Advances in Brief

Increased Expression of Inducible and Endothelial Constitutive Nitric Oxide Synthases in Rat Colon Tumors Induced by Azoxymethane

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Abstract
Nitric oxide (NO) is an important bioregulatory mediator involved in a variety of biological processes under both physiological and pathological conditions. To assess whether NO production is altered in colon carcinogenesis, the expression levels and localization of two isoforms of NO synthase, inducible NO synthase (iNOS) and endothelial constitutive NO synthase (eNOS), were examined by immunoblot and immunohistochemical methods in normal colonic mucosa and colon carcinomas induced by azoxymethane in male F344 rats. All colon carcinoma tissues examined were found to have an increased expression of iNOS and eNOS proteins as compared to normal colonic mucosa. In particular, the pronounced staining of iNOS protein localized to the luminal surface of carcinoma epithelial cells was not detectable in normal colon epithelium. The neovascularization in tumor tissues also demonstrated intense eNOS immunoreactivity in endothelial cells. These findings indicate that NO production is markedly elevated in azoxymethane-induced rat colon carcinomas, suggesting that regulatory pathways involving this mediator have some biological relevance to colon carcinogenesis in this model.

Introduction
NO is an important bioregulatory mediator involved in a variety of processes in the cardiovascular, nervous, and immune systems (1). It is synthesized from l-arginine by a family of NO synthases, three isoforms of which have so far been identified; neural constitutive NO synthase, eNOS and iNOS (2). The former two constitutive isoforms are calcium-dependent and play physiological roles in regulating vascular tone and neurotransmission, whereas iNOS is calcium-independent and induced by bacterial endotoxins and cytokines to provide a sustained release of NO, which mediates immune cell cytotoxicity (3). Chronic infection and inflammation cause overproduction of NO, and evidence is accumulating that this may contribute to multistage carcinogenesis by inducing DNA mutations and tissue damage (4). Recently, increased expression of NOS in human ovarian, uterine, breast, and brain tumor tissues has been described (5–7). In human colon carcinoma tissues, however, previous reports have indicated that the protein amount and enzyme activity may be significantly reduced in colon carcinoma tissues as compared to normal colonic mucosa (8, 9). Because the situation with regard to NOS in rodent colon tumors has not yet been reported, we compared the expression of iNOS and eNOS by immunoblot and immunohistochemical methods in AOM-induced colon carcinomas and normal colonic mucosa in rats. Contrary to the results from human colon carcinoma tissues, we found AOM-induced colon adenocarcinomas to show elevated expression of both protein species as compared to that of normal colonic mucosa.

Materials and Methods
Animals and Treatment. Male F344 rats (Charles River Japan, Inc., Kanagawa, Japan) at 6 weeks of age were treated s.c. with AOM (Sigma Chemical Co., St. Louis, MO) in sterile saline at a dose of 15 mg/kg body weight once a week for 2 weeks and then maintained with a standard diet, AIN-76A (Dyets, Inc., Bethlehem, PA) until being sacrificed 36 weeks after the first carcinogen exposure. Colon tumors and normal background colon mucosa were obtained from eight different randomly selected AOM-treated rats. The tumors were cut in two, with one half being immediately frozen and stored at −80°C, and the other fixed with formalin at 4°C overnight and embedded in paraffin. Paraffin sections were stained with H&E for histological examination and also used for immunohistochemistry. Accompanying normal mucosa from the same animals was collected and stored at −80°C for comparison by immunoblot analyses. Moreover, samples of normal colon mucosa from four saline-treated rats were prepared for immunoblotting.

