Frequent Nitric Oxide Synthase-2 Expression in Human Colon Adenomas: Implication for Tumor Angiogenesis and Colon Cancer Progression

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ABSTRACT

An increased expression of nitric oxide synthase (NOS) has been observed in human colon carcinoma cell lines as well as in human gynecological, breast, and central nervous system tumors. This observation suggests a pathobiological role of tumor-associated NO production. Hence, we investigated NOS expression in human colon cancer in respect to tumor staging, NOS-expressing cell type(s), nitrotyrosine formation, inflammation, and vascular endothelial growth factor expression. Ca^{++}-dependent NOS activity was found in normal colon and in tumors but was significantly decreased in adenomas (P < 0.001) and carcinomas (Dukes' stages A–D; P < 0.002). Ca^{++}-independent NOS activity, indicating inducible NOS (NOS2), is markedly expressed in approximately 60% of human colon adenomas (P < 0.001 versus normal tissues) and in 20–25% of colon carcinomas (P < 0.01 versus normal tissues). Only low levels were found in the surrounding normal tissue. NOS2 activity decreased with increasing tumor stage (Dukes' A–D) and was lowest in colon metastases to liver and lung. NOS2 was detected in tissue mononuclear cells (TMCs), endothelium, and tumor epithelium. There was a statistically significant correlation between NOS2 enzymatic activity and the level of NOS2 protein detected by immunohistochemistry (P < 0.01). Western blot analysis of tumor extracts with Ca^{++}-independent NOS activity revealed up to three distinct NOS2 protein bands at Mí 125,000–138,000. The same protein bands were heavily tyrosine-phosphorylated in some tumor tissues. TMCs, but not the tumor epithelium, were immunopositive using a polyclonal anti-nitrotyrosine antibody. However, only a subset of the NOS2-expressing TMCs stained positively for 3-nitrotyrosine, which is a marker for peroxynitrite formation. Furthermore, vascular endothelial growth factor expression was detected in adenomas expressing NOS2. These data are consistent with the hypothesis that excessive NO production by NOS2 may contribute to the pathogenesis of colon cancer progression at the transition of colon adenoma to carcinoma in situ.

INTRODUCTION

NO is an important bioactive agent and signaling molecule that mediates a variety of actions such as vasodilatation, neurotransmission, host defense, and iron metabolism but increased NO production may also contribute to the pathogenesis of a variety of disorders including cancer (1–6). NO is endogenously produced by a family of enzymes known as NOSs (7, 8). Ca^{++}-dependent isoforms (NOS1 and NOS3) were found to be constitutively expressed, whereas a Ca^{++}-independent isoform required induction (iNOS or NOS2). It is expressed in some tissues constitutively, e.g., bronchus and ileum (9, 10). Only the inducible isofrom (i.e., NOS2 or Ca^{++} independent) produces sustained NO concentrations in the micromolar range, which is high when compared to the pico- to nanomolar concentrations produced by the neuronal (NOS1) and endothelial isoforms (NOS3), which are Ca^{++} dependent (11, 12).

Recent studies have examined the expression and activity of the three NOS isoforms in human cancer. An increased level of NOS expression and/or activity was observed in human gynecological (13), breast (14), and central nervous system (15) tumors. In the case of human gynecological and breast cancer, the increased expression was inversely associated with the differentiation grade of the tumor. Moreover, nitrotyrosine accumulation in both the inflammed mucosa of patients with ulcerative colitis (16) and in the stomach of patients with a Helicobacter pylori gastritis (17) indicates that NO production and the formation of peroxynitrite are involved in the pathogenesis of both diseases, which predispose to cancer (18).

These observations suggest the hypothesis that NO expression may contribute to tumor development or progression. NO has several properties that might enhance carcinogenesis. For example, NO is an endothelial growth factor and specifically mediates tumor vascularization (19, 20) and tumor blood flow (21). Although high concentrations of NO induce apoptosis in susceptible cells (22), low concentrations of NO protect many cell types, including endothelial cells (23, 24), from apoptosis. Because factors like cytokines and hypoxia synergistically induce NOS2 expression (25), the micro-environmental changes in premalignant and malignant tumor tissue may establish sustained and high NO production in a variety of tumor cells, thereby supporting clonal selection and tumor growth.

