Tumor Cell Apoptosis by Irradiation-induced Nitric Oxide Production in Vascular Endothelium

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ABSTRACT

We examined the effects of X-ray irradiation on endothelial nitric oxide (NO) production in bovine aortic endothelial cells (BAECs). Single irradiation of up to 60 Gy did not affect the expression of endothelial NO synthase (eNOS) mRNA, as assessed by reverse transcription-PCR, in BAECs. The basal level of intracellular Ca\(^{2+}\) concentration and Ca\(^{2+}\)-dependent relaxation were markedly reduced in irradiated arteries obtained from dissected tumor tissues that had been treated with X-ray irradiation. These results indicate that single irradiation does not affect Ca\(^{2+}\) mobilization and eNOS expression but induces the expression of iNOS in BAECs, and the latter property would be beneficial to induce apoptosis in the adjacent tumor cells.

INTRODUCTION

The cell cycle of endothelial cells is generally considered to be much slower than that of tumor cells, and radiation is generally considered to inhibit cell cycle progression. Therefore, although the inhibition of angiogenesis, or endothelial proliferation, is a promising approach to tumor treatment (1), capillary vasculature within tumor tissues have not been considered as a target of radiation therapy. On the contrary, adverse effects of irradiation on endothelial functions have been reported. For instance, eNOS\(^{3}\) expression and endothelium-dependent relaxation were markedly reduced in irradiated arteries obtained from dissected tumor tissues that had been treated with preparative radiation therapy (2). Other groups have also reported similar results in irradiated model animals (3, 4). These authors attributed the alterations of irradiated vessels such as stenosis, fibrosis, and thrombosis to the impairment of endothelial functions (2–4). In contrast, another group reported that intravascular irradiation did not affect endothelium-dependent vasodilatation (5). Therefore, it would be important to clarify whether irradiation affects endothelial normal functions, especially NO productivity, and the possible influences of these changes on tumor treatment.

Another alteration with regard to NOS expression after radiation therapy is the induction of iNOS, which has been reported in various tissues and cells such as ileum and colon epithelium in rat (6), bone marrow stromal cells in mouse (7), lung in rat (8), and murine embryonic liver cell line (9). These reports discussed the expression of iNOS, which generates NO continuously in a Ca\(^{2+}\)-independent manner in the presence of l-arginine (10), as a pathogenesis of radiation-induced organ dysfunctions, but thus far, no reports have examined the possible contribution of iNOS expression to the validity and/or invalidity of radiation tumor therapy. NO generated by iNOS is known to show dual effects on tumor growth. For instance, immune cells, such as macrophages and neutrophils, express iNOS, and the produced NO shows cytotoxic effects by inducing DNA damage. This has been considered as one of the causes of carcinogenesis at chronic inflammatory sites (11), but also as a mechanism of the antitumor actions of macrophages (12). Furthermore, tumor cells, such as gynecological cancer (13) and breast cancer (14), express iNOS, which also has dual effects on tumor growth: i.e., low concentrations of NO promote tumor growth mainly by activating vascular growth, whereas high concentrations of NO show antitumor action by inducing apoptosis of tumor cells themselves (15). No reports, however, have examined the effects of irradiation on iNOS expression in vascular endothelium.

The aim of this study was to investigate the possible alterations of endothelial NO productivity by X-ray irradiation. For this purpose, we first examined the effects of irradiation on Ca\(^{2+}\) mobilizing properties and eNOS expression, both of which are essential for constitutive NO production in endothelium (16). Furthermore, we have also examined the effects of irradiation on the expression of iNOS in the endothelium. The results show for the first time that irradiation induces iNOS-mediated continuous NO production, which may be applicable for inducing tumor cell apoptosis.

MATERIALS AND METHODS

Cell Culture. BAECs were obtained and cultured in DMEM (Life Technologies, Inc., Rockville, MD) supplemented with 10% fetal bovine serum as described previously (17). Cells of the second subculture were used for the present study and grown on either a coverslip, a 60-mm culture dish, or a 6-cm dish. Cells were cultured in DMEM with 10% fetal bovine serum.

Irradiation of BAECs. X-ray irradiation was applied to BAECs by Pantak (Shimadzu Co., Kyoto, Japan) at 1 Gy/min at room temperature under ambient conditions. Control cells (sham-irradiated cells) were also brought to the shield room but were left nonirradiated at room temperature for the same period as the irradiation procedure.

