Inducible Nitric Oxide Synthase (iNOS) Is Expressed Similarly in Multiple Aberrant Crypt Foci and Colorectal Tumors from the Same Patients

Xing Pei Hao, Thomas G. Pretlow, J. Sunil Rao, and Theresa P. Pretlow

Abstract

Aberrant crypt foci (ACFs) are the earliest identified neoplastic lesions in the colon. Aberrant expression of inducible nitric oxide synthase (iNOS or NOS2) has been documented in colorectal tumors, but expression of iNOS has not been reported in human ACF or multiple neoplastic lesions from the same patient. Immunohistochemical expression of iNOS was evaluated in 42 ACF, 14 adenomas, and 25 carcinomas and their adjacent normal mucosa. iNOS was strongly expressed in the normal colonic epithelial cells of all patients; it was markedly reduced in 21 of 42 (50%) ACF and in 14 of 25 (56%) carcinomas. The expression of iNOS was remarkably similar in multiple lesions from the same patient (P < 0.0001). These results suggest that the reduced expression of iNOS is a very early event in the development of some human colorectal tumors, and that host factors control the expression of iNOS similarly in premalignant and malignant colonic epithelial cells.

Introduction

ACF are putative premalignant lesions found microscopically in whole-mounts of grossly normal colorectal mucosa of rodents treated with carcinogen and of humans (reviewed in Refs. 1 and 2). Human ACF display the following characteristics: (a) morphological alterations from atypia to varying degrees of dysplasia (3) and carcinoma in situ; (b) alterations of enzyme activities and the expression of tumor-associated antigens such as carcinoembryonic antigen, fragile histidine triad (4), sialyl Lewis\textsubscript{x}, and sialyl Tn antigens; and (c) genetic alterations that include mutations of K-ras and APC genes as well as genomic instability (reviewed in Refs. 1 and 2). The monoclonality of human ACF has been demonstrated (5, 6), making ACF the earliest identified neoplastic lesion in the large bowel. Nitric oxide, an important mediator in various physiological and pathological activities, is produced when L-arginine is metabolized to L-arginine to nitric oxide, an important mediator in various physiological and patholog-


cal activities, is produced when L-arginine is metabolized to L-


citrulline by nitric oxide synthase. At least three isoenzymes have been identified, i.e., Ca\textsuperscript{2+}-dependent neuronal and endothelial iso-


syntheses in the reverse reaction of nitric oxide synthase a Ca\textsuperscript{2+}-dependent neuronal and endothelial iso-


forms that are constitutively expressed and a Ca\textsuperscript{2+}-independent (iNOS or NOS2) isoform that is inducible (reviewed in Refs. 7–9). Neuronal and endothelial nitric oxide synthase usually produce small amounts of nitric oxide that last a short time (minutes), whereas iNOS generates large amounts of nitric oxide that last up to several days (reviewed in Refs. 7–9). Constitutive expression of iNOS by colon cancer cell lines was first demonstrated by Radomski et al. (10) and has subsequently been demonstrated in the epithelial cells of normal colonic mucosa and colorectal tumors (11, 12). Although several studies implicate iNOS in colon tumorigenesis (8, 11–14), none have evaluated the expression of iNOS in human ACF. Our studies demonstrate that iNOS expression is reduced in similar proportions in ACF (21 of 42, or 50%) and colorectal carcinomas (14 of 25, or 56%). Unexpectedly, the expression of iNOS was similar in multiple independent lesions (including ACF, adenomas, and carcinomas) from the same patient.