The biological effects of NO are partly the product of NO metabolites such as peroxynitrite and NO+. Protein nitration by peroxynitrite and S-nitrosylation by NO+ may modulate important signal transduction pathways (26–28). High concentrations of NO may have pathobiological effects. For example, NO causes DNA damage by nitrosative deamination (29, 30) and generates several genotoxic by-products including NO2, which cause DNA strand breakage (31), and peroxynitrite, which causes oxidative damage (32) and DNA modifications (e.g., nitration; Ref. 33). NO may also cause DNA modification by generating N-nitrosoamines (34, 35) and DNA-reactive lipid peroxidation intermediates via peroxynitrite (36). Furthermore, NO inhibits certain DNA repair activities (37, 38) and members of the cytochrome P-450 enzyme family (39). Peroxynitrite is a powerful oxidant (32) but also reacts with proteins producing 3-nitrotyrosine, which is a stable product and marker of simultaneous NO and superoxide radical generation (40, 41). Although NO is capable of reaching concentrations of NO protect many cell types, including endothelial cells (23, 24), from apoptosis. Because factors like cytokines and hypoxia synergistically induce NOS2 expression (25), the micro-environmental changes in premalignant and malignant tumor tissue may establish sustained and high NO production in a variety of tumor cells, thereby supporting clonal selection and tumor growth.

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2 The abbreviations used are: NOS, nitric oxide synthase; VEGF, vascular endothelial growth factor; PMSF, phenylmethylsulfonyl fluoride; L-NAME, L-N^6-monomethyl-L-arginine; IP, immunoprecipitation; TMC, tissue mononuclear cell; 3-NT, 3-nitrotyrosine; RT-PCR, reverse transcription-PCR.

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Deamination of DNA by NO may represent an important endo
genous mechanism of genomic alteration. For example, the NO-mediated deamination of 5-methylcytosine produces thymine. Hence, the overrepresentation of point mutations in human disorders at methylated CpG sites (46) and the high frequency of mutations at CpG sites in the p53 tumor suppressor gene in human colon and brain tumors (47, 48) may reflect the etiological contributions of NO in human carcinogenesis.

To define the role of NO production in the development of human colon cancer, we correlated NOS expression in colon tumors to stage, cell type, nitrotyrosine formation, inflammation, and VEGF expression. If NO generates the G:C to A:T transitions at CpG sites, which are so common in colon cancer, one would expect sustained and excessive NO concentration in colon adenomas. Our data demonstrate significant NOS2 activity in a high percentage of colon adenomas. We hypothesize that NO is a candidate endogenous carcinogen that either generates or selects for the high frequency of p53 mutations that arise at the transition from adenoma to carcinoma in situ (49).

MATERIALS AND METHODS

Materials. Nω-monomethyl-L-arginine, (6R,S)-5,6,7,8-tetrahydro-L-biop
terin, aprotinin, pepstatin A, and PMSF were purchased from Calbiochem (San Diego, CA); the Dowex AG 50W-X8 resin, 200–400 mesh, sodium form, was from Bio-Rad (Richmond, CA); and the BCA protein reagent was from Pierce (Rockford, IL); rabbit polyclonal anti-NOS2 antibodies were either purchased from Transduction Laboratories (Lexington, KY) or kindly provided by Merck & Co (Rahway, NJ); the rabbit polyclonal anti-VEGF (A-20) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA); the rabbit polyclonal anti-nitrotyrosine antibody and the murine monoclonal anti-phosphotyrosine antibody 4G10 were received from Upstate Biotechnology (Lake Placid, NY); L-(2,3,4,5)-arginine was obtained from Amersham Corp. (Arlington Heights, IL).

