Nitric Oxide, a Mediator of Inflammation, Suppresses Tumorigenesis

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Abstract

Inflammation influences the development of cancer. The nitric oxide synthase (NOS2) is induced by inflammatory cytokines, e.g., tumor necrosis factor α and interleukin 1β, and produces nitric oxide (NO), a critical mediator of the inflammatory response. Because p53 governs NO production by transcriptionally transrepressing NOS2, we used a genetic strategy to determine whether NO and p53 cooperatively regulate tumorigenesis. Lymphomas developed more rapidly in p53+/−/NOS2+/− or p53−/−/NOS2+/− mice than in p53−/−/NOS2++ mice that were crossbred into a >95% C57BL6 background and maintained in a pathogen-free condition. Likewise, sarcomas and lymphomas developed faster in p53+/−/NOS2−/− or p53−/−/NOS2−/− than in p53+/−/NOS2++ mice.

When compared with the double knockout mice, p53−/−/NOS2++ mice showed a higher apoptotic index and a decreased proliferation index with an increased expression of death receptor ligands, CD95-L and tumor necrosis factor-related apoptosis-inducing ligand, and the cell cycle checkpoint protein, p21−/−, in the spleen and thymus before tumor development. Furthermore, mice deficient in both p53 and NOS2 produced a high level of anti-inflammatory interleukin 10 when compared with p53-deficient mice. These studies provide genetic and mechanistic evidence that NO can suppress tumorigenesis.

Introduction

Homozygous and heterozygous p53-knockout mice are an animal model of the cancer-prone human Li-Fraumeni syndrome and develop cancer at an early age (1, 2). However, the genetic background plays a significant role in the latency of spontaneous tumor development. p53-deficient mice with pure 129/Sv background develop tumors sooner as compared with mixed (75% C57BL6 and 25% 129/Sv) genetic background (3). The majority of the tumors in homozygous p53-knockout mice are lymphomas, mostly of T-cell origin, in contrast to sarcomas in heterozygous mice. p53 transcriptionally regulates many different genes that are involved in a variety of cellular functions, including DNA repair, apoptosis, senescence, and cell cycle regulation (reviewed in ref. 4). Because inducible nitric oxide synthase (NOS2) is transpressed by p53 (5), p53−/− mice have higher basal expression of NOS2 and produce a modest and significant increase in nitric oxide (NO) as compared with the mice with intact p53 (6). NOS2 is a member of a nitric oxide synthase (NOS) family of enzymes and produces micromolar quantities of NO for a prolonged period of time. NO is a signaling molecule with diverse functions, including neurotransmission, vasodilation, immune regulation, and host defense against pathogenic micro-organism (reviewed in refs. 7, 8). However, a sustained and very high level of NO produced during chronic inflammation can be involved in pathological disorders, including cancer (reviewed in ref. 9). Previous studies by us and others have indicated both pro- and antineoplastic effects of NO (9). Here, we investigated the role of endogenous NO produced by NOS2 in regulating tumorigenicity in this animal model.

Materials and Methods

p53 and NOS2 Double Knockout Mice. To generate p53 and NOS2-deficient mice, we interbred p53−/−/NOS2++ mice (1) with p53+/−/NOS2−/− mice (10). Mice were backcrossed six times to reach inbred C57BL6/129 (with <1% of strain 129). To obtain mice homozygous for disrupted NOS2 and p53, F1 mutant heterozygotes were interbred, and their progeny were genotyped by PCR analysis (Supplemental Fig. 1). Absence of NOS2 and p53 were also confirmed by immunoblotting on lysate from the spleen (Supplemental Fig. 2) in these mice with or without treatment with heat-inactivated Corynebacterium parvum, which enhances NOS2 expression (6). NOS2 expression was also determined by immunohistochemical staining in the spleen and thymus of p53−/−/NOS2++ and p53−/−/NOS2−/− mice (Supplemental Fig. 3).

