Preferential Impairment of Nitric Oxide–Mediated Endothelium-Dependent Relaxation in Human Cervical Arteries After Irradiation

Tsuneki Sugihara, MD; Yuichi Hattori, MD; Yuhei Yamamoto, MD; Fazhi Qi, MD; Ryoichi Ichikawa, MD; Atsushi Sato, MD; Ming-Yue Liu, MD; Kazuhiro Abe, MD; Morio Kanno, MD

Background—Vascular abnormalities are a major cause of postoperative complications in irradiated tissues. Endothelial cell dysfunction characterized by diminished endothelium-dependent relaxation may be involved. We examined the endothelium-dependent relaxation and morphology of the endothelium in irradiated human cervical arteries.

Methods and Results—Irradiated arteries were taken from the neck region of patients who had radiation therapy. Arteries from patients who did not receive radiation therapy were used as controls. Endothelium-dependent relaxation to acetylcholine and A23187 was impaired in irradiated arteries. Norepinephrine-induced contraction and sodium nitroprusside–induced relaxation were unchanged. In control arteries, N^\text{v}-nitro-L-arginine and indomethacin each caused a partial inhibition of endothelium-dependent relaxation. In irradiated arteries, the impaired endothelium-dependent relaxation was unaffected by these agents, but it was abolished by high K^+ . Acetylcholine produced similar degrees of hyperpolarization in control and irradiated arteries. Immunohistochemical examination for endothelial nitric oxide synthase indicated no expression in the endothelium of irradiated arteries. Electron scanning microscopy showed morphologically intact endothelial cells in irradiated arteries.

Conclusions—In irradiated human cervical arteries, the nitric oxide– and prostacyclin-mediated endothelium-dependent relaxation, but not endothelium-derived hyperpolarizing factor–mediated relaxation, are specifically impaired, without significant morphological damage of the endothelium. The impaired nitric oxide–mediated relaxation was associated with a lack of endothelial nitric oxide synthase expression. Our results suggest the importance of impaired endothelial function in irradiated human blood vessels, which may partly explain the development of vascular stenosis and poor surgical wound healing in irradiated tissues. (Circulation. 1999;100:635-641.)

Key Words: arteries  endothelium  immunohistochemistry  vasodilation

Vascular endothelium plays an important role in the regulation of vascular tone by releasing constricting and relaxing factors, such as nitric oxide (NO), prostacyclin, and endothelium-derived hyperpolarizing factor (EDHF). Because released NO and prostacyclin relax vascular smooth muscle and inhibit platelet aggregation, thereby preventing the occurrence of ischemia and vascular occlusion, it has been suggested that endothelial dysfunction contributes to vascular disorders. Surgical wounds within irradiated tissue are subject to an increased incidence of postoperative complications, including impaired wound healing. These postirradiation complications are thought to be due to capillary damage, microvascular occlusion, and increased fibrosis. Random and progressive damage of blood vessels after irradiation has been demonstrated. This may be, in part, the result of endothelial cell dysfunction after irradiation. Several lines of evidence suggest that irradiation exerts unfavorable influences on endothelial cell function. A single dose of 45-Gy x-ray radiation blunts endothelium-dependent relaxation to acetylcholine (ACH) and substance P in rabbit ear arteries. Recently, we showed that endothelium-dependent relaxation in rabbit ear arteries exposed to radiation is impaired, without any morphological alteration in endothelial cells. However, it remains to be seen whether the conclusion obtained using animal models can be applied to irradiated human blood vessels. Therefore, the aim of this study was to determine whether endothelium-dependent relaxation and the morphology of the endothelium were altered in irradiated arteries from the neck region of patients who received radiation therapy.

