Adenoviral Gene Transfer of the Human Inducible Nitric Oxide Synthase Gene Enhances the Radiation Response of Human Colorectal Cancer Associated with Alterations in Tumor Vascularity

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ABSTRACT

Nitric oxide is a potent radiosensitizer of tumors, but its use clinically is limited by serious side effects when administered systemically. We have demonstrated previously that gene transfer of the inducible nitric oxide synthase gene (iNOS) into colorectal cancer cells enhances radiation-induced apoptosis in vitro. The objectives of this study were to further characterize the effects of iNOS gene transfer on the radiosensitivity of human colorectal cancer cells in vitro and tumors grown in athymic nude mice. Adenoviral gene transfer of iNOS (AdiNOS) into human colorectal cancer cell lines (HCT-116 and SNU-1040 cells) significantly enhanced the effects of radiation with sensitizing enhancement ratios (0.1) of 1.65 and 1.6, respectively. The radiation enhancement induced by iNOS was associated with increased iNOS expression and nitric oxide production and prevented by L-NIO, an enzymatic inhibitor of iNOS. AdiNOS treatment of HCT-116 tumors combined with radiation (2 Gy x three fractions) led to a 3.4-fold greater (P < 0.005) tumor growth delay compared with radiation (RT) alone. AdiNOS plus RT also caused significant (P < 0.01) tumor regression with 63% of tumors regressing compared with only 6% of tumors treated with RT. AdiNOS plus RT significantly (P < 0.001) increased the percentage of apoptotic cells (22 ± 4%) compared with either tumors treated with control vector plus RT (9 ± 1%), AdiNOS alone (9 ± 3%), or no treatment (2 ± 1%). These radiosensitizing effects of AdiNOS occurred at low infection efficiency (4% of tumor infected), indicating a significant bystander effect.

INTRODUCTION

Neoadjuvant therapy with radiation (RT) or combined RT and chemotherapy has advanced treatment of rectal cancer. By administering adjuvant therapy before resection, tumor downstaging occurs, allowing for greater likelihood in obtaining tumor-free surgical margins. A tumor-free surgical margin is one of the most significant variables associated with cure in rectal cancer (1, 2). By downstaging the tumor, tumors unresectable previously may be successfully removed, and there is increased salvage of the anal sphincter muscles and continence (3, 4). Despite this encouraging advance in the treatment of rectal cancer, 21–62% of rectal cancers fail to respond to irradiation (4–8). Therefore, therapies that enhance the effects of neoadjuvant therapy hold promise for improving the overall results in this disease.

We hypothesized that nitric oxide (NO) produced by gene transfer of the high output inducible nitric oxide synthase (iNOS) gene would be a useful adjunct to enhance the cytotoxic effects of RT of cancer cells. NO formation is known to interfere with cellular respiration and deplete cellular ATP by inhibiting enzymes in the glycolytic pathway, Krebs cycle, and electron transport chain (9, 10). High concentrations of NO cause DNA damage associated with inhibition of DNA ligase (11). In combination with RT, NO inhibits growth and sensitizes oxic tumor cells to irradiation (12). In hypoxic environments, similar to that seen in the central portion of tumors where cells are often most radioresistant, NO has also been shown to augment the cytotoxic effects of RT (13, 14). Our laboratory first cloned the human iNOS gene (15) and developed an adenoviral vector carrying the iNOS cDNA driven by a cytomegalovirus promoter. We have shown previously that transfection of the human iNOS gene leads to high output NO production (16). We recently demonstrated that overexpression of iNOS in human colorectal cancer cells enhances the acute cytotoxicity of RT with increased apoptosis in vitro (17). The objectives of this study were to: (a) further characterize the effects of iNOS gene transfer on the radiosensitivity of human colorectal cancer cell lines in vitro; and (b) to determine whether intratumoral injection of an adenovirus carrying the human iNOS (AdiNOS) enhances the RT response of human colorectal cancers.

MATERIALS AND METHODS

Cell Lines. The SNU-1040 cell line was obtained from the Korean Cell Bank (Seoul, Korea) and HCT-116 cell line from the American Tissue Type Culture Collection (Rockville, MD). HCT-116 cells are primary colorectal adenocarcinoma cells that express wild-type p53. All cells were maintained in DMEM with 10% FCS + 1% L-glutamine.

Viral Vectors. First generation adenoviral vectors deleted in the E1, 3a genes carrying either the human inducible nitric oxide synthase gene (AdiNOS) or LacZ gene (AdLacZ) were prepared by GenVec (Gaithersburg, MD; Ref. 16). First generation adenoviral vectors deleted in the E1, 3a genes carrying no exogenous gene (AdPsi-5) were prepared by our core viral vector facility. Cells were transduced with the human iNOS (AdiNOS) or either the control vector AdLacZ or AdPsi-5.