Materials and Methods

Specimens. Human colonic tissues were obtained from the Western Division of the National Cancer Institute’s Cooperative Human Tissue Network at Case Western Reserve University and processed as described previously (1, 3). Eleven tumors from four FAP patients were received as paraffin sections rather than as fresh tissues. We collected 27 ACF and their adjacent normal mucosa from 19 patients with sporadic colon carcinomas (12 males and 7 females; mean age, 68 ± 7.6 years) and 15 ACF from 6 patients with FAP. A maximum of three ACF per patient were analyzed, except for one patient with FAP from whom 4 ACF were used, two from the proximal colon and two from the distal colon. For comparison, 26 carcinomas (23 from sporadic carcinomas and 3 from FAP) and 17 adenomas from nine of these same patients with ACF and/or cancer were included in this study. The patients with cancer included 10 females and 16 males that had 1 Dukes’ stage A, 12 stage B, 5 stage C, and 8 stage D carcinomas that were moderately differentiated except for 4 that were poorly differentiated. The adenomas ranged in size from 2 × 3 mm to 15 × 19 mm. All tissues were fixed in phosphate-buffered 10% formalin (Fisher Scientific, Pittsburgh, PA) and embedded in paraffin. Multiple sections were cut and mounted on Superfrost/Plus slides (Fisher Scientific) and stored at 4°C. Sections near serial to those used for immunohistochemistry were stained with H&E for histopathological classification as described previously (3).

Immunohistochemical Analysis. A streptavidin-biotinylated horse radish peroxidase immunohistochemical procedure combined with tyramide signal amplification (TSA Biotin System; NEN Life Science Products, Boston, MA) was used. Briefly, slides were heated at 60°C for 75 min, de waxed in xylene twice for 7 min, rehydrated, and then heated in 0.01 M citrate buffer (pH 6.6) in a pressure cooker for 3 min after reaching full pressure. To block nonspecific staining, the sections were incubated for 15 min in a blocking solution of 10% normal horse serum in PBS [0.01 M phosphate (pH 7.4), and 0.137 M NaCl] and 1% milk in block solution. Solution of a blocking solution of 10% normal horse serum in PBS [0.01 M phosphate (pH 7.4), and 0.137 M NaCl] and 1% milk in block solution. To deplete endogenous peroxidase activity, the slides were immersed in 3% hydrogen peroxide in methanol for 30 min and washed in distilled water. The sections were incubated for 30 min in streptavidin-biotinylated horseradish peroxidase (TSA Biotin System) diluted 1:100 in blocking solution, washed in PBS, incubated for 10 min in biotinyl tyramide IgG (Vector Laboratories, Burlingame, CA) diluted 1:200 in blocking solution. To deplete endogenous peroxidase activity, the slides were immersed in 3% hydrogen peroxide in methanol for 30 min and washed in distilled water. The sections were incubated for 30 min in streptavidin-biotinylated horseradish peroxidase complex (Amersham, Arlington Heights, IL), diluted 1:100 in blocking solution, washed in PBS, incubated for 3,3′-diaminobenzidine (Sigma Chemicals, St. Louis, MO) for the chromogenic substrate. The slides were counterstained with 0.1% methyl green for 3 min, dried, and mounted with 50% Clearium/50% xylene (Surgipath Medical Industries, Inc., Richmond, IL).

Cancer Research 61, 419–422, January 15, 2001

Received 9/6/00; accepted 11/27/00.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Supported in part by Public Health Service Grants CA66725, CA57179, CA43703, and CA54031 from the National Cancer Institute.

2 To whom requests for reprints should be addressed, at Institute of Pathology, Case Western Reserve University, 2085 Adelbert Road, Cleveland, Ohio 44106. Phone: (216) 368-8702; Fax: (216) 368-1278; E-mail: tp3@po.cwru.edu.

3 The abbreviations used are: ACF, aberrant crypt foci; FAP, familial adenomatous polyposis; iNOS, inducible nitric oxide synthase.

4 The abbreviations used are: ACF, aberrant crypt foci; FAP, familial adenomatous polyposis; iNOS, inducible nitric oxide synthase.

5 The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Supported in part by Public Health Service Grants CA66725, CA57179, CA43703, and CA54031 from the National Cancer Institute.

