrease in the expression of eNOS in the vessels at 1 and 4 weeks after irradiation. It is thus very probable that the deficit in eNOS activity contributes to the impaired endothelium-dependent relaxations in the irradiated vessels. Whether irradiation downregulates the expression of eNOS by acting at the levels of transcription or translation remains the subject of ongoing studies.

In conclusion, we have shown that the endothelium-dependent relaxations were specifically impaired in the rabbit central ear artery following irradiation. This endothelial cell dysfunction which was observed within the first 4 weeks after irradiation was not accompanied by morphological damage of endothelial cells. The impaired endothelium-dependent relaxations in the irradiated vessels did not appear to be due to an overproduction of vasoconstrictor prostanoids, an excessive destruction of nitric oxide by free radicals, or a lack of substrate for the NOS, but were related to the reduced expression of eNOS. The endothelium has an important role in modulating vascular tone (Furchgott, 1983) and abnormal endothelial cell function may be a contributory factor to vascular disorders (Vane *et al*., 1990). Therefore, the irradiation-induced impairment of endothelial cell function we have observed may be one of the factors that aggravates postoperative complications when surgical procedures are performed on irradiated tissues that have normal-looking vessels.

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