After aspiration of media, cells (2.5 x 10⁴) were infected with either AdiNOS or AdLacZ diluted in Optimum solution (Life Technologies, Inc., Carlsbad, CA). After 4 h, the viral infection was stopped by aspirating the media, washing twice with DMEM, and then adding DMEM + 10% FCS.

NO Reagents and Measurement of Nitrite Production. The NO donor, S-nitroso-N-acetyl penicillamine (SNAP) was used in several experiments (Sigma Co., St. Louis, MO) to mimic the effects of gene transfer of iNOS. SNAP was decomposed by allowing oxidation to occur at 37°C for 7 days, and oxidized SNAP (ox-SNAP) served as a negative control. The NO donor DETA/NONOate (DETA/NO; Alexis Biochemical, San Diego, CA) was used in other experiments. DETA/NO was decomposed by allowing oxidation to occur at 37°C for 7 days, and oxidized DETA/NO (ox-DETA/NO) served as a negative control. In some experiments, iNOS-transduced cells were pretreated with 100 µM N-iminoethyl-L-ornithine (L-NIO), a NO inhibitor to abrogate the effects of iNOS expression.

The release of NO by SNAP and complete oxidation of SNAP were determined by measuring nitrite levels as a function of time by Griess reaction. The production of NO in iNOS-transduced cells was also measured by Griess reaction. The Griess reaction was performed under neutral conditions (18), as described previously with naphthylendiamine dihydrochloride, sulfuramidine,
and phosphoric acid. Nitrite levels were determined by measurements at 550 nm using a spectrophotometer.

**Clonogenic Assay.** Cell survival under aerobic and hypoxic conditions was determined by clonogenic assay. Plating efficiency ranged from 60 to 89%. Aerobic irradiations were performed at low cell density with cells in single cell suspension. Cells were irradiated with graded doses (0–12 Gy) of external beam irradiation (80.8 rads/min, Cesium-137 source, model 68 mark I small animal irradiator: JL Shepherd and Associates). After irradiation, cells were plated in colony dishes (n = 4 wells/group) at variable density depending on the RT dose given (100–100,000 cells/plate), and experiments were repeated a minimum of two times. Formed colonies (>50 cells) were manually counted at 10–14 days. The plating efficiency was calculated by: PE = (# colonies counted/# cells seeded) × 100 and the Surviving Fraction (SF) determined by: SF = (# colonies/# cells seeded × PE/100). Surviving fractions were calculated and linear-quadratic fits accomplished using the FIT program (version 2) for analysis of cellular survival data (developed by Dr. N. Albright, Department of Radiation Oncology, University of California at San Francisco, San Francisco CA). Error bars represent SD of the mean. The Dq and Do were determined from the survival curves using the single-hit multitarget model of RT dose survival. The Dq is the point at which the shoulder region of the curve transforms into an exponential region and the injury from RT converts from being sublethal to lethal. The Do represents the inherent radiosensitivity of cells. Sensitizer enhancement ratios (SERs) were calculated by dividing the RT dose for control conditions (AdLacZ or ox-SNAP) by the RT dose for varied treatment conditions (AdiNOS or SNAP) at the 10% survival fraction (SER 0.1). Cells were plated in glass flasks 24 h before hypoxia induction as described previously (19). SNAP or ox-SNAP was added to flask immediately before hypoxia induction. Each flask was then sealed with a white, nontoxic rubber stopper, and two hypodermic needles were inserted for humidified gas inlet and outflow. The flasks were connected in series, placed on a reciprocating platform, and were gassed with 95% nitrogen/5% carbon dioxide for 1 h at 300 ml/min at 37°C. Before irradiation, the needles were removed (outlet needle first) with the gas flowing, so no air was allowed to enter the vial. The flasks were irradiated with an Eldorado 8 60Co teletherapy unit (Theraratron International Ltd., Kanata, Ontario, Canada, formerly Atomic Energy of Canada, Ltd.) at a dose rate of ~200 μGy/min. Decay corrections were done monthly, and full electron equilibrium was ensured for all irradiations. After irradiation, the cells were plated for clonogenic cell survival as described above.

