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NOS Inhibition Modulates Immune Polarization and Improves Radiation-Induced Tumor Growth Delay

Lisa A. Ridnour¹, Robert Y.S. Cheng¹, Jonathan M. Weiss², David R. Soto Pantoja³, Debashree Basudhar¹, Julie L. Heinecke¹, Andrew Stewart², William DeGraff¹, Anastasia L. Sowers¹, Angela Thetford¹, Aparna H. Kesarwala⁴, David D. Roberts³, Howard A. Young², James B. Mitchell¹, Giorgio Trinchieri², Robert H. Wilttrout², and David A. Wink¹

¹Radiation Biology Branch, Center for Cancer Research, National Cancer Institute, Frederick, MD 21702

²Cancer Inflammation Program, Center for Cancer Research, National Cancer Institute, Frederick, MD 21702

³Laboratory of Pathology, Center for Cancer Research, National Cancer Institute, Bethesda, MD 20892

⁴Radiation Oncology Branch, Center for Cancer Research, National Cancer Institute, Bethesda, MD 20892

Abstract

Nitric oxide synthases (NOS) are important mediators of pro-growth signaling in tumor cells, as they regulate angiogenesis, immune response, and immune-mediated wound healing. Ionizing radiation (IR) is also an immune modulator and inducer of wound response. We hypothesized that radiation therapeutic efficacy could be improved by targeting NOS following tumor irradiation. Herein, we show enhanced radiation-induced (10 Gy) tumor growth delay in a syngeneic model (C3H) but not immunosuppressed (Nu/Nu) SCC tumor-bearing mice treated post-IR with the constitutive NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME). These results suggest a requirement of T cells for improved radiation tumor response. In support of this observation, tumor irradiation induced a rapid increase in the immunosuppressive Th2 cytokine IL-10, which was abated by post-IR administration of L-NAME. *In vivo* suppression of IL-10 using an anti-sense IL-10 morpholino also extended the tumor growth delay induced by radiation in a manner similar to L-NAME. Further examination of this mechanism in cultured Jurkat T cells revealed L-NAME suppression of IR-induced IL-10 expression, which reaccumulated in the presence of exogenous NO donor. In addition to L-NAME, the guanylyl cyclase inhibitors ODQ and TSP-1 also abated IR-induced IL-10 expression in Jurkat T cells and ANA-1 macrophages, which further suggests that the immunosuppressive effects involve eNOS. Moreover, cytotoxic Th1 cytokines, including IL-2, IL-12p40, and IFN- γ , as well as activated CD8⁺ T cells were elevated in tumors receiving post-IR L-NAME. Together, these results suggest that post-IR NOS inhibition improves radiation tumor response via Th1 immune polarization within the tumor microenvironment.

Correspondence to: Lisa A. Ridnour.

No Conflict of Interest

Introduction

Radiation therapy remains a primary mode of treatment for more than 50% of cancer patients in North America (1). At the molecular level, ionizing radiation (IR) exerts its anti-tumor effects by inducing direct DNA damage in the form of DNA double-strand breaks as well as indirect damage by the generation of reactive oxygen species (2). While DNA damage has a central role in radiation-induced tumor cell death, it does not fully account for tumor response to local radiation. In addition to stimulation of DNA repair, IR induces multiple cellular signaling pathways. Importantly, cell survival depends upon the ratio of activated pro- and anti-proliferative pathways, suggesting that irradiated cells, which evade death, survive and progress to more aggressive and therapeutically-resistant tumors (3). Radiation-induced signaling pathways associated with cancer progression include elevated epidermal growth factor receptor, hypoxia inducible factor-1 (HIF-1), up-regulation and/or activation of matrix metalloproteinases (MMPs), and overexpression of cytokines including vascular endothelial growth factor (VEGF) and other immunosuppressive mediators that promote cancer survival, invasion, and metastasis (4). Thus, the biology of sub-lethally irradiated tumor cells favor survival, invasion, and angiogenesis, suggesting that therapeutic efficacy could be improved by combining radiation treatment with agents that target these or other pro-growth pathways induced by radiation (5).

Nitric oxide (NO) is an important mediator of many pro-growth signaling cascades in cancer (6-9). Nitric oxide synthases (NOS) catalyze the production of NO by the five-electron oxidation of a guanidino nitrogen atom of the substrate L-Arginine, which requires NADPH, FAD, FMN, heme, and O₂ as cofactors (10). Three NOS isoforms are known to exist; neuronal NOS (nNOS or NOS1), inducible NOS (iNOS or NOS2), and endothelial NOS (eNOS or NOS3). Nitric oxide has many diverse roles in normal physiology and tumor biology, which are spatially-, temporally-, and concentration-dependent. The constitutive isoforms eNOS and nNOS are tightly regulated by Ca²⁺/calmodulin, and produce low flux (pM) NO over short periods of time. In contrast, the inducible isoform iNOS is Ca²⁺-independent and generates higher flux NO over a longer period of time that can range from nM-μM in concentration, depending upon the stimulant (11). Nitric oxide synthase has been studied extensively in carcinogenesis. While elevated NOS3 expression has a role in tumor angiogenesis, increased NOS2 expression predicts poor therapeutic response, tumor progression, and decreased patient survival (9, 12-15). To date, our molecular signatures suggest that NO-mediated pro-survival, cell migration, angiogenesis, and stem cell marker (i.e. ERK, Akt, IL-8, IL-6, S100A8, CD44) signaling in tumors and tumor cells occurs at 400 nM steady state NO (6, 9). Together, these observations suggest that the NOS enzymes are exploitable therapeutic targets.