2 To whom requests for reprints should be addressed, at Institute of Pathology, Case Western Reserve University, 2085 Adelbert Road, Cleveland, Ohio 44106. Phone: (216) 368-8702; Fax: (216) 368-1278; E-mail: tp3@po.cwru.edu.

3 The abbreviations used are: ACF, aberrant crypt foci; FAP, familial adenomatous polyposis; iNOS, inducible nitric oxide synthase.
Non-specific horse serum or mouse monoclonal anti- bromodeoxyuridine (Chemicon, Temecula, CA), an inappropriate antibody, was substituted for the primary antibody at the same dilution as a negative control in every group of slides stained.

**Evaluation of the Staining.** The extent and intensity of immunoreactivity for iNOS of all specimens were determined by two independent observers (X. P. H. and T. P. P.). The following scale was used to express the extent of positivity: 0, <5%; 1, >5–25%; 2, >25–50%; 3, >50–75%; and 4, >75% of the colonic epithelial cells expressing iNOS in the respective lesions. The intensity of iNOS expression was scored as follows: 0, negative; 1+, weak; 2+, moderate; 3+, as strong as normal mucosa from the same patient. The final score, obtained by multiplying the extent of positivity and intensity scores, ranged from 0–12. Scores of 0–4 were defined as “markedly reduced” or “no expression”; scores 5–8 were defined as “intermediate expression”; and scores of 9–12 were defined as “strong expression” (4).

**Statistical Analyses.** Fisher’s exact test was used to assess the associations between iNOS expression and pathological data. A one-way ANOVA was used to compare the iNOS expression with the size (expressed in mm$^2$) of the ACF and with the number of crypts/ACF. To evaluate the expression of iNOS in multiple lesions from the same patient, a one-sample proportion test was used where the null value being tested was 0.33 for random mixing. All tests were two-tailed and a $P < 0.05$ was considered significant.

**Results**

**iNOS Expression in Carcinomas and Their Adjacent Normal Mucosa.** Epithelial cells in normal colonic mucosa adjacent to ACF or tumors from all patients showed strong cytoplasmic expression of iNOS; this served as an internal positive control. Three adenomas and one carcinoma from one FAP patient were not included in the analysis because the adjacent normal mucosa did not stain strongly, whereas other samples with normal mucosa from this same patient did stain strongly. These specimens were among those received as paraffin sections; it is likely these specimens were overfixed. Stromal cells such as endothelial cells, smooth muscle cells, and macrophages also showed immunoreactivity for iNOS in both the normal mucosa and the neoplastic lesions. iNOS expression in the malignant epithelial cells displayed a similar cytoplasmic localization but a sharp reduction compared with adjacent normal mucosa in both the number of epithelial cells stained and their intensity (Table 1 and Fig. 1, A and B). Although both 1:500 and 1:1000 dilutions of the primary antibody demonstrated strong expression of iNOS in the normal colonic epithelial cells, the 1:1000 dilution generally demonstrated greater contrast in those lesions with reduced expression of iNOS. Forty-two (56%) carcinomas exhibited weak or no expression of iNOS; eight had intermediate expression, where some morphologically indistinguishable cells showed strong positive expression, whereas others were negative; and three carcinomas retained strong expression of iNOS. Because our carcinoma specimens included 21 of 25 with moderate differentiation, it was not possible to determine whether iNOS expression was associated with differentiation. The expression of iNOS did not appear to be associated with the sex of the patient, the location in the colon, or Dukes’ stage ($P > 0.1$; Fisher’s exact test).