**Immunohistochemistry.** For analysis of iNOS protein expression, cells were plated (5 × 10⁴ cells) on autoclaved glass coverslides placed in 24-well plates in DMEM + 10% fetal bovine serum. The cells were then fixed in paraformaldehyde 2% for 2 h at 4°C. Cells were stained with an iNOS rabbit polyclonal antibody IgG 1:500 (Transduction Laboratories, Lexington, KY). The secondary antibody was a goat-anti-rabbit antibody Alexa 488 (Molecular Probes, Eugene, OR). After the secondary antibody was washed with BSA and PBS, the slides were stained with Hoescht stain. The slides were coverslipped with gelvatol and dried overnight at 4°C overnight. For in vivo experiments, tumors were sectioned (5 μm thick) and similarly stained for iNOS. From tumor sections, tumor cells were also identified with a polyclonal human carcinoembryonic antigen antibody (Fitzgerald Industries, Cambridge, MA), and blood vessels were identified with a rat antisense cd31 antibody (Serotec, Inc., Raleigh, NC) with secondary antibodies of goat anti-rabbit cy3 3 (Jackson ImmunoResearch Laboratories, West Grove, PA) and goat antirabbit cy5 3 (Jackson ImmunoResearch Laboratories), respectively. After the secondary antibody was washed with BSA and PBS, the slides were stained with Hoescht stain. All slides were examined using an Olympus BX 51 fluorescent microscope.

**Terminal Deoxynucleotidyl Transferase-Mediated Nick End Labeling Assay.** Tumors were harvested and fixed in paraformaldehyde 2% and kept at −80°C before examination. Tumor sections (5 μm thick) were examined for apoptosis by the terminal deoxynucleotidyl transferase-mediated nick end labeling technique as described by the Apoptosis Detection System (Promega). Detection of localized green fluorescent apoptotic cells (fluorescein 12-dUTP) in a red background (propidium iodide) was performed by fluorescence microscopy (20). The apoptotic fraction was determined in a blinded fashion by manually counting the frequency of green and red fluorescent cells per five random high powered fields (×20 magnification) for each tumor section (two to three sections per tumor).

**Cell Cycle Analysis.** Cells were either maintained in log phase or rested for 72 h in serum-free media and then infected with AdLacZ or AdiNOS and harvested 48 h after. Cells were incubated in bromodeoxyuridine (1 μm/ml) and harvested as per the manufacturer’s instructions (bromodeoxyuridine flow kit; BD PharMingen, San Diego, CA). Flow cytometric analysis (CoulterXL flow cytometer) was used to measure cellular incorporation of bromodeoxyuridine (stained with FITC anti-bromodeoxyuridine). Total DNA content was measured with 7-amino-actinomycin D (21).

**Tumorigenicity.** The effect of iNOS overexpression on the tumorigenicity of colorectal cancer cells was examined in HCT-116 cells. HCT-116 cells were infected with AdiNOS or Ad-CTL (Ad-Psi5) in vitro and 24 h after cells were injected into the right hind limb of athymic nude mice. The effect of iNOS transduction on the ability of colorectal cancer cells to form tumors was examined for 18 days.

**Measurement of Tumor Hypoxic Fraction.** To detect hypoxic regions within the tumor, a pentfluorinated derivative (EFS) of etanidazole was injected i.v. into mice 3 h before tumor harvest. Tumors were sectioned and stained with ELK3–51 antibody conjugated with Cy3 dye (22).

**In Vivo RT Experiments.** Athymic nude mice (Harlan Laboratories, Indianapolis, IN) were injected with 1 × 10⁶ tumor cells in the right hind limb. Tumor size was measured daily in two dimensions using Vernier calipers. When tumors reached 3–4 mm in diameter, tumor volume (V) was calculated according to the following equation: V (mm³) = (π/6) (mean diameter)^2. Mice were randomized to treatment groups to ensure equivalent starting tumor volumes. Tumors were injected with no vector, AdiNOS, or a control vector. The control vector (Ad-CTL) was AdLacZ and Ad-Psi5, respectively, for single and multifractionated irradiation experiments.

**Single or multifractionated irradiation was administered to the tumor-bearing leg using photons (6 mV) with 250 cGy fractions (106 monitor units/100 cGy) at a rate of 250 monitor units/s with 1 cm bolus using a Varian Clinac 600C irradiator. Mice were anesthetized, and the tumor was centered in a 30 × 1.8 cm circular irradiation field. In all experiments, mice were followed for 18 days with serial tumor measurements for a minimum of tumor doubling times. The time for three doublings in each tumor volume (tumor doubling) was determined. The growth delay (GD) for each treated tumor was determined by the formula GD = time for three doubling times (each treated tumor) – mean time for three doubling times (control, untreated tumors). Tumor regression (>10% volume decrease sustained for a minimum of 2 days) was determined. The radiopotentiation was calculated as GD (AdiNOS + RT)/GD (AdiNOS) + GD (RT alone), as described by others (23).