Nitric oxide produced by the constitutive eNOS isoform controls blood flow and is a key mediator of the pro-angiogenic effects of vascular endothelial growth factor (VEGF) (16). A clinical study demonstrated reduced tumor blood volume within one hour of administration of the competitive NOS inhibitor nitro-L-arginine (L-NNA), which lasted for twenty-four hours in all patients studied (17). Side effects of NOS inhibition included bradycardia and hypertension, which were not study limiting and suggest that NOS inhibition may be a beneficial therapeutic option for combined modalities (17). Advances in radiotherapy have

included the co-administration of anti-angiogenic (AA) drugs, which radiosensitize endothelial cells (18). Ionizing radiation also activates constitutive NOS, as well as ERK1/2 kinase pro-survival signaling, both of which were blocked by L-NNA (19). In addition, the administration of L-NNA 24 hr prior to 10 Gy irradiation of tumor-bearing mice demonstrated reduced tumor blood flow and increased tumor cell apoptosis when compared to mice receiving radiation or L-NNA alone, further supporting NOS inhibition as a target to improve radiation therapeutic response (20).

In addition to targeting tumor vasculature, IR modulates host immunity and mimics vaccine response by enhancing the release of damage associated molecular patterns (DAMPs) from dying cells, which then activate cytotoxic lymphocytes (CTLs) through toll-like receptor activation (21). Ionizing radiation facilitates antigen-presenting cell and T cell penetration into the tumor (22), and also impacts host immunity through modulation of both pro- and anti-tumor responses depending upon the Th1 (cytotoxic) vs Th2 (immunosuppressive) cytokine milieu and associated immune cell mediators (21). Macrophages exposed to Th1 cytokines exhibit increased levels of pro-inflammatory cytokine production, antigen presentation, and cytotoxic activity. In contrast, macrophages exposed to Th2 cytokines exhibit an immunosuppressive phenotype associated with blocked CTL activity, increased angiogenesis, tissue restoration and wound healing response (23). T-regulatory cells (Tregs) are pivotal mediators of immune suppression and the development of immunologic tolerance through their ability to limit antitumor immune responses (24). Tregs mediate tumor immune tolerance in part through the secretion of IL-10, TGF- β , or IL-35 immunosuppressive molecules (24). Indeed, IL-10 and TGF- β derived from Tregs promote tumor progression through antitumor immune suppression (25). Nitric oxide also mediates both cGMP-dependent and -independent Th1-Th2 immune transition and may be important in the tumor response to radiation-induced injury (26, 27). To explore this hypothesis, we examined cytokine expression profiles and T cell activation during radiation-induced tumor growth delay in a syngeneic model treated post-IR with the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME).

Materials and Methods

Cell Culture

Jurkat cells (Jurkat clone E6-1) were obtained from the ATCC and maintained in 5% CO₂, RPMI-1640 culture medium with 10% fetal bovine serum and 100 IU/ml Pen Strep antibiotics (Life Technologies). Jurkat cells were treated with ionizing radiation (0, 1, or 5 Gy) in the presence or absence of various concentrations of DETA/NO (0, 30, 60, 100, 300, 500 & 100 μ M), L-NAME (0, 500 or 1000 μ M), or the guanylyl cyclase inhibitor ODQ (1 μ M) or thrombospondin-1 (TSP-1: 1 μ g/ml). The NONOate donors including DETA/NO are stable when maintained in basic conditions (10 mM NaOH) but release NO at defined rates at physiologic pH (28); 10 mM NaOH served as vehicle control in experiments utilizing the NO donor DETA/NO. The ANA-1 macrophage cellline used in this study was established by immortalization of bone marrow macrophages from C57BL/6 mice with J2 recombinant retrovirus-expressing v-myc/v-raf oncogenes (29). ANA-1 cells were grown in Dulbecco's

modified eagle's medium (DMEM) supplemented with 10% FBS and 1% penicillin-streptomycin, plated at a density of 5×10^5 per well in a 12 well plate and grown overnight.

Suppression of IL-10

Silencing of IL-10 protein translation was accomplished by using an antisense 25-mer oligo (Gene Tools, Philomath, OR) designed specifically to block the AUG translational start site of mouse IL-10 (GenBank accession no. NM_010548: oligo sequence, 5'-AGCTCTCTTTTCTGCAAGGCTGCTT). This oligo complements the sequence from -31 to -6 relative to the initiation codon. Suppression of secreted IL-10 protein levels were verified in LPS-stimulated Raw 267.4 (30) cells pre-treated with control or IL-10 morpholino. Cell culture media was collected at 24 and 48 hr and IL-10 protein levels were measured by ELISA assay (R&D Systems, Minneapolis MN) according to the manufacturers recommendations.