Methods

Tissue Source
Lumps of the neck tissue including cervical arteries were harvested intraoperatively from 17 patients who had a previously irradiated
malignant tumor requiring a neck dissection. The radiation therapy used at our institution as preoperative or primary treatment is 40 to 65 Gy in 14 to 25 fractions over 4 to 6 weeks to the tumor itself or the lymph nodes in the neck. The dose to the neck region is given in daily doses of 2.5 Gy. The total preoperative dose of radiation averaged 47.9±2.8 Gy. Surgery was done 4 to 6 weeks after irradiation. All irradiated blood vessels were located in the irradiated neck zone. No attempt was made to dissect out the blood vessels into nonirradiated zones. Nonirradiated (control) arteries were obtained from lumps of neck tissue from 15 patients with a malignant tumor who did not have radiation therapy. The age of the patients ranged from 25 to 79 years, with a mean of 58.8±3.3 in the control group and 60.5±2.2 in the irradiated group. None of the patients showed any signs of cardiovascular disease or diabetes mellitus.

Tissue samples were placed in ice-cold oxygenated physiological salt solution (PSS) immediately after excision. On arrival at the laboratory, human cervical arteries (HCAs) were freed of surrounding tissue under a dissection microscope. Care was taken to ensure that the endothelium was not damaged during tissue preparation. Excised HCAs contained facial arteries, superior thyroid arteries, and cervical horizontal arteries.

Experimental procedures were performed according to the ethical guidelines of the 1989 modified Helsinki declaration, and written informed consent from all patients was obtained before the study.

Mechanical Experiments
HCAs were cut into rings 4 mm in length (diameter, ~1.5 mm). Each ring was suspended under a resting tension of 2 g in a bath filled with 25 mL of PSS gassed with 95% O₂ and 5% CO₂ at 37°C. The composition of PSS was (in mmol/L): NaCl 118.2, KCl 4.7, MgCl₂ 1.2, KH₂PO₄ 1.2, CaCl₂ 2.5, NaHCO₃ 25.0, and glucose 10.0. After an equilibration period of at least 60 minutes, the contractile capacity of each ring was examined through repeated exposure to 40 mmol/L isotonic K⁺ PSS, which was prepared by substituting KCl for NaCl on an equimolar basis.

Concentration-response relationships for norepinephrine were determined by the cumulative addition of increased agonist concentrations. After HCAs were precontracted to 80% of maximal contraction with 1 µmol/L norepinephrine, different relaxant agents (ie, ACh, A23187, and sodium nitroprusside [SNP]) were cumulatively added. Three or 4 concentration-response curves for ACh were successively separated by a washout period of 30 minutes. No significant differences existed between curves without any other treatment, and the first curve constructed in normal PSS was used as the control. The second or third curve was constructed in the presence of either 100 µmol/L L-NNA and indomethacin. The incubation period was at least 15 minutes. The last curve was established in 30 mmol/L K⁺ PSS in the presence of L-NNA and indomethacin.

At the completion of the experiment, the preparations were carefully blotted dry and weighed. Contractions of each preparation to norepinephrine were expressed as milligrams of developed tension per milligram of tissue weight. Relaxations were expressed as the percentage of decreased tension of contractile force elicited by 1 µmol/L norepinephrine.

Electrophysiological Experiments
Transverse strips were prepared by cutting along the longitudinal axis of the rings. The strip was pinned down, intimal side upward, on the bottom of an organ chamber and superfused at a constant flow rate of 7 mL/min with oxygenated PSS at 37°C. Glass microelectrodes filled with 3 mol/L KCl (tip resistance 40 to 80 megohms) were inserted into the smooth-muscle cells from the intimal side. Electrical signals were monitored continuously on an oscilloscope and recorded on a chart recorder. After stable membrane potentials lasted for 2 minutes, the ACh hyperpolarizing response was determined by continuous recordings of membrane potential from a single cell. Further details of the experimental procedure have been described elsewhere.13

Figure 1. Contractile response to norepinephrine in control (C) and irradiated (O) HCAs. Data are shown as mean±SEM of 8 to 12 experiments.

Histological Evaluation
For light microscopy, control and irradiated HCAs were fixed in 10% formalin, embedded in paraffin, and sectioned for hematoxylin and eosin staining or Weigert elastic fiber staining.

For scanning electron microscopy, HCAs were immersed with 4% lidocaine perivascularly and perfused with heparinized saline (1000 U heparin in 500 mL PSS). The specimens were split longitudinally and immersed in 2% buffered glutaraldehyde and 2% osmium tetroxide for at least 4 hours. The specimens were washed with 0.1 mol/L phosphate buffer, electron-stained with 2% titanic acid for 3 hours at room temperature, washed with distilled water several times, and fixed in 1% OsO₄ for 2 hours. After repeated washes with the buffer, the specimens were dehydrated in graded alcohol solutions, dried in CO₂ at the critical point, mounted on stubs, and examined by scanning electron microscopy.