**Statistical Analysis.** The survival curves were determined for each condition from the results of the clonogenic assay. The Dq and Do were calculated. Statistical comparisons were performed by one-way ANOVA using computerized software (SPSS, Inc., Chicago, IL). The results of the tumorigenicity experiments were analyzed by y^2. Tumor regrowth curves and apoptosis of varied treatments were compared by one-way ANOVA andχ² where appropriate. Tumor blood vessel counts were compared by t test. Significance was defined as a minimum of P = 0.05 for each comparison.

**RESULTS**

**AdiNOS Infection of Cancer Cells Results in High Output NO Production.** Cell lines were infected with AdiNOS at variable multiplicities of infection to determine the optimal concentration of viral vector required to induce high output NO production. AdiNOS induced the most significant (P < 0.001) increase in nitrite levels (Fig. 1A) at 1,10 plaque-forming units (pfu) for SNU-1040 and HCT-116 cells, respectively. There was no increase in nitrite levels for the corresponding control vector. Both cell lines expressed iNOS protein at 24 h after iNOS gene transfer (Fig. 2). There was no detectable iNOS protein expression in cells treated with either control vector or untreated control.

**Overexpression of iNOS Enhances Radiosensitivity of Colorectal Cancer Cells.** The effects of iNOS overexpression on the radiosensitivity of colorectal cells were characterized in HCT-116, SNU-1040 cells (Figs. 1, B–D). Cells were infected with AdiNOS or
AdLacZ at multiplicities of infection demonstrated previously to induce high output NO production (1, 10 pfu/cell for SNU-1040, HCT-116 cells, respectively). Cells were then exposed to a single dose of external beam irradiation (0–12 Gy) at 48 h after viral infection.

Overexpression of iNOS by AdiNOS infection significantly enhanced the effects of RT (Fig. 1, B–D). The SERs (0.1) were 1.65 and 1.6 for HCT-116 and SNU-1040 cells, respectively. The RT enhancement induced by iNOS overexpression was the result of both a decrease in the Dq and Do of the survival curve (Fig. 1D). For both cell lines, the radiosensitizing effect of iNOS gene transfer was predominantly a result of a decrease in the Dq. For HCT-116 cells, iNOS overexpression resulted in a 80-fold decrease in the Dq and for SNU-1040 cells a 6.3-fold decrease in the Dq.

NO Mediates the Radiosensitizing Effects of AdiNOS. To determine whether NO mediates the radiosensitizing effects of iNOS gene transfer, additional experiments were performed with an iNOS inhibitor and NO donors. HCT-116 cells were transduced with AdiNOS with or without 1-h preincubation with 1 mM L-NIO and then irradiated (Fig. 3A). Controls included cells incubated with AdLacZ, L-NIO alone or no treatment. Colony counts were determined and compared relative to no treatment (control). iNOS gene transfer resulted in enhancement (P < 0.001) in cytotoxicity at both 1 and 2 Gy of RT. Preincubating cells with L-NIO significantly (P < 0.001) prevented the radiosensitizing effects of iNOS gene transfer and was associated with suppression of NOS activity (48-h nitrite levels of 3.0 and 2.9 ± 0.1 for cells infected with iNOS plus L-NIO pretreatment or untreated cells compared with 22.5 ± 0.1 for AdiNOS-infected cells).