Spontaneous Tumor Incidence. Nine different groups of mice (17 to 45 mice per group), each representing a specific genotypic combination of p53 and NOS2, wild-type or null, were housed under pathogen-free conditions (Supplemental Table 1). The percentage of mice with cancer in each group was compared at different time points. Kaplan-Meier survival curves were generated for each group of mice and the Wilcoxon test was used to estimate statistical differences between survival curves.

Apoptosis Assay. Five-micron sections of the spleen and thymus on formalin-fixed paraffin blocks were cut, deparaffinized, dehydrated, and rehydrated. The sections were then incubated with either normal serum (negative controls) or with antibodies against monoclonal antimouse CD95 (BD Biosciences, San Jose, CA), polyclonal antimouse CD95-L (Dako, Carpinteria, CA), or antibodies against monoclonal antimouse CD95 (BD Biosciences, San Jose, CA), followed by horseradish peroxidase-conjugated avidin-biotin complex (Vector Laboratories, Burlingame, CA); diaminobenzidine tetrahydrochloride (Pierce, Rockford, IL) was the chromogen. Immunohistochemical staining was repeated twice with positive and negative controls. Positive controls included irradiated mouse tissue for p21−/−, UV-treated mice for CD95-L, and rat intestine for Ki67. Coded slides were evaluated by two independent observers.

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Note: Supplementary data for this article can be found at Cancer Research Online (http://cancerres.aacrjournals.org).

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Student’s *t* test was used to analyze the statistical significance for the immunohistochemical staining.

**Interleukin (IL)-10 Expression and Quantitation.** RNase protection assay and Cytometric Bead Array (BD Biosciences) were used for IL-10 analysis. The detail procedure is provided as supplemental data.

**Results**

**Genetic NOS2 Deficiency Enhanced Tumorigenesis in p53-deficient Mice.** *p53*−/−*NOS2*−/− or *p53*−/−*NOS2*+/+ mice developed tumors more rapidly than *p53*−/−*NOS2*+/+ mice (*P* < 0.001; Fig. 1 and Supplemental Table 1). The majority of the tumors in *p53*−/− mice, irrespective of NOS2 status, were lymphomas with a dominance of T-cell lymphomas (Supplemental Fig. 4). Furthermore, *p53*+/−*NOS2*−/− or *p53*+/−*NOS2*+/+ mice also developed tumors faster when compared with *p53*+/−*NOS2*+/+ mice (*P* = 0.001 and *P* = 0.003). Most of the tumors in the heterozygous *p53* mice were sarcomas, irrespective of the genetic status of NOS2 (Supplemental Fig. 4). These results indicate that homozygous- or haploinsufficiency of NOS2 had similar effects in the *p53*−/− mice and *p53*+/− mice. At 2 years of age, 25% of the *p53*+/+*NOS2*−/− mice developed cancer, whereas, only 15% of wild-type mice (*p53*+/+*NOS2*+/+) showed tumors in the 2 years of observation (Fig. 1). Because the tumor tissue and histologic spectra were unchanged in both *p53* and NOS2 double knockout mice, as well as *p53*-deficient mice with intact NOS2, a systemic effect of NO on tumorigenesis was investigated.

**Apoptosis Is Increased in NOS2-proficient p53-null Mice.** Spleen and thymus of 8 to 9-week-old *p53*−/−*NOS2*−/− and *p53*−/−*NOS2*+/+ mice were analyzed for apoptotic cells before the development of any cancer. Both spleen and thymus from *p53*−/−*NOS2*+/+ mice showed a significantly higher apoptotic index when compared with *p53*−/−*NOS2*−/− mice (*P* < 0.0001; Fig. 2A and Supplemental Fig. 5).