Immunohistochemistry for Endothelial Nitric Oxide Synthase
For immunohistochemical analysis of endothelial nitric oxide synthase (eNOS), we used mouse monoclonal antibody to human eNOS (Transduction Laboratories). HCAs were fixed in 10% formalin, embedded in paraffin, and cut in slices 5-µm thick. After deparaffinization, the sections were immersed in 0.5% periodic acid for 15 minutes and treated with pepsin (Dako Corporation) in 0.2 mol/L HCl at 37°C for 10 minutes. Rabbit serum (10%) was used for blocking the nonspecific binding of protein for 30 minutes. Anti-eNOS antibody was applied at a dilution of 1:4000 and incubated overnight at 4°C in a humidified environment. After the sections were washed in phosphate-buffered saline containing 0.1% Tween 20, biotinylated rabbit anti-mouse IgG was applied for 60 minutes at room temperature. Staining was visualized with a avidin-biotin-peroxidase complex at room temperature for 30 minutes using a Histfine SAB-PO(M) Kit (Nichirei Corporation). Omission of the primary antibody served as negative control. Immunoreactive level of eNOS was evaluated with light microscopy. Anti-factor VIII–related antigen mouse monoclonal antibody (Nichirei) was used as a positive specificity control to identify endothelial cells.

Statistical Analysis
Data are presented as mean±SEM. Groups of data were compared by 2-tailed Student’s t test. Comparisons of data between >2 groups were performed by 1-way ANOVA. P<0.05 was considered statistically significant.

Results
Contraction and Relaxation Responses
The contractile response of irradiated arteries to norepinephrine was almost identical to that of the control arteries (Figure 1). No significant difference existed in the maximum con-
traction or the sensitivity, as reflected by the EC₅₀ of irradiated arteries compared with controls (177±6 versus 189±34 mg/mg wet weight; 0.73±0.06 versus 0.72±0.06 μmol/L).

After precontraction with norepinephrine, ACh produced concentration-dependent relaxation in both control and irradiated arteries. However, the response of irradiated arteries was markedly attenuated (Figure 2A). The maximum response was significantly diminished in irradiated arteries compared with controls (33±6% versus 100±4%; P<0.001); it was also associated with a decrease in sensitivity (EC₅₀: 218±90 versus 22±8 mmol/L). The relaxant response to A23187 was also impaired in irradiated arteries (Figure 2B). Irradiated arteries exhibited a lesser maximum response (65±8%) compared with controls (98±4%; P<0.01), with a tendency to decrease the sensitivity (EC₅₀: 304±137 versus 13±4 mmol/L). In contrast, the relaxant response to SNP in irradiated arteries was virtually identical to that of control arteries (Figure 2C); the EC₅₀ value was 0.17±0.07 μmol/L in control and 0.32±0.12 μmol/L in irradiated arteries.

In control arteries, the ACh-relaxant response was partially inhibited by 100 μmol/L L-NNa (Figure 3A). L-NNa caused a significant reduction in maximum response from 100±4% to 72±7% (P<0.01). The combination of 10 μmol/L indomethacin and 100 μmol/L L-NNa further decreased the ACh response (Figure 3A). However, ACh still induced significant relaxation (maximum response, 46±8%) in the presence of indomethacin and L-NNa. The remaining relaxation was completely inhibited when the external K⁺ concentration was increased to 30 mmol/L (Figure 3A). In irradiated arteries, the combination of L-NNa and indomethacin did not affect the concentration-response curve for ACh-induced relaxation (Figure 3B). Exposure to 30 mmol/L K⁺ produced complete inhibition of the ACh response (Figure 3B). Raising the extracellular K⁺ concentration had no effect on SNP-induced relaxation in either control or irradiated arteries (data not shown).