The effect of exogenously added NO on the radiosensitivity of colorectal cancer cells (SNU-1040, HCT-116 cells) was examined with the addition of NO donors, SNAP (500 μM) for 1 h, or DETA/NO (50, 100 μM) for 16 h before irradiation. Incubation of SNU-1040 cells with SNAP resulted in a significant (P < 0.001) increased radiosensitivity of cells with an SER (0.1) of 2.53 (Fig. 3, B and E). Addition of the NO donor SNAP to HCT-116 cells led to enhanced radiosensitivity only under hypoxic conditions (Fig. 3, C and E). The SER was 1.6 for HCT-116 cells exposed to SNAP under hypoxic conditions compared with 0.97 for oxic cells. HCT-116 cells (oxic conditions) exposed to DETA/NO (50, 100 μM) for 16 h exhibited a significant dose-dependent increase in radiosensitization with SERs of 1.16–1.6 (Fig. 3E). The enhancement of radiosensitivity was predominantly the result of a decrease (2.5–20.3-fold) in the Dq.
Cell Cycle Changes Do Not Account for the Radiosensitizing Effects of AdiNOS. To determine whether cell cycle changes could account for the RT enhancement of AdiNOS, cell cycle analysis was performed on cells 48 h after AdiNOS infection (just before irradiation). HCT-116 cells in log phase or growth arrest were infected with either AdiNOS (10 pfu) or AdLacZ (10 pfu). Cell cycle distribution was determined by flow cytometry. For HCT-116 cells growing in log phase, the percentage of cells in subG0, G0-G1, S phase, and G2-M were 0.7, 59.5, 10.3, and 24.5%, respectively, for cells infected with AdiNOS, which was similar to the distribution after AdLacZ treatment (0.3, 61.1, 16.6, and 21.5%, respectively). For HCT-116 cells that were growth arrested by serum deprivation, the percentage of cells in subG0, G0-G1, S phase, and G2-M were 7.4, 49.4, 9.5, and 20.9%, respectively, for cells infected with AdiNOS, which was similar to the distribution after AdLacZ treatment (0.9, 63.8, 10.1, and 17%, respectively). AdiNOS infection (1 pfu) also did not alter the cell cycle distribution of SNU-1040 cells (48-h postinfection) with respective subG0, G0-G1, S phase, and G2-M of 1.1, 46, 13.3, and 32.4% compared with 0.6, 50, 10.9, and 31% for cells infected with AdLacZ (1 pfu).

AdiNOS Has No Effect on Tumorigenicity. To determine whether decreased tumorigenicity could account for the RT enhancement induced by AdiNOS, HCT-116 cells were infected with AdiNOS and then injected into athymic nude mice. HCT-116 cells were infected with either AdiNOS (1 or 10 pfu/cell), AdLacZ (1 or 10 pfu/cell), or no treatment. AdiNOS and AdLacZ infected cells (1 × 106 cells) were injected into 32 athymic nude mice with equal frequency of the mice injected with cells infected with 1 and 10 pfu/cell of viral particles. Untreated HCT-116 cells were injected into an additional 9 mice. Cells infected with AdiNOS demonstrated significantly increased nitrite production in vitro (data not shown). There was no significant (P = 1) effect of AdiNOS infection on tumorigenicity. At 18 days after injection, all mice injected with tumor cells transduced with the iNOS gene had grown tumors. Tumors were not present in only 3 mice: (a) 1 mouse injected with cells treated with Ad-CTL; and (b) 2 mice injected with untreated cells.

AdiNOS Infection Efficiency in Vivo. HCT-116 tumors grown in athymic nude mice were injected with Ad-CTL or AdiNOS for two consecutive days using equivalent volumes (20 μl/day) and frequency of viral particles (9 × 107 pfu). Immunohistochemical analysis was performed for iNOS protein expression (Fig. 4, A and B). Tumors were sectioned, and the total tumor area and area of iNOS-positive cells were determined at 48 h after intratumoral injection with either Ad-CTL or AdiNOS. For tumors (n = 3) injected with AdiNOS, iNOS protein expression was present in 4.4 ± 1.4% of the tumor and persisted for 72 h after intratumoral gene injection. In contrast, iNOS protein expression was not detectable in tumors infected with Ad-CTL (n = 3).

Tumor Hypoxia. In mice injected with EF-5, a large central area of the tumor (HCT-116) stained positive for EF-5, indicating a significant hypoxic center (Fig. 4D), Tumors of mice injected with saline alone had no autofluorescence (Fig. 4C).

Intratumoral AdiNOS Injection Enhances Radiosensitivity of Colorectal Cancers. AdiNOS treatment of HCT-116 tumors significantly (P ≤ 0.005) delayed tumor doubling time and growth when combined with single or multifractionated RT (Fig. 5).
For single fraction RT experiments, 40 tumor-bearing mice were randomized to the following four groups (n = 10 mice/group): (a) no treatment (CTL); (b) RT (2 Gy) alone; (c) tumor injection with Ad-CTL plus RT (2 Gy); or (d) tumor injection with AdiNOS plus RT (2 Gy). Tumors were injected with adenovirus for five consecutive days with equivalent volumes (20 μl/day) and frequency of viral particles (9 × 10⁷ pfu). Tumors were irradiated with a clinically relevant single fraction of 2 Gy on day 3 of adenoviral injection. AdiNOS combined with irradiation delayed tumor growth 3.6-fold greater (P = 0.001) than RT alone (Fig. 5, A and B). RT was not enhanced (P = 0.7) by tumor injection with Ad-CTL.