**iNOS Expression in ACF.** Twenty-one of 42 (50%) ACF showed a marked reduction in iNOS expression compared with adjacent normal mucosa (Fig. 1, C–F). The expression of iNOS was not related to the presence or degree of dysplasia or to the size (expressed in mm$^2$) or to the number of crypts in the ACF (Table 2). No differences of iNOS expression were found, either between carcinomas and ACF ($P > 0.05$; Table 1) or between ACF from sporadic cancer patients and those from FAP patients. Multiple ACF, i.e., a total of 27 ACF, were available from 10 of our patients. In six of these patients, iNOS expression (graded as strong, intermediate, or weak) was the same in all of the ACF analyzed from that patient, i.e., three patients had seven ACF with strong expression and three patients had eight ACF with weak expression of iNOS. In three additional patients, the iNOS expression was somewhat more variable. Each of two patients had two ACF with weak expression and one ACF with intermediate expression; the third patient had two ACF with weak expression, one ACF with intermediate expression, and one ACF with strong expression. In these three patients, 6 of 10 ACF stained alike in the same patient. A total of 21 of 27 ACF expressed iNOS like the other ACF from the same patient; this pattern differs from what would be observed with random mixing in each patient ($P < 0.0001$).

**iNOS Expression in Multiple Lesions.** Because this was unexpected for independent monoclonal lesions, we looked to see how many different types of colonic tissues (ACF, adenomas, and carcinomas) were available from each patient. We evaluated two or three different types of tissues from each of 13 patients (Fig. 2). This provided a total of 44 different lesions, with between 2 and 6 lesions/patient, and consisted of 22 ACF, 14 adenomas, and 8 carcinomas. Two patients (Fig. 2, patients 8 and 9) had two lesions each that stained differently from one another. Six patients (Fig. 2, patients 1, 2, 3, 6, 11, and 12) had all 15 of their lesions stain like the others from the same patient; these lesions were as diverse as carcinoma and ACF with atypia. The remaining five patients (Fig. 2, patients 4, 5, 7, 10, and 13) had a total of 25 lesions with iNOS expression that was somewhat variable. For example, one patient (Fig. 2, patient 13) had six lesions: five lesions including one carcinoma, two adenomas, and two ACF that all retained strong expression of iNOS and one adenoma that had intermediate expression. Sixteen of 25 lesions from these five patients (Fig. 2, patients 4, 5, 7, 10, and 13) expressed iNOS similar to other lesions from the same patient. A total of 31 of 44 lesions from 13 patients each expressed iNOS similar to the other lesions from the same patient ($P < 0.0001$).

**Discussion**

The immunohistochemical demonstration of iNOS expression in normal colonic epithelial cells and stromal cells including endothelial cells, macrophages, and smooth muscle cells is similar to that reported previously (11). A complete absence of iNOS in malignant colonic epithelial cells in 12 of 12 tumors was reported in those studies (11), whereas iNOS expression was weak or absent in only 14 of 25 (56%) of our cancers, reduced to an intermediate level in an additional 8 (32%), and like normal epithelial cells in 3 (12%). Different tumor samples may account for some of these differences. In addition, our immunohistochemical method appears to be more sensitive. We observed strong iNOS expression in all our normal mucosal samples; the previous work (11) reported no strong colonic epithelial cell staining, i.e., most of their normal mucosa scored 2 or 3 of a possible 7. In the previous study (11), any reduction of staining from normal would appear as a complete loss of expression. Our results reflect the variability of iNOS activity reported by others in colon cancer cell lines (10, 15) and primary tumors (8, 12). When tissue homogenates were analyzed for iNOS expression by Western blot or for iNOS enzymatic activity, only very low levels of iNOS were detected in normal colonic epithelium; but iNOS was highly expressed in 60% of adenomas and 20–25% of carcinomas (8). Although we did not detect increased expression of iNOS in our lesions, we observed strong

---

**Table 1** iNOS expression in human colorectal cancer and ACF

<table>
<thead>
<tr>
<th>iNOS expression</th>
<th>Strong</th>
<th>Intermediate</th>
<th>Weak or absent</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carcinoma</td>
<td>3</td>
<td>8</td>
<td>14</td>
<td>25</td>
</tr>
<tr>
<td>ACF</td>
<td>14</td>
<td>7</td>
<td>21</td>
<td>42</td>
</tr>
</tbody>
</table>

$P = 0.0989$; Fisher’s exact test.
iNOS expression in the epithelial cells of 33% of our ACF and 12% of our colorectal cancers. In the reported study (8), iNOS activity appeared primarily in tissue mononuclear cells rather than in epithelial cells when the tissues were examined immunohistochemically with polyclonal antibodies (8). In a later study of 118 colon cancers from this same group, iNOS activity decreased with increasing stage and was lowest in metastatic lesions (12).