In Vivo Mouse Tumor Model

The Animal Care and Use Committee (National Cancer Institute, NIH, Bethesda, MD) approved mouse protocols. Female C3H/Hen or athymic nude mice were supplied by the Frederick Cancer Research and Development Center Animal Production Area (Frederick, MD). The animals were received at 6 weeks of age, housed five per cage, and given autoclaved food and water *ad libitum*. Experiments were performed at 9-10 weeks of age and in accordance with principles outlined in the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, National Research Council). Squamous cell carcinoma VII/SF tumor cells (SCC) were derived from spontaneous abdominal wall squamous cell cancer (obtained from Dr. T. Phillips, UCSF, San Francisco, CA) and propagated in C3H/Hen mice (31). For growth delay studies, 2×10^5 viable SCC cells were injected into the subcutaneous space of the right hind leg of eight-week-old C3H/Hen or nude mice and grown for one week when tumor size reached approximately 200 mm³ in size. Similarly, human colon carcinoma HT29 tumor xenografts were grown in nude mice injected with 1×10^6 cells. C57BL/6 WT or eNOS^{-/-} (The Jackson Laboratory Stock No. 002684) mice on the same background were injected with 1×10^6 B16 melanoma cells. SNP analysis (DartMouse, The Geisel School of Medicine at Dartmouth, Dartmouth, NH) demonstrated background purities of C57BL/6 WT and eNOS^{-/-} mice to be 99.8% and 98.9%, respectively, when compared to the in-house control. Tumor volume was measured by caliper and calculated as $\text{mm}^3 = [\text{width}^2 \times \text{length}] / 2$ where width was the smaller dimension. Tumor irradiation was accomplished by securing each animal in a specially designed Lucite jig fitted with lead shielding that protected the body from radiation while allowing exposure of the tumor-bearing leg. A Therapax DXT300 X-ray irradiator (Pantak, Inc., East Haven, CT) using 2.0 mm Al filtration (300 KVp) at a dose rate of 2.53 Gy/min was used as the X-ray source. Irradiated tumors received one 10 Gy dose. Designated groups of animals were treated with NOS inhibitor L-NAME or IL-10 suppressing agents. NOS inhibition was achieved by administering L-NAME post-IR in the drinking water at a concentration of 0.5g/L for the duration of the experiment (20). IL-10 protein levels were suppressed using an IL-10 morpholino; mice were injected with a 750 μl volume of 10 μM IL-10 morpholino (Gene Tools, Philomath, OR) or a 4 base-mismatched control morpholino in saline 48 hr prior to irradiation. After irradiation, the mice were returned to their cages,

and tumors were measured three times each week thereafter to assess tumor growth. Animals were euthanized when tumor growth approached the maximum allowable limit.

Cytokine Screen

Control and irradiated tumors (+/- L-NAME) were collected at 0, 0.25, 1, 2, 3, 4, and 7 days post-irradiation. Cytokine protein expression was evaluated by Q-Plex multiplex ELISA arrays (QUANSYS Biosciences, Logan UT).

Isolation of Leukocytes from Spleen

Spleens were harvested from tumor-bearing animals, placed in sterile saline, and filtered through a two-chamber sterile Filtra-Bag (Fisher Scientific). Splenocytes were counted by Sysmex KX-21 (Roche Diagnostics, Indianapolis, IN).

Isolation of Tumor-infiltrating Leukocytes

Tumors were dissected and filtered through a two-chamber sterile Filtra-Bag (Fisher Scientific), then digested in RPMI containing 5% fetal calf serum, 700 units/ml collagenase (Invitrogen, Carlsbad, CA), 100 µg/ml DNase I (Boehringer Mannheim, Mannheim, Germany), and 1mM EDTA (pH 8.0), at 37°C for 45 min. The homogenate was then processed in a tissue stomacher-80 (Seward, West Sussex, UK) for 30 sec, washed with HBSS (BioWhittaker, Walkersville, MD), and resuspended in 40% Percoll (Amersham Pharmacia, Piscataway, NJ) in DMEM medium (BioWhittaker). The suspension was underlaid with 80% Percoll and centrifuged for 25 min at 1000g. Leukocytes were collected from the interphase, washed and counted.

Flow Cytometry

Cells (1×10^6) were incubated in cell staining buffer (0.1% BSA, 0.1% sodium azide) containing 250 µg/ml 2.4G2 ascites, which blocks non-specific Fc receptor antibody binding, for 15 min. Cells were stained with fluorescently-conjugated antibodies (BD Pharmingen, San Jose, CA) for 20 min. Labeled cells were washed twice in cell staining buffer and analyzed on a BD FACS Canto II flow cytometer (Becton Dickinson, Mountain View, CA).

RNA extraction, Reverse Transcription and Quantitative Real Time PCR

Total RNA was extracted with TRIzol (Invitrogen, Grand Island NY) according to the manufacturer's protocol. RNA samples were reverse transcribed into cDNA using Sprint RT Complete 8-well strips (Clontech, Mountain View CA) according to the manufacturer's recommendation. Primer pairs were designed for IL-10 that recognized F: TTAAGGGTTACCTGGGTTGC and R: GCCTGAGGGTCTTCAGGTTC sequences using the IL-10 gene Ref Seq sequences by Primer3 (32). All quantitative real time PCR reactions were designed to follow a universal real time PCR condition: 94°C 2 min, 45 cycles of 94°C 30 sec and 60°C 30 sec in PowerSYBR Master Mix (Applied Biosystems, Grand Island NY). Amplicon specificity was checked by BLAST search and on a 2% FlashGel (Lonza, Walkersville MD) to ensure that neither non-specific amplicons nor primer-dimer complexes were formed. Relative expression was calculated using the ddCt formula.