For multifractionation RT experiments, 48 tumor-bearing mice were randomized to six groups (n = 8 mice/group): (a) no treatment (CTL); (b) RT alone (2 Gy × three fractions); (c) Ad-CTL injection; (d) AdiNOS injection; (e) Ad-CTL injection plus RT (2 Gy × three fractions); and (f) AdiNOS injection plus RT (2 Gy × three fractions). Tumors were injected with adenovirus for two consecutive days using equivalent volumes (20 μl/day) and frequency of viral particles (9 × 10⁷ pfu). RT was initiated on the 2nd day of viral injection with three consecutive days of treatment with clinically relevant fractions of 2 Gy.

AdiNOS combined with RT delayed tumor growth 3.4-fold greater (P < 0.01) than RT alone (Fig. 5, C and D). The mean GD for tumors treated with AdiNOS plus RT was 8.5 days, which was greater than the combined mean GDs of tumors treated with AdiNOS alone (1.9 days) and RT alone (2.5 days). As a result, tumor injection with AdiNOS caused a 1.9-fold radiopotentiation. RT was not enhanced (P = 0.5) by tumor injection with Ad-CTL.

AdiNOS injection combined with RT also caused significant enhancement (P < 0.01) tumor regression (24–72-h postirradiation) with 63% of tumors regressing compared with only 6% of tumors treated in the RT control groups. The mean tumor volume decrease was 40% in tumors treated with AdiNOS combined with RT.
AdiNOS Plus RT Induces Tumor Cell Apoptosis in Vivo. The apoptotic fraction was measured at 24 h after either single fraction or multifractionated RT. AdiNOS plus single fraction RT was associated with an apoptotic fraction of 0.22 ± 0.04, which was significantly greater (P < 0.001, by ANOVA) than either AdiNOS alone (0.09 ± 0.03), Ad-CTL plus RT (0.09 ± 0.01), or no treatment (0.02 ± 0.01). AdiNOS injection plus three fractions of RT was associated with an apoptotic fraction of 16.9 ± 0.03%, which was significantly (P < 0.001) greater than the apoptotic fraction (2.3 ± 0.01%) of tumors injected with Ad-CTL plus three fractions of RT (Fig. 6).

To determine the cell type undergoing apoptosis in vivo, tumor sections were stained immunohistochemically with carcinomaembryonic antigen to localize the tumor cells and CD31 to localize the endothelial and terminal deoxynucleotidyl transferase-mediated nick end labeling to detect apoptotic cells. Apoptosis was only demonstrated in carcinomaembryonic antigen-positive cells, indicating that AdiNOS plus RT induced apoptosis in tumor cells in vivo (Fig. 7A).

Apoptosis occurred as early as 24 h after RT, which was before tumor regression, suggesting that apoptosis is an important mechanism accounting for tumor regression and delayed tumor growth induced by iNOS plus RT.

AdiNOS Injection Enhances Tumor Vascularity. Because NO may increase tumor vascularity, which could enhance tumor radioresponsiveness (24), we determined whether AdiNOS injection increased the vascularity of tumors. Blood vessel counts were quantified (µm²) by staining tumor sections with CD31. Tumors from experiments examining the effects of AdiNOS in combination with multifractionated irradiation (Fig. 5, C and D) were used. Tumors from mice injected with AdiNOS but not irradiated were compared with untreated mice on days in which RT was administered to other tumors. Tumors (n = 4) injected with AdiNOS had a 2.9-fold increase (P < 0.001) in tumor vascularity compared with untreated tumors (n = 4). Blood vessel counts (µm²) for AdiNOS-treated tumors were 0.045 ± 0.005 compared with 0.0155 ± 0.002 for untreated tumors. The blood vessels in AdiNOS-treated tumors were associated with surrounding cells overexpressing iNOS (Fig. 7B), suggesting a causal relationship between AdiNOS injection and enhanced tumor vascularity.

**DISCUSSION**

Although NO is a promising radiosensitizer, the use of NO donors to augment the effects of RT in vivo has significant limitations. In vivo administration of these agents results in systemic hypotension and may increase tumor perfusion and oxygenation, potentially promoting tumor growth (25). Overexpression of iNOS in tumors by localized direct intratumoral injection of the iNOS gene has the potential of minimizing the systemic side effects of NO while maintaining the salutary tumoricidal effects of high output paracrine NO release.