Immunoblot Analysis. The tissues were homogenized at 4°C in radioimmunoprecipitation assay buffer (50 mM Tris-HCl (pH 7.5), 150 mM sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.02% sodium azide, 1 mM phenylmethylsulfonl fluoride and 10 μM leupeptin) and centrifuged. Protein concentrations of soluble fractions of tissue lysates were determined using Bio-Rad protein assay reagents (Bio-Rad Laboratories, Hercules, CA). Aliquots of 100 μg of total cellular protein were denatured by boiling with SDS and DTT, electrophoresed on 8% SDS-polyacrylamide gels, and then transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore Corporation, Bedford, MA). After blocking with blocking solution (Block Ace; Dai-nippon Pharmaceutical Corp., Osaka, Japan), the membranes were probed with monoclonal anti-iNOS or anti-eNOS antibodies (Transduction Laboratories, Lexington, KY; 1:2500 dilution with 10% blocking solution) for 1 h, washed with Tris-buffered saline containing 0.1% Tween 20, and then incubated with anti-mouse immunoglobulin, a horseradish peroxidase-conjugated F(ab')2 fragment from sheep (Amersham, Buckinghamshire, England; 1:2000 dilution with blocking solution) for 1 h. After several washes, the membranes were developed using a chemiluminescence system (enhanced chemiluminescence detection reagents; Amersham) and exposed to Hyperfilm-MP (Amersham). To verify the relative protein amounts in each lane, actin levels were determined using Bio-Rad Protein Stain Reagents (Bio-Rad Laboratories, Hercules, CA) at 6 weeks of age. The tumors were cut in two, with one half being immediately frozen and stored at −80°C, and the other fixed with formalin at 4°C overnight and embedded in paraffin. Paraffin sections were stained with H&E for histological examination and also used for immunohistochemistry. Accompanying normal mucosa from the same animals was collected and stored at −80°C for comparison by immunoblot analyses. Moreover, samples of normal colon mucosa from four saline-treated rats were prepared for immunoblotting.

Immunohistochemical Staining. All immunohistochemical analyses were performed with the avidin-biotin complex immunoperoxidase technique. Tissue sections (5 μm) were mounted on poly-L-lysine-coated slides, deparaffinized with xylene, and rehydrated with graded alcohol. Slides were then immersed in 10 mM citrate buffer (pH 6) and microwaved for 5 cycles of 5 min at 500 W to enhance antigen exposure. After blocking endogenous peroxidase with 0.3% hydrogen peroxide in methanol for 30 min and nonspecific protein binding with 2% normal horse serum in PBS containing 0.3% Triton X-100 for 30 min, sections were incubated overnight at 4°C in a humidified chamber with monoclonal mouse anti-iNOS or anti-eNOS antibodies (Transduction Laboratores).
**Fig. 1.** Immunoblot analysis of tissue lysates from AOM-induced colon carcinomas and their adjacent normal colonic mucosa. Aliquots (100 μg) of total cellular protein were electrophoresed on an 8% SDS-polyacrylamide gel and transferred to a membrane filter. Lanes T1–8, AOM-induced colon carcinomas; Lanes N1–8, paired normal colonic mucosa samples. The filters were incubated with specific monoclonal antibodies: A, anti-iNOS; B, anti-eNOS; C, anti-actin.

**Fig. 2.** Immunohistochemical staining for iNOS. A, no iNOS immunoreactivity is apparent in normal colon mucosa. B, immunoreactive iNOS is prominent at the luminal surfaces of colon carcinoma epithelial cells. A and B, ×100.
tories) diluted 50× or 250× in PBS containing 2% horse serum and 0.3% Triton X-100. Biotinylated anti-mouse IgG (H+L) raised in a horse, affinity-purified, and absorbed with rat serum (Vector Laboratories, Inc., Burlingame, CA) was diluted 200× in PBS containing 2% horse serum and used as the secondary antibody. Staining was performed using avidin-biotin reagents (Vectastain ABC reagents; Vector Laboratories, Inc.), 3,3′-diaminobenzidine and hydrogen peroxide. The sections were counterstained with methylgreen. As a negative control, duplicate sections were immunostained without exposure to the primary antibody.

**Results**

**Expression Level of iNOS and eNOS Proteins in Rat Colon Tumors.** iNOS and eNOS protein abundance in eight samples of AOM-induced adenocarcinomas and their normal colonic mucosa was determined by immunoblot analysis (Fig. 1). iNOS and eNOS immunoreactive bands were hardly detectable in any of the eight normal colon mucosa samples from AOM-treated rats examined (Fig. 1, A and B, Lanes N1–8) or in any of four normal mucosa samples from saline-treated rats (data not shown). In contrast, strong expression of iNOS, Mr 130,000, and eNOS, Mr 140,000, was demonstrated in all eight adenocarcinoma samples (Fig. 1, A and B, Lanes T1–8). The immunoreactive bands of actin, demonstrated as an internal control protein, were of almost equal intensity in all tissue samples (Fig. 1C).