Amplification efficiencies for primers were checked against the 18S housekeeping gene to ensure signals were detected in PCR exponential phase and that primers had similar amplification efficiencies.

Jurkat and CD47-deficient JinB8 Jurkat cells ($\sim 1 \times 10^6$) (33) were plated on 12-well plates (Corning) using RPM1 medium + 2% FBS at 37 °C in 5% CO₂ and treated with 1 µg/ml TSP1 for 6 h. Untreated cells were used as controls. Total RNA was extracted using TriPure Isolation Reagent (Roche). The first strand cDNA was made using Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific). Primer sequences for HPRT1 (5'-ATT GTA ATG ACC AGT CAA CAG GG-3'/5'-GCA TTG TTT TGC CAG TGT CAA-3') and IL-10 (5'-AAA TTA GCC GGG CAT GGT GG-3'/5'-CTG CAA CTT CCA TCT CCT GGG T-3') were used for real time PCR performed using SYBR Green (Roche) on an MJ Research Opticon I instrument (Bio-Rad) with the following amplification program: 95 °C for 15 min, followed by 40 cycles of 95 °C for 15 s, 58 °C for 20 s, 72 °C for 25 s, and 72 °C for 1 min. Melting curves were performed for each product from 30 to 95 °C, reading every 0.5 °C with a 6-s dwell time. Fold change in mRNA expression was calculated by normalizing to HPRT1 mRNA level. Two-factor ANOVA with replication was used for statistics analysis.

Automated Capillary Western Blot (WES)

Western blots were performed using WES, an automated capillary-based size sorting system (ProteinSimple, San Jose CA) (34). All procedures were performed with manufacturers reagents according to their user manual. Briefly, 8µL of diluted protein lysate was mixed with 2µL of 5× fluorescent master mix and heated at 95°C for 5 min. The samples (1 µg), blocking reagent, wash buffer, primary antibodies, secondary antibodies, and chemiluminescent substrate were dispensed into designated wells in a manufacturer provided microplate. The plate was loaded into the instrument and protein was drawn into individual capillaries on a 25 capillary cassette provided by the manufacturer. Protein separation and immuno detection was performed automatically on the individual capillaries using default settings. The data was analyzed using Compass software (ProteinSimple, San Jose CA) (34). Primary antibodies used were nNOS, eNOS (Cell Signaling, Beverly MA), and iNOS (Santa Cruz Biotech, Santa Cruz, CA) HPRT was used as loading control (rabbit, Santa Cruz Biotech, Santa Cruz CA).

Statistics

All results are expressed as the mean $\pm$ SEM. The differences in means of groups were determined by the Student's *t*-test with the minimum level of significance set at *P* 0.05.

Results

NOS Inhibition Enhances Radiation-Induced Tumor Growth Delay in Syngeneic Mice

Tumor growth delay is described by the substance enhancement ratio (SER), which is the ratio of time required for treated vs. control tumors to reach a defined size (1000 mm³). The effect of NOS inhibition by L-NAME, a NOS inhibitor that is more selective for the constitutive isoforms (35) (eNOS and nNOS), on radiation-induced tumor growth delay was

examined in a syngeneic murine model of SCC tumor-bearing C3H mice. L-NAME was administered in the animals' drinking water (0.5 g/L) following tumor irradiation (post-IR, 10 Gy). Because NO regulates vascular tonicity, we chose post-IR administration of L-NAME to minimize vascular constriction and maintain tumor pO₂ prior to and during tumor irradiation while targeting vascular constriction post-IR. Figure 1A demonstrates enhanced radiation-induced tumor growth delay in mice that received post-IR L-NAME (SER ~ 3.3) when compared to tumors treated with 10 Gy IR alone (SER ~ 1.8). The iNOS-specific inhibitor aminoguanidine was also tested, and yielded an SER of 2.1. Interestingly, L-NAME-mediated NOS inhibition had no effect on the radiation-induced tumor growth delay of HT29 human adenocarcinoma cells or SCC xenografts in immunosuppressed nude mice lacking T cells (Figure 1B, 1C, respectively). These results indicate a requirement of T cells for L-NAME potentiation of radiation-induced tumor growth delay.