Measurement of [Ca\(^{2+}\)]\(_i\) and [Ca\(^{2+}\)]\(_{ex}\). [Ca\(^{2+}\)]\(_i\) was measured as described previously (18). The average of [Ca\(^{2+}\)]\(_i\), from 20–30 cells on one coverslip was regarded as one data point. Experiments were performed at room temperature (20°C–25°C).

Measurement of Intracellular Production of NO. NO was measured by using DAF-2, a NO-sensitive fluorescent dye (19), as described previously (20). The average of the data from one coverslip containing 20–30 cells was regarded as one data point.
RT-PCR Analysis of NOS mRNA. Expression of eNOS and iNOS mRNA in BAECs was estimated with semiquantitative RT-PCR using the primers described in Table 1. 

The obtained PCR products were quantified with electrophoresis by using an image analysis system EDAS 290 (Eastman Kodak Co., Rochester, NY). The expression levels of these mRNA were normalized with that of GAPDH (Table 1).

ECL Western Blot Analysis. Expression of iNOS protein was assessed by ECL Western blotting. Cells were irradiated as described above, and cell lysate was prepared after the proper incubation period. Western blot analysis for iNOS protein was performed by using anti-iNOS polyclonal antibody (Stress-Gen Biotechnologies, Co., San Diego, CA) and ECL system (SuperSignal; Pierce, Rockford, IL). Emitted chemiluminescence was then detected and analyzed by a luminoimage analyzer (FAS-1000; Toyobo, Osaka, Japan).

MCLA Chemiluminescence Assay. Cellular production of superoxide anion (O$_2^-$) was detected by using an O$_2^-$-sensitive luciferin derivative, MCLA (Tokyo Kasei Kogyo, Tokyo, Japan), as described previously (21).

BrdUrd Incorporation Assay. Proliferation of HepG2 cells was assessed by ELISA of BrdUrd incorporation into the nucleus. BAECs were grown to confluence on a cell culture insert with a membrane pore size of 0.4 μm (Millicell; Millipore Corp., Bedford, MA) and irradiated. HepG2 cells were seeded on 6-well culture plates and cocultured with 2-Gy-irradiated or sham-irradiated BAECs for 72 h, and BrdUrd was added to the culture medium at a concentration of 10 mM during the last 6 h. HepG2 cells were then harvested and put into a 96-well microtiter plate at a density of 12,000 cells/well. The incorporation of BrdUrd was measured as an absorption of 450 nm wavelength by using a commercial kit (Biotrak cell proliferation ELISA system; Amersham Pharmacia Biotech, Piscataway, NJ).

Analysis of DNA Damage of HepG2 Cells with Comet Assay. For analysis of cellular apoptosis, HepG2 cells were coincubated with BAECs for 72 h as described above. After harvesting HepG2 cells, DNA damage was assessed by single-cell gel electrophoresis (comet assay) as reported previously (22). In this assay, fragmented DNA of apoptotic cells is shifted out of the nucleus and shows the microscopic electrophoretic pattern as a "comet tail." We quantified the degree of DNA damage with five categories according to the percentage of DNA in the tail: (a) grade 0, no change or <5%; (b) grade 1, low-level damage (5–20%); (c) grade 2, medium-level damage (20–40%); (d) grade 3, high-level damage (40–95%); and (e) grade 4, total damage (>95%; Ref. 22).

Solutions and Drugs. The standard extracellular solution for measuring [Ca$^{2+}$]i and NO was a modified Krebs solution (1.5 mM Ca$^{2+}$ solution)
Fig. 2. Irradiation did not affect ATP-induced Ca\textsuperscript{2+} oscillations in BAECs. A, control cells showed Ca\textsuperscript{2+} oscillations in response to 3 \textmu M ATP. A trace from a representative cell is shown. B, cells 24 h after 2 Gy of irradiation also showed ATP (3 \textmu M)-induced Ca\textsuperscript{2+} oscillations. C, analysis of ATP (3 \textmu M)-induced Ca\textsuperscript{2+} oscillations in control and 2-Gy-irradiated BAECs. Both oscillation frequency (a) and net increment of peak Ca\textsuperscript{2+} increment (b) did not differ significantly between control and 2-Gy-irradiated cells. n.s., P > 0.05. Numbers in parentheses indicate the number of experiments.