**ACh-Induced Hyperpolarization**

The average resting membrane potential of smooth-muscle cells in irradiated arteries (−52.3±3.6 mV; n=4) did not differ from that in controls (−48.5±1.7 mV; n=4). ACh (1 μmol/L) produced similar degrees of hyperpolarization in control and irradiated arteries (Figure 4). The peak membrane potentials changed by ACh were −74.3±3.2 mV (n=3) in control arteries and −71.0±3.0 mV (n=3) in irradiated arteries.
Morphology

Arteries from the 2 groups examined in this study were 1.5-mm thick and the muscular type, showing a wavy internal elastic lamina between the tunica intima and media. Some control arteries showed thickened tunica intima, but most appeared normal (Figure 5A). In contrast, most irradiated arteries clearly showed thickening of the tunica intima and media (Figure 5B). Thus, the mean intimal thickness tended to be larger in irradiated arteries ($27.9 \pm 10.9 \mu m$; $n=6$) compared with controls ($9.6 \pm 2.8 \mu m$; $n=6$), although this difference was not statistically significant ($P = 0.13$).

The mean medial thickness of irradiated arteries ($315.3 \pm 115.5 \mu m$) was significantly greater than that of controls ($225.3 \pm 9.0 \mu m$; $P < 0.001$). The thickened irradiated arteries revealed an increase in smooth-muscle cells and massive elastic fibers in the tunica media (Figures 5C and 5D). Neither significant fibrosis nor neutrophil infiltration was identified in the adventitia of the 2 groups.

Scanning electron microscopy indicated structurally intact endothelium in irradiated arteries (Figure 6). In both control and irradiated arteries, endothelial cells were flat, well defined, and rhomboid in shape, had their long axes lying parallel to that of the vessel, and had tight junctions with neighboring cells. The luminal surface of the cells was covered with microvillous projections.

Immunohistochemistry for eNOS

Control and irradiated arteries were examined for expression of eNOS protein by immunohistochemistry (Figure 7). Positive staining for eNOS was observed in the endothelium of control arteries, but no expression was detectable in arteries after irradiation.

Discussion

The present study is the first to demonstrate that endothelium-dependent relaxation induced by ACh and A23187 are markedly impaired in irradiated HCAs. This finding is
consistent with the results from animal models reported by us and other investigators. The endothelium-independent relaxant substance SNP produced similar degrees of relaxation in control and irradiated HCAs, excluding the fact that irradiation might have induced a general impairment of the relaxing capacity of arterial smooth muscles. Furthermore, the finding that norepinephrine-induced contraction was unaffected by irradiation indicates that no damage to the vascular smooth-muscle contractile mechanism occurred after irradiation. It thus seems likely that the relaxant responses to endothelium-dependent vasodilators are specifically impaired in irradiated HCAs.

In irradiated HCAs, intimal and medial thickening was observed. The thickened intima and media may be promoted by cytokines and growth factors known to cause smooth-muscle proliferation. These cytokines and growth factors play an important role in the pathogenesis of the short- and long-term effects of radiotherapy. Alternatively, it may be that the intimal and medial proliferation is due, in part, to the reduced release of NO from the endothelium. NO-generating vasodilators inhibit vascular smooth muscle proliferation in vitro. Endothelial dysfunction after irradiation may, thus, lead to an imbalance between growth-promoting and growth-inhibiting factors and favor vascular occlusion, which is a major problem associated with surgical procedures performed on irradiated tissues. However, scanning electron microscopy showed that irradiated endothelial cells were morphologically intact. We suggest that impaired endothelium-dependent relaxation of irradiated HCAs is not accompanied by an altered appearance of endothelial cells.

The present results indicate that different mechanisms may contribute to endothelium-dependent relaxation in HCAs. In control HCAs, ACh-induced relaxation was partially inhibited by the NO synthase inhibitor L-NNA, suggesting that the relaxation was mediated, at least in part, by NO. The cyclooxygenase inhibitor indomethacin produced further inhibition of ACh-induced relaxation in the presence of L-NNA. Thus, part of the non-NO response to ACh was due to prostacyclin. However, the ACh-relaxant response was not abolished by the combination of L-NNA and indomethacin. This remaining relaxation was completely eliminated by raising the extracellular K⁺ concentration. Raising the extracellular K⁺ concentration can be used to prevent endothelium-dependent hyperpolarization and its associated relaxation. The existence of a L-NNA- and indomethacin-resistant but K⁺-sensitive component of vasorelaxation indicates the involvement of an additional mechanism, possibly EDHF, in ACh-induced relaxation in HCAs. EDHF produces hyperpolarization and subsequent relaxation through the activation of K⁺ channels in vascular smooth-muscle cells. The types of K⁺ channels activated by EDHF and the mechanism by which the subsequent hyperpolarization mediates smooth-muscle relaxation in HCAs remains to be elucidated.