Effect of L-NAME on Radiation-Induced Cytokine Expression in Syngeneic Mice

T cells are lymphocytes that direct cell-mediated immunity and are distinguished from other lymphocytes by the presence of cell surface T-cell receptors. There are several subsets of T cells, each with a distinct function; proliferating helper T cells differentiate into two major types of effector T cells known as Th1 and Th2 cells, which secrete specific cytokines that mediate different immune responses. Th1 cells are pro-inflammatory, mediate host immunity to foreign pathogens, and are induced by IL-2, IL-12, and their effector cytokine IFN- γ (36). In contrast, Th2 cells are immunosuppressive, secrete IL-4, IL-5, IL-10 as well as TGF- β , and mediate wound resolution following pro-inflammatory assault (37). To explore a potential role for NOS-derived NO during radiation-induced T-cell response, QPlex was used to examine alterations in tumor cytokine expression. Supplemental Table I summarizes the impact of NOS inhibition by L-NAME, on the trend of Th1 vs. Th2 cytokine protein expression induced by 10 Gy tumor irradiation, while Supplemental Table II summarizes pg/mg cytokine levels as well as p-values and fold-change, as a function of time after irradiation. The trend of Th1 vs. Th2 cytokine protein expression summarized in Supplemental Table I suggests that tumors receiving radiation alone rapidly acquire (within 24 hr) an overall Th2 signaling profile as defined by early elevation of IL-10 (Day 1) followed by increased IL-5, IL-3, and IL-4 tumor expression (Day 2-4). In contrast, tumors from animals that received post-IR L-NAME exhibited a Th1 profile as defined by elevated IL-2 (6 Hr, Day 1), IL-12, and IFN- γ (Day 3, 4, 7) tumor expression. The most profound observation pertained to the dramatic early induction of IL-10 24 hrs post-IR, which was abated by L-NAME (Figure 2A). These results suggest the rapid induction of an IL-10-mediated immunosuppressive phenotype in response to tumor irradiation, which was abolished by post-IR NOS inhibition. We also examined tumor NOS isoform protein expression 24 hr Post-IR. When compared to control, Figure 2B shows increased iNOS protein expression in the 10 Gy and 10Gy + L-NAME tumors. This is an interesting observation considering that constitutive NOS inhibition by L-NAME was more effective in extending the radiation-induced tumor growth delay. This may be explained by the findings of Connelly et al. who demonstrated that eNOS is required for the full activation of iNOS (38).

Nitric Oxide-Induced IL-10 Expression in Jurkat T Cells and ANA-1 Macrophages

IL-10 is generally produced by differentiated monocytes and lymphocytes (i.e. macrophages and T cells, respectively). To further examine the involvement of NO during radiation induced IL-10 expression, we used Jurkat cells, which are T lymphocytes that express IL-10 and are commonly employed to study T cell signaling. Cytokine expression profiles were examined in cells exposed to the slow releasing NO donor DETA/NO, which mimics NO flux under inflammatory conditions. Figure 3 demonstrates NO concentration-dependent induction of IL-10 mRNA (A) and protein (B) in Jurkat cells, which peaked at 300 μ M DETA/NO. Next, Jurkat cells were exposed to 1 Gy irradiation, then treated with or without L-NAME and incubated overnight to mimic the tumor xenograft irradiation protocol. Figure 3C shows a greater than 4-fold increase in Jurkat IL-10 expression 24 hr after 1 Gy irradiation, which was abated by L-NAME and is similar to the L-NAME effect on radiation-induced tumor IL-10 expression shown in Figure 2. Interestingly, the L-NAME suppressed IL-10 levels re-accumulated to that induced by 1 Gy irradiation in the presence of the exogenous NO donor DETA/NO at concentrations of 100-500 μ M or $\sim$ 400 nM steady state NO (6, 7, 12) (Figure 3C). To date, our breast cancer biomarker signatures suggest that NO-mediated pro-survival, cell migration, angiogenesis, and stem cell marker (i.e. ERK, Akt, IL-8, IL-6, S100A8, CD44) signaling in tumors and tumor cells occurs at 400 nM steady state NO (6, 7, 9, 12, 39). When considering this molecular signature, the results shown in Figure 3A-C are consistent with our earlier reports and suggest that $\sim$ 400nM steady state NO modulates radiation-induced IL-10 expression in Jurkat cells. Also, the NO flux-dependent regulatory trend of IL-10 shown in Figure 3C resembles a bell shaped curve, which is consistent with low flux NO regulation of wound response vs. high flux NO-mediated toxicity (11, 40-42).

L-NAME is more selective for the constitutive NOS isoforms, which implicates possible eNOS/cGMP-dependent signaling (35, 43). To explore the potential of cGMP-dependent signaling during radiation-induced IL-10 expression, Jurkat cells were exposed to 1 Gy IR +/- the guanylyl cyclase inhibitor ODQ, which completely abolished IL-10 expression induced by 1 Gy IR (Figure 3D). Thrombospondin-1 (TSP-1) inhibits NO signaling through its receptor CD47 by inhibiting eNOS activation and abating NO-dependent cGMP synthesis and cGMP-dependent protein kinase signaling in vascular cells and Jurkat T cells (41, 42). Exogenous TSP-1 also blocked radiation-induced Jurkat IL-10 expression, suggesting eNOS/cGMP-dependence for this process (Figure 3D). Inhibition of IL-10 expression by TSP-1 is CD47-dependent because inhibition of basal IL-10 mRNA expression was lost in the CD47-deficient Jurkat mutant JinB8 (Figure 3E). The 2-fold stimulation of IL-10 mRNA by TSP-1 in the CD47 mutant is consistent with reported positive effects of TSP-1 on IL-10 expression mediated by the TSP-1 receptor CD36 (44). Radiation also induced IL-10 in murine ANA-1 macrophages, which was abated by L-NAME, ODQ and TSP-1, indicating that cGMP-dependent regulation of IL-10 is not restricted to T cells (Figure 3F). Collectively, these results indicate that radiation-induced IL-10 expression in T cells is tightly controlled by low constitutive NOS-derived NO flux.