Tissue Collection and Preparation of Soluble Tissue Extracts. With the approval of local boards governing research on human subjects, surgically resected frozen colon tumors [25 adenomas, 42 carcinomas (Dukes' stages A–D), and 5 colon metastases to lung and liver] and surrounding nontumor tissues (n = 68) were obtained from the Cooperative Human Tissue Network (Birmingham, AL; Columbus, OH; Philadelphia, PA) or the University of Maryland, Department of Pathology (Baltimore, MD) and stored at −70°C for less than 3 months. Tissue fragments (<500 mg) were crushed with a pestle and mortar under liquid nitrogen and homogenized on ice in RIPA buffer [50 mM HEPES, 1 mM EDTA, 1 mM L-citrulline, 1 mM MgCl2, 5 mg/ml pepstatin A, 0.1 mM PMSF, and 10 mg/ml aprotinin]. Supernatant was prepared by centrifugation at 120,000 × g for 10 min, and protein concentrations were determined with the BCA protein reagent. For NOS2 and phosphotyrosine, 300 µg of soluble tissue extract protein was loaded on a SDS/7% polyacrylamide gel and separated at 150 V for 2 h. VEGF expression was determined by IP-Western. Five µg of rabbit polyclonal anti-VEGF antibody (Santa Cruz) were added to 1 mg of protein extract and incubated for 1 h at 8–10°C. Ten µg of protein A-Sepharose (Pharmacia, Piscataway, NJ) were added and mixed for 1 h at room temperature, and the samples were spun at 10,000 × g. The pellet was washed with RIPA buffer, heated at 95°C (plus 5× SDS/DTT loading buffer; 5 Prime-3 Prime, Inc., Boulder, CO) and loaded on a 13% gel. After electrophoretic transfer to an Immobilon-P membrane (Millipore, Bedford, MA), unspecific binding was blocked by incubation in TBST [10 mM Tris (pH 8), 150 mM NaCl, and 0.05% Tween 20] plus 4% BSA for 4 h at room temperature. The membranes were probed either with a polyclonal anti-human NOS2 antibody (Merck), diluted 1:40,000 in TBST, or with a monoclonal anti-phosphotyrosine antibody (Upstate Biotechnology). 1 µg/ml, or with a rabbit polyclonal anti-VEGF antibody, diluted 1:1000 in TBST/2% BSA. After washing three times in TBST, the membrane was probed with an anti-rabbit/mouse immunoglobulin perox
idase-coupled antibody (Amersham) diluted 1:10,000 in TBST/2% BSA. Blots were developed using the Renaissance Western blot chemiluminescence system (DuPont,Boston, MA) and exposed to Hyperfilm-ECL (Amersham).

RT-PCR and Sequencing. RNA [prepared according to Chomczynski and Sacchi (51)] from normal and tumor tissues from colon cancer patient 1 was submitted to RT-PCR using murine macrophage NOS cDNA primers (5′-primer CGCATATCATCCTTCAGCCTC, and 3′-primer TGGCCGAAAACT-TCGGAAGGG). Briefly, 1 µg of total RNA was added to RT buffer [67 mM Tris/HCl (pH 8.8), 6.7 mM MgCl2, 16.6 mM (NH4)2SO4, 6.8 mM EDTA, 10 mM 2-mercaptoethanol, and 1 mM deoxynucleotide triphosphate] and incubated (45 min at 42°C) in the presence of 40 units of RNasin (Promega Corp., Madison, WI), 200 units SuperScriptTM Reverse Transcriptase (BRL, Gaithersburg, MD), and the 3′ primer (final concentration, 1 µM). The 5′-primer and DMSO (final concentration, 1 µM and 12%, respectively) were added, and the samples were subjected to 35 cycles of denaturation (30 s at 94°C), annealing (1 min at 56°C), and extension (30 s at 78°C) in the presence of Taq polymerase (1 unit; Perkin-Elmer, Norwalk, CT). The cDNA fragment obtained was gel purified (QuickPrep; Schleicher and Schuell, Keene, NH), and 50 ng were used for sequencing using Exo-Pfu Cyclist (Stratagene, La Jolla, CA).

Statistical Analysis. Comparisons between two characteristics were car
ried out either by the Mann-Whitney U test or the Spearman rank sum test. Relationships were considered statistically significant when P < 0.05.