Several investigators have examined the potential beneficial effects of intratumoral iNOS gene transfer. Direct injection of iNOS cDNA led to decreased tumor growth associated with necrosis in a medullary thyroid cancer model (26). Tumor cell growth was inhibited using a retroviral vector engineered to overexpress iNOS selectively in carcinomaembryonic antigen-producing cells (27). Intratumoral injection of human kidney cells engineered to overexpress iNOS significantly delayed colon and ovarian cancer growth (28). Direct intratumoral injection of iNOS cDNA has been reported to augment the effects of RT on a radioresistant murine sarcoma in vivo (29). We have demonstrated previously that overexpression of the iNOS gene using an adenoviral vector enhances RT-induced apoptosis in human colorectal cancer cells via a caspase-dependent mechanism (17).

Given the salutary effects of NO in colorectal cancer, we examined...
the overexpression of the human iNOS gene in preclinical colorectal cancer models. In vitro, we demonstrated that overexpression of iNOS in two colorectal cancer cell lines results in significant radiosensitization with SERs (0.1) of 1.6–1.65 and is mediated by high output of NO production. This degree of radiosensitization compares favorably to results with 5-FU, the current radiosensitizer used in treating rectal cancer. In one study, 5-FU radiosensitized two colorectal cancer cell lines with SERs of 1.1–1.2 (30). Another report also found that 5-FU significantly radiosensitized three colorectal cancer cell lines with SERs of 1.1, 1.3, and 2.1 (31). The radiosensitizing effects of iNOS are also comparable with results with CPT-11, a topoisomerase I inhibitor being explored as a promising radiosensitizer for rectal cancer. CPT-11 radiosensitized HT-29 colon cancer cells, with an SER of 1.5 (32). This degree of radiosensitization is clinically relevant as evidenced by significant downstaging of locally advanced rectal cancers in a recent clinical trial using CPT-11 as a radiosensitizer (33).

To test the efficacy of iNOS gene transfer as a radiosensitizer, we further examined the effects of direct intratumoral gene delivery of iNOS combined with both single and multifractionated irradiation on colorectal cancer growth. Gene transfer of iNOS combined with a single fraction of irradiation resulted in a 3.6-fold greater tumor GD compared with treatment with RT alone. Gene transfer of iNOS combined with multiple fractions of RT resulted in even more pronounced effects than with a single dose of irradiation. In experiments using multifractionated RT, mice received 60% less AdiNOS compared with experiments with a single RT fraction (two injections versus five injections of AdiNOS, respectively). Despite significantly less iNOS gene, there was a similar tumor GD in mice treated with iNOS plus multifractionated RT (3.4-fold GD) compared with iNOS plus single fractionated RT (3.6-fold GD).

The overall radiopotentiation with AdiNOS injection was 1.9 in HCT-116 colorectal tumors, which is close to the radiopotentiation (2.8) achieved by intratumoral injection of iNOS into a murine fibrosarcoma (29). The radiopotentiation that we observed with AdiNOS injection also compares favorably to recent studies examining other promising radiosensitizers. In SW620 colon cancers, adenoviral gene transfer of p53 resulted in a radiopotentiation of 2.2 (34). Iressa, an epidermal growth factor receptor tyrosine kinase inhibitor, has been shown to potentiate RT (1.6) in LoVo colon cancers (35). Another promising radiosensitizer NS-398, a cox-2 inhibitor, radiosensitized H460 lung cancers but had no effect on HCT-116 colorectal tumors (36). We observed a significant bystander effect with iNOS gene transfer. AdiNOS injection plus RT significantly prolonged tumor growth and was associated with partial tumor regression and increased apoptosis compared with all other treatments. This occurred despite the fact that only a minority of the tumor (4% of tumor) was transduced.
with iNOS. This bystander effect may be explained in part by increased tumor vascularity. Although gene transduction was low in tumors treated with AdiNOS, these tumors were 3-fold more vascular. The blood vessels in tumors injected with AdiNOS were in close proximity to the cells overexpressing iNOS, suggesting that the NO produced in these cells may have diffused out locally and stimulated angiogenesis. NO is known to enhance angiogenesis by up-regulating endothelial growth factors, such as vascular endothelial growth factor (24). Although we did not specifically measure tumor oxygenation after AdiNOS injection, a 3-fold increase in tumor vascularity most likely improved tumor oxygenation and contributed to the enhanced radiosensitivity of tumors. These data suggest that overexpression of iNOS enhances aerobic radiosensitization by increasing tumor vascularity. These findings are significant in light of the fact that intrinsic tumor radiosensitivity has been recently linked to NO-induced tumor blood flow changes, which enhance tumor oxygenation (37).