Pretreatment with L-NNA and indomethacin did not further suppress impaired endothelium-dependent relaxation in irradiated HCAs. The ACh-relaxant response of irradiated HCAs was almost identical to that of controls incubated with L-NNA and indomethacin. These results suggest that the production of both NO and prostacyclin is severely impaired in irradiated HCAs. In support of this notion, our immunohistochemical examination indicated that no expression of eNOS occurred in the endothelium of irradiated HCAs. This fact agrees with our recent results using Western blot analysis, which showed that eNOS expression is greatly reduced in irradiated rabbit ear arteries. The present experiments also reconcile with earlier observations that the capacity for prostacyclin production is reduced in irradiated vascular rings. It is, therefore, most likely that a deficit in eNOS activity and, possibly, cyclooxygenase activity contributes to impaired endothelium-dependent relaxation in irradiated HCAs.

The residual endothelium-dependent relaxation in HCAs after irradiation was attributed to the release of EDHF from the endothelium. This was confirmed by the discovery that raising the extracellular K⁺ concentration blocked relaxation.
in irradiated HCAs. Thus, the production of EDHF in the endothelium seems to be well preserved, even after irradiation. In support of this idea, ACh produced similar degrees of hyperpolarizations in control and irradiated HCAs. The identity of EDHF has not yet been established. Recent studies suggest that EDHF is 1 of the cytochrome P450–derived arachidonic acid metabolites.20 However, we preliminarily observed that ACh-relaxant responses in the presence of L-NNA and indomethacin were unmodified by 30 μmol/L SKF525A, a cytochrome-P450 inhibitor, in both control and irradiated HCAs, although the existence of different EDHFs cannot be ruled out.

In conclusion, the endothelium-dependent relaxation of HCAs involve NO, prostacyclin, and possibly EDHF. In HCAs from the patients who had been irradiated, the relaxation was markedly attenuated, without significant morphological damage to the endothelium; this attenuated relaxation resulted from the impaired production of NO and prostacyclin. The impaired NO-dependent relaxation was associated with the lack of eNOS expression. However, the EDHF-like component of relaxation was less sensitive to irradiation, suggesting that the mechanisms for the generation of EDHF may function normally even after irradiation. However, even if EDHF acts as a backup mechanism for NO and prostacyclin in HCAs, the lack of the NO- and prostacyclin-mediated components of endothelium-dependent relaxation would reduce local blood flow and favor thrombus formation. Therefore, the irradiation-induced impairment of endothelium-dependent relaxation observed in this study implicates the potential development of stenosis, which does occur in irradiated arteries.21 Finally, our results could partly account for the reason why microvascular surgery in irradiated human blood vessels has a high risk of postoperative complications, including impaired wound healing.

Acknowledgments
This work was supported in part by the Special Grant-in-Aid for Promotion and Science at Hokkaido University provided by the Ministry of Education, Science, Sports, and Culture of Japan. The authors wish to acknowledge the technical support and patience of the staff of the Head and Neck Surgical Team at Hokkaido University Hospital.

References
Preferential Impairment of Nitric Oxide–Mediated Endothelium-Dependent Relaxation in Human Cervical Arteries After Irradiation
Tsuneki Sugihara, Yuichi Hattori, Yuhei Yamamoto, Fazhi Qi, Ryoichi Ichikawa, Atsushi Sato, Ming-Yue Liu, Kazuhiro Abe and Morio Kanno

Circulation. 1999;100:635-641
doi: 10.1161/01.CIR.100.6.635

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1999 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/100/6/635

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org//subscriptions/