Fig. 3. Semiquantitative RT-PCR analysis of eNOS mRNA expression in control and irradiated BAECs. A, dose-dependent effects of irradiation on eNOS mRNA expression. The intensity of the eNOS PCR product (34 cycles) was expressed relative to that of GAPDH, an endogenous control. B, absence of time-dependent change in eNOS expression after 2 Gy of irradiation. In both A and B, values in the graph were obtained from four independent experiments. Representative pictures of PCR bands are shown on top.
containing 132 mM NaCl, 5.9 mM KCl, 1.2 mM MgCl₂, 1.5 mM CaCl₂, 11.5 mM glucose, and 11.5 mM HEPES; the pH was adjusted to 7.3 with NaOH. Ca²⁺-free solution was made by replacing CaCl₂ of Krebs solution with 1 mM EGTA.

All drugs were purchased from Sigma Chemical Co. (St. Louis, MO).

**Data Analysis.** Pooled data are given as mean ± SE, and statistical significance was determined using Student’s unpaired t test. Probabilities < 5% (P < 0.05) were regarded as significant.

**RESULTS**

**Effects of Irradiation on Ca²⁺ Mobilizing Properties in BAECs.** First we examined the effects of irradiation on endothelial Ca²⁺ mobilizing properties in BAECs. The basal level of [Ca²⁺], was not altered 6 or 24 h after 2 or 4 Gy of irradiation (Fig. 1A).

Because Ca²⁺ entry from extracellular space rather than Ca²⁺ release from the intracellular Ca²⁺ stores plays an essential role in Ca²⁺-dependent NO production in endothelial cells (20, 23), we then observed the effects of irradiation on Ca²⁺ entry. In control cells that were sham-irradiated, thapsigargin, a selective inhibitor of endoplasmic/sarcoplasmic reticulum Ca²⁺-ATPase (24), induced a transient [Ca²⁺] elevation in Ca²⁺-free solution due to a Ca²⁺ leak from the intracellular store sites. Subsequent application of Ca²⁺-containing solution induced a further increase in [Ca²⁺], due to the capacitative Ca²⁺ entry mechanism (Ref. 25; Fig. 1B). These thapsigargin-induced changes in [Ca²⁺], were not altered by 2 (Fig. 1C) or 4 Gy (Fig. 1D) of irradiation. The time integral of the thapsigargin-induced initial Ca²⁺ transient in Ca²⁺-free solution, calculated as an indicator of the amount of intracellularly stored Ca²⁺, did not differ significantly between control and irradiated cells (Fig. 1E, a). Furthermore, the net increment of [Ca²⁺], induced by capacitative Ca²⁺ entry also did not differ significantly between control and irradiated cells (Fig. 1E, b).

It is known that ATP is an autocrine/paracrine mediator that induces Ca²⁺ transients (17) and NO synthesis (26). Therefore, we then examined the effects of exogenous ATP on [Ca²⁺], in control and irradiated BAECs. ATP induced Ca²⁺ oscillations in both sham-irradiated (Fig. 2A) and 2-Gy-irradiated cells (Fig. 2B). Both the frequency and peak amplitude of Ca²⁺ oscillations did not differ significantly between control and irradiated cells (Fig. 2C).

**Effects of Irradiation on eNOS Expression in BAECs.** Single irradiation (up to 60 Gy) also did not affect the expression level of eNOS mRNA, as assessed by semiquantitative RT-PCR (Fig. 3A). Late onset alteration in eNOS mRNA expression was also not observed until 120 h after 2 Gy of irradiation (Fig. 3B). Therefore, these results may imply that agonist-induced NO production is not affected by irradiation in BAECs. We then tried to examine agonist-induced NO production in irradiated cells with DAF-2, a NO-sensitive fluorescent dye (19). However, this was difficult to evaluate because of the continuous production of NO in irradiated cells as described below.
Spontaneous NO Production in Irradiated BAECs. When 3 mM L-arginine was perfused to sham-irradiated cells, only a small increase in DAF-2 fluorescence was observed (Fig. 4, A and D). In contrast, 2-Gy-irradiated cells showed a marked increase in DAF-2 fluorescence without any Ca\(^{2+}\)-mobilizing agent (Fig. 4, B, C, and D). This was not observed in the presence of 0.1 mM L-NAME, thereby indicating that DAF-2 fluorescence properly reflected the production of NO (Fig. 4, B, C, and D). NO production in 2-Gy-irradiated cells was significantly greater than that in control cells from 6–24 h after irradiation, and this slightly higher level of NO production persisted for at least up to 72 h (Fig. 4C).