**Enhanced Expression of CD95-L and TRAIL in NOS2-proficient p53-null Mice.** To study a possible mechanism that contributes to the enhanced apoptosis in *p53*−/−*NOS2*+/+ mice, we analyzed the expression of the CD95 receptor, its ligand, and TRAIL by immunohistochemistry in the spleen and thymus of 8 to 9-week-old, tumor-free, *p53*−/−*NOS2*−/− and *p53*−/−*NOS2*+/+ mice. *p53*−/−*NOS2*+/+ mice showed an enhanced expression of CD95-L and TRAIL, as compared with *p53*−/−*NOS2*−/− mice in the spleen (*P* < 0.05) and thymus (*P* < 0.01 and *P* < 0.05; Fig. 2, B and C, and Supplemental Fig. 6). We did not observe any difference in CD95 expression in *p53*-deficient mice, irrespective of their NOS2 status.

**Reduced p21*waft* and Increased Ki-67 Expression in Mice De-ficient in p53 and NOS2.** To determine the NOS2-associated *p21* independent expression of *p21*waft, we analyzed the spleen and thymus of 8 to 9-week-old *p53*−/−*NOS2*−/− and *p53*−/−*NOS2*+/+ mice by immunohistochemistry, *p53*−/−*NOS2*+/+ mice showed an enhanced expression of *p21*waft in both spleen and thymus as compared with *p53*−/−*NOS2*−/− mice (*P* < 0.05 and *P* < 0.01; Fig. 3A and Supplemental Fig. 7). We also analyzed the expression of Ki-67, a proliferation marker antigen. *p53*−/−*NOS2*−/− mice showed a higher frequency of Ki-67-immunopositive cells compared with *p53*−/−*NOS2*+/+ mice in the spleen (*P* = 0.04) and thymus (*P* = 0.06; Fig. 3B and Supplemental Fig. 7). Furthermore, the thymus of *p53*−/−*NOS2*−/− mice weighed more than that of *p53*−/−*NOS2*+/+ mice (*P* < 0.05; Supplemental Fig. 8).

**Enhanced Expression of NOS2 in p53-deficient NOS2-proficient Mice.** To determine the expression of NOS2, we analyzed the spleen and thymus of *p53*−/−*NOS2*−/− and *p53*−/−*NOS2*+/+...
mice by immunohistochemistry. Only p53−/−NOS2+/+ mice showed a higher expression of NOS2 in both spleen and thymus (Supplemental Fig. 3).

**Increased Expression of IL-10 in Mice Deficient in p53 and NOS2.** To study the possible systemic effect of NO, such as immune modulation, in the regulation of tumorigenesis, we measured the panel of cytokine expression in the splenocyte, either with or without the treatment of IFN-γ, and/or IL-12 in 14 to 16-week-old mice. Mice deficient in both p53 and NOS2 showed a higher RNA and protein expression of IL-10 as compared with p53-deficient mice with intact NOS2 (Fig. 4).

**Discussion**

The balance of cellular proliferation and apoptotic cell death can influence tumor development. High but physiologically relevant concentrations of NO can induce apoptosis, whereas lower concentrations can be antiapoptotic (9). Different cell types also vary in their sensitivity to NO. The redox status and transition metal complexes in a specific cell type can determine the apoptotic response of NO. Furthermore, increased intracellular non-heme iron converts NO from a proapoptotic molecule to an antiapoptotic molecule (11). We reported earlier that p53 transrepresses the transcription of NOS2 in mouse and human cells (5) and p53−/−NOS2+/+ mice showed a modest increase in basal expression of NOS2 and NO production when compared with p53+/+NOS2+/+ mice (6). We first backcrossed the mice into a >99% C57BL6 background, which is more resistant to spontaneous tumor formation than either the 129/Sv or C57BL6 (75%)/129 (25%) mice (3). NOS2 expression additionally protected the p53-deficient mice from tumor formation, which is consistent with the tumor-suppressive effect of NO. Before the development of lymphoma, we found that p53−/−NOS2+/+ mice showed a significantly higher apoptotic index in both the spleen and thymus when compared with p53−/−NOS2−/− mice. These results are consistent with in vitro studies indicating that NO induces apoptosis in murine thymocytes and splenocytes (12, 13).