RESULTS

NOS Activity. Colonic NOS activity was measured in 72 colon
tumors and 68 surrounding normal tissues. The activity was deter
mined as the L-NMA-sensitive conversion of arginine to citrulline.
Initial experiments with several colon tissues showed that this activity was more than 85% dependent on the addition of NADPH to the assay (data not shown). The Ca\(^{2+}\)-independent NOS activity, indicating inducible NOS (NOS2), was markedly expressed in 60% of human colon adenomas \((P < 0.001)\), whereas it was low or undetectable in the surrounding normal tissue (Fig. 1B). The activity decreased with increasing stage of the disease and was lowest in colon metastases to liver and lung (Fig. 1B). Ca\(^{2+}\)-dependent NOS activity was detected in normal colon and tumors (Fig. 1A) but was significantly decreased in adenomas \((P < 0.001)\) and carcinomas \((\text{Dukes’ A–D}: P < 0.002; \text{Dukes’ A and B}: P < 0.02; \text{Dukes’ C}: P < 0.03; \text{Dukes’ D}: P < 0.24\) versus normal tissue). This result suggests a stage-dependent reduction in the abundance of NOS1- and NOS3-expressing cells, which are mainly endothelial cells (NOS3) and neurons of the autonomous nervous system (NOS1).

NOS2 Western and Tyrosine Phosphorylation of NOS2. Western blot analysis of tumor extracts with Ca\(^{2+}\)-independent NOS activity revealed up to three distinct NOS2 bands at approximately \(M_r 125,000–138,000\) (Fig. 2A). Tissue pairs, consisting of an adenoma and surrounding normal tissue, were analyzed. NOS2 protein was detected in every tumor but not in the surrounding normal tissue. A comparison with human NOS2 protein in an extract from a NOS2-transfected human colon carcinoma cell line (positive control, Fig. 2A) revealed that these cells express mainly the \(M_r 130,000\) NOS2 protein but also one or two smaller NOS2 proteins, which migrate at approximately \(M_r 110,000–120,000\). These last two bands did not comigrate with the \(M_r 125,000–138,000\) bands found in the protein extracts of adenomas (Fig. 2A). Because the posttranslational activation of NOS2 by tyrosine phosphorylation has been reported, we examined the extent of this modification in vivo. We found that a \(M_r 125,000–138,000\) protein, migrating exactly with the position of the NOS2 bands, was heavily tyrosine-phosphorylated in some tumor tissues (B). The phosphotyrosine labeling at \(M_r 125,000–138,000\) was not obtained with extracts from normal tissues.

Fig. 2. Western blot analysis of NOS2 protein expression and tyrosine phosphorylation in colon adenomas. Tissue pairs consisting of an adenoma and the surrounding normal tissue were analyzed. Three hundred \(\mu\)g of protein were separated on a 7% polyacrylamide gel. After transfer to an Immobilon-P membrane, the Western blots were probed with either a polyclonal anti-human NOS2 antibody (Merck) or a monoclonal anti-nitrotyrosine antibody. Protein extracts of tumors with Ca\(^{2+}\)-independent NOS activity contained up to three distinct NOS2 bands at approximately \(M_r 125,000–138,000\) (A). NOS2 protein was detected in every tumor (7) but not in the surrounding normal tissue (X). One hundred \(\mu\)g of a protein extract from a NOS2-transfected human colon carcinoma cell line were loaded as a NOS2-positive control (A). A \(M_r 125,000–138,000\) protein band, migrating exactly with the position of the NOS2 bands, was heavily tyrosine phosphorylated in some tumor tissues (B). The phosphotyrosine labeling at \(M_r 125,000–138,000\) was not obtained with extracts from normal tissues.

**Immunohistochemical NOS2 Protein Expression.** The source of Ca\(^{2+}\)-independent NOS activity was determined by immunohistochemistry with two different polyclonal anti-NOS2 antibodies. Both antibodies generated the same labeling pattern. Whereas NOS2 was detected in several cell types such as TMCs, endothelium, and tumor epithelium (Table 1), it was most commonly found in TMCs (Fig. 3, A and C). However, the appearance of NOS2-expressing TMCs was not associated with the amount of tissue inflammation. To further validate the staining specificity, we investigated the quantitative correlation between NOS2 immunohistochemistry and NOS2 assay in 21 tissues (Fig. 4). We found a statistically significant correlation between NOS2 activity and immunohistochemical staining \((P < 0.01)\). This result also underlines the usefulness of the NOS activity assay in human colonic tissues, in particular, because the Western blot analysis confirmed the presence of NOS2 protein in tissues with Ca\(^{2+}\)-independent NOS activity.
Table 1  NOS2 expression in human colon tumors