IL-10 Suppression Enhances Radiation-induced Tumor Growth Delay

Post-IR NOS inhibition by L-NAME enhanced radiation-induced tumor growth delay and abated radiation-induced IL-10 expression (Figure 1A and Figure 2, respectively). To examine a role of IL-10 in the recovery from post-IR tumor growth delay, IL-10 protein translation was suppressed by treatment with an IL-10 morpholino (45, 46). Confirmation of the morpholino efficacy for IL-10 protein suppression was verified using LPS-stimulated Raw 267.4 cells because LPS is a strong inducer of IL-10 in these cells (30) as shown in Figure 4A. Next, tumor-bearing animals were treated with IL-10 or control morpholino 48 hr prior to tumor irradiation. IL-10 morpholino treatment enhanced the radiation-induced tumor growth delay (SER ~2.7) as shown in Figure 4B in a manner similar to that observed by L-NAME (Figure 1A) but had no effect on tumor growth in the absence of radiation. These results suggest that radiation-induced tumor growth delay can be improved by inhibiting IL-10-mediated immunosuppressive signaling in the C3H/SCC syngeneic model.

L-NAME Increases Tumor-associated CD8⁺ Cytolytic T Cells Post-IR

Cytotoxic T lymphocytes are a subgroup of T cells that when activated kill invading pathogens and tumor cells. These cells are commonly referred to as CD8⁺ T cells because they express cell surface CD8 glycoprotein. Importantly, immunosuppressive molecules including IL-10 can inactivate CD8⁺ T cells. To identify the presence of CD8⁺ T cells, markers of tumor lymphocyte infiltration were examined in control and 10 Gy ± L-NAME tumors, as well as spleen from tumor-bearing mice. Figure 5A shows increased tumor-associated CD8⁺T cells in irradiated tumors treated with L-NAME but not spleen taken from the same animals (Figure 5B). CD69 is a marker of T cell activation. Figure 5C demonstrates increased CD8⁺ CD69⁺ mean fluorescence intensity in infiltrating lymphocytes from irradiated tumors treated with L-NAME but not in spleen taken from the same animals (Figure 5D). Importantly, these results implicate a localized tumor response culminating in the elevation of activated cytotoxic T cells in post-IR NOS inhibited tumors. Neutrophils, dendritic cells, and immature myeloid cells from post-IR treated L-NAME tumors were elevated on day 3, when compared to irradiated tumors (Figure 5 E-G). In contrast, Tregs and natural killer cells did not change (Figure 5 H, I). Collectively, these results demonstrate that altered NO flux via L-NAME-mediated NOS inhibition can improve the efficacy of therapeutic radiation by immune polarization favoring a pro-inflammatory phenotype within the tumor microenvironment in a SCC/C3H syngeneic model.

L-NAME is more selective for inhibiting the constitutive NOS isoforms (eNOS and nNOS) and our cell culture results implicate eNOS/cGMP-dependent signaling (Figure 3) in the immune polarization shown in Figure 5. To further explore a role of eNOS in the potentiation of radiation therapeutic efficacy, we examined the radiation-induced tumor growth delay of murine B16 melanoma xenografts in wild type (WT) and eNOS knockout (eNOS^{-/-}) mice on the C57BL/6 background (Figure 6A). When compared to control, the irradiated tumor in WT mice yielded an SER of ~ 2 (Figure 6A), which is consistent with the SCC/C3H syngeneic model shown in Figure 1A. Remarkably, the irradiated tumor in eNOS^{-/-} animals exhibited an SER of ~ 5.5 when compared to the WT irradiated tumor (Figure 6A). Interestingly, IL-10 protein was not detected in B16 xenografts grown in WT

C57BL/6 or eNOS^{-/-} on the same background under these conditions, however the cytokine protein expression profile of irradiated tumors in eNOS^{-/-} mice exhibited significantly elevated Th1 cytokines including IL-2, TNF- α , and IFN- γ as shown in Figure 6 panels B-D. Importantly, radiation has been shown to induce eNOS expression, which promotes tumor recovery from radiation injury (47). Together, these results suggest that eNOS has a vital role in acute tumor radiation response and that improved pharmacology of NOS inhibitors in combination with radiation may be therapeutically beneficial.

Discussion

The role of NO flux within the tumor microenvironment as it relates to therapeutic efficacy is complex. Studies have shown that steady state NO modulation within the tumor microenvironment leads to improved radiation therapeutic efficacy (20, 47, 48). Nitric oxide mediates numerous effects at the cellular and physiological levels. Similar to molecular O₂, the outer π orbital of NO has an unpaired electron, which imparts a high affinity for other radicals including radiation-induced carbon radicals on DNA, which leads to fixed DNA damage and enhanced NO-mediated radiosensitivity (49). In support of this early observation, a recent study demonstrated that the presence of low steady state NO dramatically enhanced radiosensitization as well as increased the time of DNA repair, when compared to anoxic and aerated control tumors (50). Similarly, site-specific iNOS transgene expression driven by the radiation-inducible pE9 promoter demonstrated enhanced tumor radiation response under hypoxic conditions (51). In addition to these *direct effects* of NO-mediated radiosensitization, altered NO gradient *prior* to tumor irradiation have been shown to normalize tumor vasculature, which increased tumor oxygen tension and tumor response to radiation (52). In contrast, administration of the NOS inhibitor L-NAME before and after radiation minimized the cytotoxic effect of NO under conditions of hypoxia (48). In this context, NO improved radiation therapeutic efficacy by enhanced tumor perfusion and oxygen effect (53). Thus, NO modulation prior to and at the time of radiation is therapeutically beneficial. Together, these studies demonstrate the contextual dependence of timing and distinct mechanisms directed by NO flux for improved tumor response to radiation therapy.