We then explored the mechanism of NO-induced apoptosis that can be either p53 dependent or independent (9). One of the extrinsic apoptotic pathways involves the binding of ligand CD95L to the CD95 receptor (14) and the formation of a signaling complex by recruiting Fas-associated death domain and FLICE, which triggers the caspase cascade leading to apoptosis. NO-induced apoptosis in human T- and B-lymphoid cell lines positively correlated with an increase in the expression of CD95 and CD95L (15). The tumor necrosis factor-related apoptosis-inducing ligand (TRAIL/APO-2) is also a component in the extrinsic apoptotic pathway. The binding of TRAIL receptors, DR4 and DR5, to the TRAIL ligand induces apoptotic signals involving the Fas-associated death domain– and caspase-8–dependent pathways (14). Spontaneous tumor development in p53-mutant mice is enhanced by the inhibition of TRAIL using anti-TRAIL monoclonal antibody (16). NO can increase TRAIL mRNA expression and TRAIL-mediated apoptosis (15). Consistent with these in vitro data, in the present study, p53−/−NOS2+/+ mice showed an enhanced expression of CD95L and TRAIL in the spleen and thymus when compared with p53−/−NOS2−/− mice. However, consistent with the p53-dependent induction of CD95 (9), the expression of the CD95 receptor in these mice was unchanged (data not shown).

A schematic representation of the proposed model of spontaneous tumorigenesis in p53-deficient mice indicating NO-mediated regulation of cellular proliferation and apoptosis is shown in Supplemental Fig. 7. p21+/−/+ mice lack the negative feedback loop of p53 transcriptional transrepression of NOS2. The resultant enhanced production of NO increases the expression of death receptor ligands TRAIL and CD95L and the cell cycle check point protein, p21+/−/+ to decrease tumorigenicity.

**Fig. 2.** Number of apoptotic cells/100 normal cells in the spleen and thymus of p53−/−NOS2−/− and p53−/−NOS2+/+ mice as determined by staining with anti-digoxigenin peroxidase (A). Mean scoring intensity of immunopositive cells in the spleen and thymus of p53−/−NOS2−/− and p53−/−NOS2+/+ mice stained for CD95-L (B) and TRAIL (C). Photomicrographs of stained tissue sections are provided as Supplemental Figs. 5 and 6.

**Fig. 3.** p21+/−/+ (A) and Ki-67 (B) expression in the spleen and thymus of p53−/−NOS2−/− and p53−/−NOS2+/+ mice. p21+/−/+ is represented as mean scoring intensity of immunopositive cells/100 high power field (Supplemental Fig. 7, A–D) and Ki-67 is represented as immunopositive cells/100 high power field in the spleen and thymus. C. schematic representation of the proposed model of spontaneous tumorigenesis in p53-deficient mice indicating NO-mediated regulation of cellular proliferation and apoptosis. p53−/−NOS2+/+ mice lack the negative feedback loop of p53 transcriptional transrepression of NOS2. The resultant enhanced production of NO increases the expression of death receptor ligands TRAIL and CD95-L and the cell cycle check point protein, p21+/−/+ to decrease tumorigenicity.
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Fig. 4. RNase protection assay showing an enhanced expression of IL-10. Ribosomal protein L32 was used as internal control for normalization (A). Quantitation of cytokines showing an increased level of IL-10 in mice lacking both p53 and NOS2. Cytokine quantification was done by Cytometric Bead Array. A single cell suspension of splenocytes from p53−/−NOS2+/+ and p53−/−NOS2−/− mice was treated with IL-12 (100 ng/mL) or IFN-γ (100 units/mL) for 6 (B) and 18 (C) hours.

shown). NO can increase TRAIL by enhancing its mRNA expression, which contributes to the NO-mediated apoptosis (15). IFN-γ also induces TRAIL in a number of different cells, including natural killer cells (17). Disruption of IFN-γ sensitivity, due to the lack of either IFN-γ receptor α chain or signal transducers and activators of transcription 1, increases the tumor formation in p53-deficient mice (18). Our results suggest that CD95-L and TRAIL cooperate in a NO-mediated apoptotic mechanism to reduce spontaneous tumorigenesis in p53-deficient mice.