<table>
<thead>
<tr>
<th>Case</th>
<th>Tissue type</th>
<th>NOS2 activity</th>
<th>NOS2 IHC</th>
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<td>195</td>
<td>3</td>
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<td>96</td>
<td>3</td>
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<td>TE</td>
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<td>2</td>
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<tr>
<td>21</td>
<td>AD</td>
<td>0</td>
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*AD, adenoma; CIS, carcinoma in situ; CA, carcinoma.
*0, not detectable; 1, few cells are positive, focal or scattered; 2, marked focal staining or frequent scattered staining; 3, very intensive staining in a large focus or intensive staining throughout the tumor tissue.
*MN, mononuclear inflammatory cells; ENDO, endothelium; TE, tumor epithelium.

RT-PCR and Sequencing. In support of the conclusion regarding an increased expression of NOS2 in colon tumors, the NOS2 mRNA concentration was determined by RT-PCR in two pairs of colon tumor and surrounding normal tissue. Both tumors had a high NOS2 activity, whereas the activity was low in the surrounding normal tissue. After amplification with NOS2-specific primers, the RT-PCR product was sequenced. As shown in Fig. 5 for one tissue pair, the tumor tissues contained higher levels of NOS2 message than the surrounding normal tissues. Sequencing revealed that the PCR products encoded a peptide sequence (62 amino acids) with 96% identity of human NOS2 reported in GenBank.

3-NT Formation. We investigated whether 3-NT, a marker of peroxynitrite, is detectable in tumors with high NOS2 expression. 3-NT, which evolves through protein nitration, was determined by immunohistochemistry with a polyclonal anti-nitrotyrosine antibody.

3-NT was detected in TMCs and some polymorphonuclear neutrophils, but only a subset of the NOS2-expressing cells stained positive for 3-NT (Fig. 3, C and D). Moreover, 3-NT was not detected in the tumor epithelium, yet NOS2 protein was present in some of the tumor epithelial cells (Fig. 3, A and B). The specificity of this staining was confirmed by the addition of 1 mm 3-NT to the staining protocol, which reduced binding of the anti-nitrotyrosine antibody by 80—100% (Fig. 6). We conclude that some of the TMCs produce peroxynitrite, and that the reactivity of this species is restricted to those cells.

VEGF Expression. We tested the hypothesis that NO may stimulate VEGF expression in developing tumors and analyzed VEGF expression by Western blot analysis in adenomas positive for NOS2. There was more VEGF protein in the four adenomas than in the surrounding normal tissues, as determined by IP-Western and densitometry (Fig. 7). This distribution correlates with the NOS2 protein content, which was high in the adenomas and undetectable in the normal tissues.
We investigated NOS expression in human colon cancer in respect to tumor staging, NOS-expressing cell type, nitrotyrosine formation, inflammation, and VEGF expression. NOS2 was markedly expressed in approximately 60% of human colon adenomas, whereas it was either low or absent in the surrounding normal tissue. NOS2 was found in TMCs, endothelium, and tumor epithelium. Our results differ from a recent report (52), which did not detect human NOS2 and concluded that NOS expression is low or absent at all stages of colon carcinogenesis. Although we found reduced Ca\(^{2+}\)-dependent NOS activity, there was markedly increased NOS2 activity in adenomas that decreased to a baseline level with cancer progression. We attribute our new findings to either dissimilarities in the studied population (United States versus Singapore) or to technical changes toward an improved detection of human NOS2, such as a more specific antibody used in our study.