**Localization of iNOS and eNOS Proteins in Rat Colon Tumors.** To determine which tissue or cells express iNOS and eNOS in colon carcinomas, an immunohistochemical analysis of paraffin-embedded

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**Fig. 3.** Immunohistochemical staining for eNOS. A, immunoreactive eNOS is localized to the blood vessels in the submucosa of normal colonic mucosa. B, intense positive staining for eNOS is evident in the endothelial cells of the neovasculature in a colon carcinoma. A and B, ×100.
specimens prepared from the same tissue samples as examined by immunoblotting was performed.

In normal colon mucosal tissue, iNOS expression was hardly detectable in either epithelial or stromal cells (Fig. 2A). In contrast, positive staining for iNOS was clearly observed in the carcinoma epithelial cells, predominantly at the luminal surfaces of carcinoma cells forming glandular patterns (Fig. 2B). iNOS immunoreactivity was also occasionally observed in inflammatory cells in the stroma, but no positive staining of other cells was observed.

With eNOS, positive staining was limited to the endothelial cells of blood vessels. In normal mucosal tissue, these were mainly observed in the submucosa and subserosa (Fig. 3A). In contrast, tumor tissue showed a marked increase of blood vessels intermingled with the epithelial structure, with intense positive staining for eNOS observed in the endothelial cells of the neovascularure (Fig. 3B). Negative control sections showed no positive staining when the primary antibody step was omitted (data not shown).

Discussion

The present study demonstrated that AOM-induced colon adenocarcinomas in rats show increased expression of both iNOS and eNOS proteins. Because NO plays an important role in the regulation of vascular tone and blood flow, it is possible that the production by eNOS in endothelial cells of the neovasculature may cause vasodilation and increase blood flow to the tumor tissues, supporting their growth. Indeed, it has been reported that inhibitors of NOS selectively reduce blood flow in tumor-associated neovascularure (10). The biological significance of increased iNOS in cancer cells is, however, more difficult to specify. Abberant iNOS expression may be one of the phenotypical changes in gene expression associated with carcinogenesis. iNOS is known to be induced by various stimuli in a variety of cell types, such as macrophages, liver cells, and smooth muscle cells, and therefore, another possible explanation for the abberant iNOS expression is that the iNOS gene is induced by some factors especially produced in tumor tissues. It is also possible that luminal components contribute to the induction of iNOS.

It has been reported earlier that iNOS and eNOS are expressed to an appreciable extent in the epithelium of the normal human colon and that levels are in fact reduced in colonic neoplasms (8, 9). However, Singer et al. recently found iNOS to be undetectable in normal colonic epithelium but induced in cases of inflammatory bowel disease (11). Expression of iNOS and eNOS has been described for colon cancer cell lines (12), and modulation of NOS gene expression by several cytokines in colon cancer cells has also been reported (12). Therefore, there seems to be some uncertainty as to the relationship between NOS proteins and colon cancer in men that needs to be clarified.

Aberrant or altered expression of NOS proteins has been reported in a variety of neoplasms. For example, neural constitutive NOS may be up-regulated in tumor cells of the ovary, uterus (5), and brain (7) and in the vascular endothelium and myoepithelial cells of breast tumors (6). iNOS has been reported to be expressed in squamous cell carcinoma of the head and neck (13) and tumor-associated macrophages in breast tumors (6). Therefore, it is conceivable that NO production has a wide involvement in carcinogenesis.

With regard to the possible relationship between overexpression of iNOS and colon carcinogenesis, the characteristic localization at the luminal surfaces of cancer cells is clearly of interest, although its significance remains to be determined. Among the biological effects of NO, interactions with COX pathways seem to be important. NO has been found to enhance the activity and expression of COX-2, an inducible form, in a variety of cell types (14–17). COX-2 is in fact known to be increased in colorectal tumors in men and rodents (18, 19), as confirmed by a separate study using the same tumor samples examined in the present study (data not shown). Overexpression of COX-2 has been demonstrated to render tumor cells resistant to apoptosis and growth advantage (20, 21), and nonsteroidal anti-inflammatory drugs, such as aspirin and sulindac, which are inhibitors of COX, are reported to be associated with decreased development of colon cancers in humans as well as in rodent studies (18, 22). Therefore, it may be possible that NO production is involved in the overexpression of COX-2 in colon cancer cells, thus conferring a survival advantage. NO has also been found to enhance neovascularization (23). Furthermore, NO is known to cause DNA damage (4), and the increased production in cancer cells may facilitate the accumulation of sequential mutations, resulting in tumor development. Thus, from the available data, inhibition of NO production could be a possible mechanistic approach to cancer prevention.

References


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