In addition to apoptosis, NO-mediated modulation of cellular proliferation may regulate tumorigenesis. Although NO can enhance, as well as suppress cellular proliferation, most studies have supported its antiproliferative function (9). NO-induced suppression of cellular proliferation can be mediated by the induction of p21WAF1 through the activation of either the mitogen-activated protein kinase or the p53 pathway (19). Our previous study showed that NO increased p21WAF1 by both p53-dependent and -independent mechanisms to induce a G1 cell cycle arrest (20). We found an enhanced expression of p21WAF1 in both the spleen and thymus of p53−/−NOS2+/+ mice when compared with p53−/−NOS2−/− mice. We also found that p53−/−NOS2−/− mice showed a higher frequency of Ki-67-immunopositive cells, an indicator of cellular proliferation, when compared with p53−/−NOS2+/+ mice in the spleen and thymus. Consistent with these combined proliferation and apoptotic results, we found that thymuses of p53−/−NOS2−/− mice weighed more than p53−/−NOS2+/+ mice.

NO selectively enhances the differentiation of Th1 cells by transactivation of the IL-12 receptor β2 tumor suppressor (21, 22), which increases the expression of IFN-γ (21). IFN-γ can additionally activate macrophages to produce high levels of NO by NOS2. Therefore, a positive feedback loop may operate in p53−/−NOS2+/+ mice between NO-induced Th1 cell differentiation and production of IFN-γ, which contributes to the availability of proapoptotic mediators and thus, diminishes spontaneous tumor development. Cytokines can both enhance and inhibit tumor development (reviewed in ref. 23), and IL-10 is an excellent example in this regard. A modest increase in the level of IL-10 expression has been reported in p53-deficient (24) or NOS2-deficient mice (25). A 4-fold higher expression level of IL-10 in untreated mice, deficient in both p53 and NOS2, as compared with p53-deficient mice with intact NOS2, may contribute to the rapid tumor development. IL-10 favors Th2 response and induces antigen-activated CD4+ cells to become CD4+CD25+ T-regulatory cells (26). T-regulatory cells also produce IL-10 and are potentially immunosuppressive. IL-10 also inhibits the maturation of dendritic cells (27). Therefore, the loss of NO-related Th1 antitumor response and the gain of IL-10-mediated inhibition of specific immune recognition by impairment of dendritic cells function and suppression of immune function through T-regulatory cells can enhance tumorigenesis. Our results and those of others indicate that further study is warranted to understand the mechanism of NO-mediated regulation of IL-10.

We propose a model of spontaneous tumorigenesis in the p53-deficient mice involving NO regulation of cellular proliferation and apoptosis (Fig. 3C). The complex role of NO and reactive nitrogen oxide species in a wide variety of physiologic functions suggests the involvement of several different pathways that may collectively contribute to the delayed development of cancer in p53−/−NOS2+/+ mice when compared with p53−/−NOS2−/− mice. Although we focused on apoptosis and cell cycle control by NO as antineoplastic mechanisms, additional studies are warranted to elucidate other possible pathways, including NO-dependent protein-protein interaction (28), immune modulation (29), and mitochondrial dysfunction (30). In addition, chromosomal stability was found to be important in the suppression of spontaneous tumorigenesis when p53-mediated apoptosis is compromised (31). Because Li-Fraumeni families with germ-line missense p53 mutations vary in their tumor spectrum (32), future studies of NO regulation of tumorigenicity of p53−/− versus versus missense p53-knockin mice (33) may also have clinical implications for these families. Future studies should also examine if much higher NO concentrations observed during chronic inflammation, e.g., in-
duced by *C. parvum* or *Helicobacter pylori*, have a different effect, e.g., an increase in cancer risk (9).

**Addendum**

Although the genetic background varied among the mice, Ohshima and coworkers (34) have reported results consistent with those reported here.

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