The appearance of NOS2 in inflammatory cells and tumor endothelium coincides with reduced expression of NOS1 and/or NOS3. Consistent with a previous report (52), our results suggest that the abundance of endothelial cells, which express NOS3, and autonomic neurons, which express NOS1, decreases in colon tumors. Because tumor-associated NOS2 also is located in the blood vessel walls, cytokines may induce NOS2 in vascular smooth muscle cells and at the same time down-regulate endothelial NOS3. Such a differential expression pattern has been observed in human vascular smooth muscle and endothelial cells treated with cytokines (53). However, a general decrease in vascularity and autonomic innervation of tumor tissues may also produce the decrease in Ca\(^{2+}\)-dependent NOS activity. A recent analysis of colon microvasculature found that adenomas have a lower capillary density than primary and metastatic tumors (54). This is consistent with our observation that NOS3 expression is significantly reduced in adenomas compared to carcinomas or normal colonic mucosa.

The statistically significant correlation between NOS2 activity and NOS2 immunohistochemistry underlines the usefulness of the NOS activity assay in human colonic tissues, in particular, because Western blot analysis and RT-PCR also confirmed the presence of NOS2 protein in tissues with high Ca\(^{2+}\)-independent NOS activity. Both of the antibodies that we used generated the same labeling pattern, and the specificity of one of the antibodies for human NOS2 has already been documented (16). NOS2 expression was primarily observed in tissue mononuclear cells, and only this cell type as well as some polymorphonuclear cells, but not the tumor epithelial cells, were labeled by a polyclonal anti-nitrotyrosine antibody. However, only a subset of the NOS2-expressing cells stained positive for 3-nitrotyrosine, which is a marker for peroxynitrite formation. Additionally, other mechanisms of NO-mediated protein modification have been reported, suggesting alternative pathways for 3-NT formation that do not involve peroxynitrite (55). Nitrotyrosine formation has been...
shown previously in ulcerative colitis (16) and Helicobacter pylori gastritis (17). In ulcerative colitis, most NOS2 was expressed in colon epithelial cells, indicating that the physiology of NOS2 expression in colon carcinogenesis may differ from that in ulcerative colitis. A colocalization of NOS2 and nitrotyrosine has been observed in ulcerative colitis (16). Others found nitrotyrosine in both epithelial cells and infiltrating macrophages in Helicobacter pylori gastritis (17) or juxtaposed to NOS2-expressing macrophages and not distant to the source of NO (41). We found that only a variable percentage of NOS2-expressing inflammatory cells stained positive for nitrotyrosine, but we did not find nitrotyrosine labeling in epithelial cells. Our observations might be explained in part by hypoxia within tumors, which reduces the concentration of reactive superoxide radicals and the subsequent formation of peroxynitrite. Only stoichiometric fluxes of NO and \( \text{O}_2^- \) interact to yield potent oxidants such as ONOO\(^-\), whereas excess production of either radical inhibits these oxidative reactions (56).

A \( M_r 125,000\text{–}138,000 \) protein band, consistent with NOS2, was heavily tyrosine-phosphorylated in some tumor tissues. The band was absent in normal tissues and tumors without detectable NOS2 expression. Tyrosine phosphorylation is a posttranslational modification that activates NOS2 (57). The specific phosphorylated tyrosine residue(s) has not been identified yet, nor do we know which tyrosine kinase(s) phosphorylates human NOS2. Our result suggests that posttranslational activation of human NOS2 by tyrosine phosphorylation might occur commonly in human lymphocytes and macrophages, because this modification was detected in tumors that had abundant NOS2 expression in tissue mononuclear cells.

The pathobiological functions of NOS2 in tumors are not well defined. Recent reports have shown that NO induces endothelial cell growth, mediates tumor vascularization, and regulates the tumor blood flow (19–21). Tumors may modulate the synthesis of NO in their vasculature by secretion of factors that induce NOS2 (25, 58). In addition, we have found that NOS2 is expressed in proliferative but not in differentiated intestinal cells in culture (59). In this respect, NO production may be a part of the angiogenic switch in developing tumors, without which the size of the developing tumor would be limited by the lack of vascularization. This view has been confirmed by the finding that VEGF-stimulated angiogenesis is dependent on NO production (60). To indirectly address the hypothesis that NO can stimulate VEGF synthesis, we studied expression of VEGF in several adenomas with high NOS2 expression. VEGF expression was increased in all of the adenomas when compared to the VEGF protein level in the surrounding normal tissues that were low in NOS2. We conclude that NO may participate in the induction of VEGF or, alternatively, VEGF and NOS2 may have common induction pathways. Regardless, this finding requires additional studies, and we are presently investigating the effect of NO on VEGF expression in a colon carcinoma model.