While the modulation of tumor NO flux *prior* to irradiation improves tumor oxygenation and radiation efficacy, NO also promotes angiogenesis in the context of immune-mediated wound response (40-42), which may facilitate post-irradiation recovery of a sub-lethally irradiated tumor. Indeed, macrophages employ NO generated by both eNOS and iNOS during wound response (40, 54) and *in vivo* models have shown delayed wound closure in iNOS knockout mice (55). Toward this end, ionizing radiation-induced angiogenesis (56) through NO signaling (47), which promoted tumor recovery following radiation injury. These observations suggest that *post-irradiation* inhibition of angiogenesis may be beneficial. Thus we hypothesized that improved radiation therapeutic efficacy and extended tumor growth delay may be achievable by targeting NO flux through NOS inhibition following tumor irradiation.

Interestingly, post-IR administration of the constitutive NOS inhibitor L-NAME extended radiation-induced tumor growth delay and was more effective than the selective iNOS

inhibitor aminoguanidine (Figure 1A). Moreover, L-NAME extended the radiation-induced tumor growth delay only in syngeneic mice but not nude mice. This observation implicates the involvement of innate immunity and cytotoxic T cells in enhanced radiosensitivity, which is regulated by NO flux, and further supported by the cytokine expression profile of post-IR NOS-inhibited tumors that expressed high levels of cytotoxic Th1 cytokines including IL-2, IFN- γ , and IL-12p40 as summarized in Figure 7. In contrast, tumors receiving radiation alone exhibited immunosuppressive Th2 signaling, as indicated by increased IL-10, IL-5, and IL-4 cytokine expression (Supplemental Table I and II). Moreover, tumor cytokine expression analysis revealed enhanced IL-10 protein levels 24 hr following tumor irradiation in SCC-tumor bearing C3H mice, which was abolished by L-NAME (Figure 2) and confirmed in irradiated Jurkat T lymphocytes, ANA-1 macrophages (Figure 3). Importantly, *in vivo* IL-10 protein suppression extended radiation-induced tumor growth delay in C3H mice in a manner similar to that of L-NAME. These findings implicate a novel role for NO as a stimulator of IL-10-mediated tumor immunosuppressive signaling, which accelerates tumor recovery and regrowth in response to radiation injury in the C3H model.

Cytokine expression analysis of ANA-1 macrophages, and Jurkat T cells demonstrated increased IL-10 expression 24 hr after 1 Gy irradiation, which was abated by L-NAME, suggesting that radiation-induced IL-10 could come from these cell types. Although we used a variety of sensitive detection methods including flow cytometry analysis of IL-10 associated with markers of specific immune cell populations, as well as flow cytometry analysis of GFP-IL-10-tagged mice, we were unable to confirm the specific cellular source of IL-10 in our experiments. In addition, no significant changes in Treg cell populations were observed that might account for the cellular source of increased IL-10 levels following irradiation. Ongoing studies are aimed at identifying the relative contributions of leukocyte subsets to IL-10 following tumor irradiation.

Flow cytometry analysis of immune cell mediators demonstrated increased CD8⁺ expression and CD8⁺/CD69⁺ MFI (indicative of cytotoxic CD8⁺ T cell activation) in post-IR NOS-inhibited C3H tumor xenografts but not spleen (Figure 5 A-D), implicating a localized immune response at the irradiated tumor site. The results herein further support a key role for NO flux-dependent regulation of IFN- γ and cytotoxic T cell activation for improved radiation therapeutic efficacy (Supplemental Tables I and II; Figures 5 and 6). Indeed, cytotoxic CD8⁺ T cells mediate cell killing through increased IFN- γ (57), and inhibition of either IFN- γ or CD8⁺ T cells abolished the therapeutic efficacy of radiation in colon adenocarcinoma tumor-bearing mice (58). In addition, our results indicate that L-NAME potentiation of radiation treatment efficacy is eNOS/cGMP-dependent. Suppression of eNOS/cGMP-dependent signaling by the guanylyl cyclase inhibitors ODQ or TSP-1 abolished radiation-induced IL-10 expression in Jurkat T lymphocytes and ANA-1 macrophages (Figure 3). Also, radiation-induced tumor growth delay was dramatically enhanced in eNOS^{-/-} tumor xenografts, which exhibited increased Th1 cytokine expression (Figures 6). Thus, radiation-induced tumor injury promotes a Th2 immunosuppressive profile that is eNOS/cGMP-dependent and involves low NO flux.

The post-IR NOS-inhibited tumor exhibited enhanced expression of IL-2, IFN- γ , and IL-12p40 Th1 mediators (Supplemental Table I). IL-2 is a pleiotropic cytokine that has pivotal roles during immune regulation in response to foreign pathogens (59). IL-2 is produced primarily by CD4⁺ T cells and promotes the differentiation, expansion, and cytolytic activation of cytotoxic T cells. Importantly, IL-2 effects are receptor-mediated; IL-2 interaction with IL-12R β 2 leads to up-regulation of IFN- γ and IL-12 during Th1 cell differentiation (59). Interestingly, a cGMP-dependent role of low flux NO in the selective upregulation of IL-12R β 2 has been reported (26). IL-12 is also important for sustaining memory/effector T cells, which promote long-term protection against pathogens and tumors. IL-2 also interacts with IL-2R α to promote CD8⁺ T cell differentiation and activation (59). Importantly, these cytokine activation profiles are consistent with the time course analysis showing elevated IL-2, IFN- γ , and IL-12p40 in the post-IR NOS-inhibited tumors summarized in Supplemental Table I, as well as CD8⁺ T cell regulation shown in Figure 5. In contrast, tumors receiving irradiation alone demonstrated increased IL-10 followed by elevated Th2 mediators IL-5, IL-3, and IL-4. Interestingly, IL-2 was also elevated in these irradiated tumors and may have played a role in the upregulation of IL-4 and IL-5 Th2 cell differentiation, which is IL-4R α dependent (59). The pro-inflammatory cytokine IL-1 α was also observed in the irradiated tumor. Despite its pro-inflammatory status, IL-1 α released by solid tumors acts as a chemoattractant to facilitate malignancy-associated inflammatory responses (60).