Because a major limitation of cancer gene therapy is the low viral infection efficiency into tumors, iNOS gene transfer with its significant bystander effect has great clinical potential. This bystander effect is also valuable because NO production may occur only to a limited extent in the hypoxic center of tumors. This hypoxic region is also the most radioresistant part of the tumor. Because NO is highly diffusible, we hypothesized that significant radiopotentiation could occur even if iNOS was only produced at the periphery of this hypoxic region. We found that HCT-116 tumors had large central hypoxic regions at the time of treatment and iNOS was produced predominantly in the peripheral portion of the tumor. Despite the fact that the most radioresistant portion of the tumor was at a significant distance from the site of iNOS expression, iNOS gene transfer significantly enhanced the effects of RT. This suggests that either the diffusibility of NO can compensate for a nonuniform distribution of NO production within tumors or that overexpression of iNOS enhances tumor oxygenation by increasing tumor vascularity, thus leading to enhanced radioresponsiveness of even hypoxic tumors.

A second potential limitation of adenoviral gene therapies is destruction and clearance of the virus itself by the immune system. The immune response is characterized by class II MHC-dependent activation of T helper and B cells to caspid proteins of the virus, which leads to antibodies neutralizing the virus (38). Transient depletion of CD4 lymphocytes improves the efficacy of adenoviral therapy (38, 39). Current studies in our lab are focused on determining whether the immune system limits the RT-enhancing effects of AdiNOS on colorectal cancer and alternative adjuncts,
such as transient CD4 depletion, are required to maintain the efficacy of AdiNOS.

Multiple studies examining potential radiosensitizers in colorectal cancer have demonstrated that apoptosis is a significant mechanism accounting for RT enhancement. In HT-29 colorectal cancer cells treated with 2′,2′-difluoro-2′-deoxycytidine added to RT, apoptosis accounted for 60% of the decrease in clonogenic survival (40). In two studies examining radiosensitizing therapies in HCT-116 cells, apoptosis was also a significant mechanism of cell and tumor death (41, 42). We have demonstrated previously that AdiNOS increases RT-induced apoptosis 4-fold in HCT-116 cells (17). Similarly in this study, direct intratumoral injection of INOS enhances the RT response associated with a 2-fold increase in apoptosis. Because there was no effect of AdiNOS on either cell cycle distribution or tumorigenicity, apoptosis seems to be a primary mechanism for the enhanced radiosensitivity induced by INOS gene transfer.

We have demonstrated previously that adenoviral delivery of the iNOS gene enhances RT-induced apoptosis in colorectal cancer cells (17). This current study is the first to examine the use of adenoviral iNOS gene delivery to radiosensitize human cancer in vivo. We have demonstrated that overexpression of the human inducible NO synthase gene by adenoviral gene delivery radiosensitizes both human colorectal cancer cells and tumors associated with increased apoptosis. The apoptosis occurs before tumor regression in iNOS plus RT-treated tumors suggesting that apoptosis plays a causal role in the radiosensitization induced by AdiNOS. The radiosensitizing effects of iNOS occur at low infection efficiency, indicating a significant bystander effect. Our data and those of others indicate that overexpression of iNOS by gene transfer has enormous potential as a radiosensitizer of hypoxic cancers (29). Our data further suggest that the radiosensitizing effects may partly be mediated by enhanced tumor vascularity and oxygenation induced by NO, enabling significant effects despite low gene transduction.

Fig. 7. A, localization of apoptosis in tumors. Representative section from tumor treated with adenoviral gene transfer of inducible nitric oxide synthase injection plus radiation. Tumor was stained with antibody to carcinoembryonic antigen (red, tumor cells), antibody to CD31 (blue, endothelial cells), and terminal deoxynucleotidyl transferase-mediated nick end labeling (green cells). Terminal deoxynucleotidyl transferase-mediated nick end labeling colocalized only with carcinoembryonic antigen-positive cells (inset at bottom right), indicating that the apoptosis occurred in tumor cells and not endothelial cells at 24 h after adenoviral gene transfer of inducible nitric oxide synthase plus radiation treatment. B, tumor vascularity. Representative section from tumor treated with adenoviral gene transfer of inducible nitric oxide synthase. Tumor was stained with an antibody to CD31 (red, endothelial cells) and inducible nitric oxide synthase (green cells). The blood vessels are associated with surrounding cells overexpressing inducible nitric oxide synthase, suggesting a causal relationship between nitric oxide production and enhanced tumor vascularity in adenoviral gene transfer of inducible nitric oxide synthase-treated tumors.
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Adenoviral Gene Transfer of the Human Inducible Nitric Oxide Synthase Gene Enhances the Radiation Response of Human Colorectal Cancer Associated with Alterations in Tumor Vascularity

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