Another hypothesis can be developed from the observation that peroxynitrite activates prostaglandin synthesis (61, 62). Peroxynitrite increases cyclooxygenase activity by serving as a substrate for the peroxidase activity of the enzyme (61) or may modify the protein via S-nitrosylation (62). In this way, coproduction of NO and superoxide may increase the production of some mitogenic eicosanoids by TMC, which then exert antiapoptotic activity in the surrounding tumor epithelium. The relevance of this pathway is suggested by the protective effect of nonsteroidal anti-inflammatory drugs against the development of colon cancer (63), which is thought to be mediated through the inhibition of cyclooxygenase-2 (64). However, the effect of NO production in tumor biology may change during tumor progression. This hypothesis is supported by data indicating that NO production in a metastatic murine melanoma cell line reduces survival of tumor cells in the circulation and inhibits tissue invasion (65). Thus, NO may support tumor growth at an early stage but may oppose development of metastases. This notion is supported by our finding that NOS2 activity declines as tumor stage advances and is low or undetectable in metastases to lung and liver.

NO can exert cytotoxicity (2). High NO concentrations are required to induce apoptosis in some mammalian cells (22). Just as compelling is the accumulating evidence that low concentrations of NO protect from apoptotic cell death (23, 24, 66, 67). NO chemistry is complex (40, 42) and is still incompletely understood in the cellular context. The concentration ratios between NO, \( \text{O}_2^- \) and \( \text{O}_3^- \) primarly determine the fate of NO (42). Hypoxia, arising during cancer development, may favor nitrosative modifications by NO. This phenomenon is explained by the NO oxidation rate, which increases with the square of the NO concentration (42) but decreases linearly as the \( \text{O}_2^- \) concentration falls during hypoxia. Therefore, a partial decrease of the tissue \( \text{O}_2^- \) concentration in the presence of a high NO concentration may favor the formation of deaminating nitrogen species such as \( \text{N}_2\text{O}_3 \). Inflammation can generate superoxide radicals that modulate the NO pathway toward peroxynitrite formation, \( \text{OH}^- \)-like oxidative damage, and nitration of DNA and proteins (40). However, high concentrations of NO may quench superoxide anion radicals because the reaction product peroxynitrite can rearrange to nontoxic nitrate. NO also can scavenge an oxidizing intermediate generated from peroxynitrite (42), which may account for NO-mediated protection against cytotoxic superoxide radicals generated by xanthine oxidase (68) and for protection from tissue damage in a rat liver inflammation model (69). Therefore, NO can either cause or protect against oxygen radical-induced lipid-peroxidation and DNA damage.

NO mutagenicity has been demonstrated in Salmonella typhimurium, in murine lymphoma cells in vivo, and in human cells (29, 30, 70). We would expect that this would lead to an accumulation of p53 protein (43, 44). We have reported recently the accumulation of p53 in human cells exposed to NO, generated either by exposure to a NO donor or by overexpression of NOS2 protein (45). Furthermore, expression of p53 results in down-regulation of NOS2 expression and activity by inhibition of NOS2 promoter activity (45). Hence, NO may be a possible candidate that selects cells carrying mutant p53 at the transition from colon adenoma to carcinoma \( \text{in situ} \) because it has both genotoxic and angiogenic properties. Mutant p53 cells may be less susceptible to NO-induced growth arrest, and low to moderate NO concentrations may exert antiapoptotic properties specifically in those cells. Only clones with nonfunctional p53 would tolerate the genotoxicity of sustained NO production and escape growth arrest and apoptosis while tumor growth is supported. Not surprisingly, hypoxia, arising with tumor size, has been reported to select for mutant p53 cells, which are resistant to hypoxia-induced apoptosis (71).

Our data demonstrate increased NOS2 activity in a high percentage of colon adenomas. Therefore, we hypothesize that NO is a candidate endogenous carcinogen that either generates or selects for the high frequency of mutations in the \( p53 \) gene and other cancer-related genes that arise at the transition from adenoma to carcinoma \( \text{in situ} \) (49).

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