Collectively, the results herein suggest a novel mechanism of low flux NO during Th1-Th2 transition, tumor immunosuppressive signaling, and accelerated wound recovery in the tumor response to ionizing irradiation. Importantly, CD8⁺ T cell regulation and IFN- γ expression seem to be determined by NO flux-dependent IL-10 versus IL-2 signaling cascades, which can be modulated by pharmacological NOS inhibition and may provide a novel immunotherapeutic approach for improved radiation therapeutic efficacy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Radiation-induced tumor growth delay is enhanced by post-IR administration of L-NAME and requires cytolytic T-cells in SCC tumor xenografts grown in female syngeneic C3H/Hen mice. Post-IR aminoguanidine, a selective iNOS inhibitor, was less effective than L-NAME at extending radiation-induced tumor growth delay (A). Human HT29 colon carcinoma (B) and SCC (C) xenografts grown in female athymic nude mice show no effect of post-IR L-NAME on radiation-induced tumor growth delay. Mice were injected with 2×10^5 SCC (A and C) or 1×10^6 HT29 (B) tumor cells in the right hind leg and grown for one week to allow formation of palpable tumors of uniform size (~ 200 mm³). On day seven animals received tumor irradiation +/- post-IR L-NAME (or aminoguanidine) in the animals drinking water (0.5g/L). Data are presented as mean $\pm$ SEM, *n* = 5 animals per group.

Figure 2.

Radiation-induced tumor IL-10 protein expression is abolished by post-IR L-NAME. Data plotted as mean $\pm$ SEM of two tumor lysate samples each measured in triplicate (A). NOS isoform protein expression in control, 10 Gy, and 10 Gy + L-NAME tumors 24 hr following tumor irradiation (B).

Figure 3.

IL-10 mRNA (A) and protein (B) is induced in Jurkat T-cells following overnight exposure to the NO donor DETA/NO. Post-IR (24 hr) NOS inhibition by L-NAME suppressed IL-10 expression induced by 1 Gy radiation of Jurkat T-cells, which rebounded in the presence of DETA/NO (C). Post-IR (24 hr) L-NAME treatment as well as guanylyl cyclase inhibition by guanylyl cyclase inhibitors ODQ or TSP-1 also suppressed IL-10 expression induced by 1 Gy radiation in Jurkat T-cells (D). TSP-1 treatment for 6 hr decreased IL-10 mRNA expression in WT but not CD47 deficient Jurkat cells (E). Radiation-induced IL-10 expression is suppressed in ANA-1 macrophages treated post-IR with L-NAME, ODQ, or TSP-1 (F). Jurkat cell viability in response to DETA/NO (G) or 1 Gy IR (H). Data are presented as mean $\pm$ SEM of $n = 3$ per treatment group.

Figure 4.

A) LPS is a strong inducer of IL-10 protein in Raw 267.4 cells, which is abated by IL-10 morpholino (IL-10 M). B) IL-10 morpholino was administered i.p. to SCC tumor-bearing C3H mice 48 hr prior to tumor irradiation. IL-10 suppression by IL-10 morpholino enhanced radiation-induced tumor growth delay of SCC xenografts. Data are presented as mean $\pm$ SEM of n = 5 animals per group.

Figure 5.

Post-IR NOS inhibition by L-NAME increased %CD8+ T cells and activation measured by CD8 CD69 MFI in tumors (A, C) but not spleen (B,D) in SCC xenografts. Elevations in the percentage of tumor neutrophils, dendritic cells, and immature myeloid cells were also observed on day 3 in post-IR+L-NAME tumors, when compared to tumors receiving 10 Gy radiation alone (E-G). Cell surface marker expression was measured by flow cytometry 2-4 days (A-G) or 24 hr (H,I) post-IR in SCC xenografts.

Figure 6.

A) Radiation-induced tumor growth delay is enhanced in eNOS^{-/-} mice. C57BL/6 WT or eNOS^{-/-} mice on C57BL/6 background were injected with 2×10^5 B16 tumor cells in the right hind leg and grown for one week to allow formation of palpable tumors of uniform size (~ 200 mm³). On day seven animals received tumor irradiation. Data are presented as mean $\pm$ SEM, $n > 5$ animals per group. B-D) Irradiated tumors from eNOS^{-/-} mice exhibit elevated protein levels of IL-2, TNF- α , and IFN- γ pro-inflammatory Th1 cytokines.

Figure 7.
Post-IR NOS inhibition improves tumor response to ionizing radiation by increased Th1 immune polarization within the tumor microenvironment.