It is well known that the expression of iNOS leads to continuous NO production in a Ca\(^{2+}\)-independent manner (27). We then measured DAF-2 fluorescence in cells treated with 250 units/ml IFN-\(\gamma\)/LPS, which are known to induce the expression of iNOS (28). As shown in Fig. 4E, IFN-\(\gamma\)/LPS-treated BAECs induced spontaneous NO production in the presence of 3 mM L-arginine. Therefore, the above-mentioned results suggest that irradiation stimulated the expression of iNOS in BAECs.

**Induction of iNOS by Irradiation in BAECs.** We then examined the expression of iNOS in irradiated BAECs with RT-PCR and Western blotting. Expression of iNOS mRNA, as assessed with semiquantitative RT-PCR, was induced by irradiation with a dose of 2 Gy (Fig. 5A) and persisted for more than 5 days after a single 2-Gy irradiation (Fig. 5B). Western blotting revealed that sham irradiation slightly induced the expression of iNOS protein (Fig. 6, sham), whereas cells that were not brought to the shield room and kept in the CO\(_2\) incubator throughout did not show iNOS expression (Fig. 6, untreated). This may indicate that the manipulation for irradiation itself induced the expression of iNOS protein in BAECs. However, it is obvious that 2-Gy-irradiated cells showed a significantly higher level of iNOS expression than sham-irradiated cells, and the expression was maximal at 12 h after irradiation (Fig. 6).

It has been suggested that NOS produces superoxide anion (O\(_2^-\)) instead of NO when insufficient concentrations of L-arginine or tetra-
rahydrobiopterin are supplied (27). Therefore, we examined whether irradiation-induced iNOS expression in BAECs led to the production of O$_2^\cdot$ under our experimental conditions by using MCLA chemiluminescence. However, no MCLA chemiluminescence was observed in irradiated BAECs in either normal culture medium containing 0.4 mM L-arginine or culture medium plus 3 mM L-arginine. Therefore, this suggests that the induction of iNOS did not lead to the generation of O$_2^\cdot$ in irradiated BAECs in the present study.

Effects of NO Generated from Irradiated BAECs on Tumor Cell Growth. The results above clearly indicate that irradiation induces the expression of iNOS, which leads to the continuous production of NO in the presence of l-arginine in BAECs. Vasculature is densely developed in tumor tissues, and therefore endothelial cells are located close to tumor cells. Endothelium-derived NO might therefore affect the adjacent tumor cells. We then examined the effects of NO released from the irradiated endothelial cells on tumor cell growth.

For this purpose, we cocultured human hepatoma cell line HepG2 with 2-Gy-irradiated BAECs for 72 h. As shown in Fig. 7, cell proliferation, as assessed by BrdUrd incorporation into the nucleus, was significantly inhibited in HepG2 cells that were cocultured with irradiated BAECs compared with cells that were cultured alone or with nonirradiated BAECs. This was also observed in HepG2 cells cocultured with BAECs that were treated with IFN$\gamma$/LPS. In contrast, BrdUrd incorporation was restored in HepG2 cells cocultured with irradiated BAECs in the presence of 0.1 mM L-NAME. Hence it is clear that NO generated from irradiated BAECs by iNOS does not stimulate but rather suppresses the proliferation of HepG2 cells.

Effects of NO Generated from Irradiated BAECs on Tumor Cell Apoptosis. We then examined whether NO generated from irradiated BAECs induced apoptosis in cocultured HepG2 cells with a comet assay. Cells showing DNA damage of grade 3 and grade 4 were regarded as apoptotic cells. As shown in Fig. 8, apoptosis was only observed in a few percent of HepG2 cells that were cultured alone (0.4 mM l-arginine, 3.5 ± 0.3%, $n = 4$; 3 mM l-arginine, 4.2 ± 0.3%, $n = 4$). When apoptosis was induced by 15 ng/ml tumor necrosis factor $\alpha$ and 40 $\mu$M cycloheximide (29), 38.9 ± 3.4% ($n = 4$) of HepG2 cells showed DNA fragmentation. Apoptosis was not observed in HepG2 cells that were coincubated with sham-irradiated
BAECs (0.4 mM L-arginine, 4.6 ± 0.5%, n = 4; 3 mM L-arginine 5.9 ± 1.1%, n = 5). In contrast, HepG2 cells cocultured with 2-Gy-irradiated BAECs showed apoptosis depending on L-arginine concentration (0.4 mM L-arginine, 14.4 ± 1.4%, n = 4; 3 mM L-arginine, 26.9 ± 2.1%, n = 7). This was significantly suppressed by L-NAME (4.0 ± 0.3%, n = 4), thereby indicating that NO was responsible for the induction of apoptosis. Furthermore, HepG2 cells cocultured with BAECs pretreated with IFN-γ/LPS also showed marked apoptosis (28.4 ± 0.5%, n = 4).

Effects of Irradiation on iNOS Expression in HepG2 Cells. Finally, we examined the effects of irradiation on the expression of iNOS protein in HepG2 cells. However, Western blot analysis revealed that the expression level of iNOS in HepG2 cells was not increased at 12 h after 2 Gy of irradiation; i.e., expression of iNOS protein in irradiated HepG2 cells was 0.96 ± 0.01X (n = 4) that in control cells.

DISCUSSION

It has been suggested that undesirable vascular side effects of radiation therapy, such as stenosis or fibrosis, could be attributed to the impairment of constitutive NO production in endothelium (2–4). In contrast, another group reported that intravascular irradiation did not affect endothelium-dependent vasodilation (5). The present study may support the latter argument because single X-ray irradiation did not induce significant changes in Ca²⁺ mobilization and expression of eNOS mRNA in BAECs (Figs. 1–3). However, we have examined eNOS mRNA only with semiquantitative RT-PCR, and we were unable to measure Ca²⁺-dependent NO production directly with DAF-2 because of the Ca²⁺-independent continuous NO production in irradiated BAECs.

We have shown that NO was generated continuously from irradiated BAECs in the presence of L-arginine and that L-NAME suppressed it (Fig. 4). We have also clearly demonstrated that a single irradiation of 2 Gy, a clinically used dose, induced strong expression of iNOS in BAECs (Figs. 5 and 6). The time course of NO production after irradiation (Fig. 4C) was similar to that of iNOS protein expression (Fig. 6). Sham-irradiated control cells also showed a marginal level of iNOS protein expression (Fig. 6) and NO production (Fig. 4A). However, iNOS expression and NO production in irradiated BAECs was much greater than those in sham-irradiated cells (Fig. 4D and 6). Therefore, we conclude that X-ray irradiation induces continuous NO production because of iNOS expression in BAECs. Induction of iNOS by irradiation has been reported in various tissues (6–9), and this is the first report demonstrating the expression of iNOS by irradiation in vascular endothelium.

NO produced by iNOS generally has a dual effect on tumor growth; i.e., it shows anti- and pro-tumor actions, and the former are due to the induction of tumor cell apoptosis (15). We have shown in this study that BrdUrd incorporation into HepG2 cells was not increased but suppressed by irradiated BAECs (Fig. 7). Because we have used an ELISA assay to measure BrdUrd incorporation, or DNA synthesis, averaged over whole cell population, this indicates that the proliferation of HepG2 is inhibited as a whole by iNOS-derived NO. It should be noted, however, that apoptosis was markedly induced in HepG2 cells cocultured with irradiated BAECs (Fig. 8); therefore, whether iNOS-derived NO also inhibited DNA synthesis in nonapoptosing cells cannot be concluded from the present results. Apoptosis increased according to the concentration of L-arginine and was inhibited by L-NAME, thereby indicating that NO was responsible (Fig. 8). This was supported by the fact that coinucbation with IFN-γ/LPS-treated BAECs also induced apoptosis of HepG2 cells (Fig. 8). It is noted that KOS produces O₂⁻ when L-arginine and/or tetrahydrodibi-


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