The finding of reduced expression of iNOS in the colonic epithelial cells of similar proportions of ACF (50%) and carcinomas (56%) suggests that the loss of iNOS expression may be a very early event in human colorectal tumorigenesis. Because iNOS expression is not associated with the presence or degree of dysplasia in ACF (Table 2), the decreased expression of iNOS in colonic epithelial cells does not appear to be strongly associated with progression in human colorectal tumorigenesis. This is in contrast with the increased expression of iNOS in diseases, such as ulcerative colitis (16) and Barrett esophagus (17), that predispose to cancer. It is interesting that in skin carcinogenesis studies, iNOS and reactive nitrogen intermediates are produced in the dermis during tumor promotion at the same time that iNOS is down-regulated in the epidermis and in the papillomas that form (18). In experimental rat colon carcinogenesis, all eight carcinomas induced with azoxymethane expressed increased levels of iNOS in the epithelial cells compared with the very low levels found in the adjacent normal colonic epithelial cells (13). Additional evidence that overexpression of iNOS may play a role in rodent colon tumorigenesis is provided by experiments in which the addition of an iNOS-specific inhibitor to the diet reduced the number of ACF that developed in rats treated with azoxymethane (14).

Perhaps the most unexpected finding in our studies is the very similar expression of iNOS (graded as strong, intermediate, or weak) among the epithelial cells of multiple lesions from the same patient. ACF (5, 6) and colon tumors (19) generally have been reported to be monoclonal lesions. Numerous studies have supported the independent expression of genetic and phenotypic markers among different lesions from the same patient (Refs. 3 and 5; reviewed in Ref. 2). The coordinate expression of iNOS in the epithelial cells of multiple independent lesions suggests that host factors regulate iNOS differently in normal colonic epithelial cells from those that are altered as ACF, adenomas, or carcinomas in these patients. The regulation of iNOS in these diverse lesions may provide important clues regarding a possible genetic alteration(s) that occurs very early in the development of this group of colon tumors.

iNOS activity and/or expression has been reported altered in several other human tumors such as breast, ovarian, brain, bladder, lung, prostate, and gastric cancers (reviewed in Refs. 7 and 9). Although increased expression of iNOS is common in tumors, it is not universal; and different studies on the same types of tumors report different results both regarding the source of iNOS and the levels of expression.

### Table 2: iNOS expression in human ACF

(iNOS expression in ACF with atypia or with dysplasia was not different (P = 0.916; Fisher’s exact test).

<table>
<thead>
<tr>
<th>iNOS expression</th>
<th>Strong</th>
<th>Intermediate</th>
<th>Weak or absent</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>14</td>
<td>7</td>
<td>21</td>
<td>42</td>
</tr>
<tr>
<td>Atypia</td>
<td>9</td>
<td>4</td>
<td>12</td>
<td>25</td>
</tr>
<tr>
<td>Dysplasia</td>
<td>5</td>
<td>3</td>
<td>9</td>
<td>17</td>
</tr>
<tr>
<td>Mild</td>
<td>4</td>
<td>2</td>
<td>6</td>
<td>12</td>
</tr>
<tr>
<td>Moderate</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Severe</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Mean ACF size</td>
<td>2.2 ± 1.5 mm²</td>
<td>2.0 ± 1.1 mm²</td>
<td>2.3 ± 2.2 mm²</td>
<td>2.2 ± 1.8 mm²</td>
</tr>
<tr>
<td>Number of crypts/ACF</td>
<td>61 ± 52</td>
<td>65 ± 83</td>
<td>58 ± 56</td>
<td>60 ± 58</td>
</tr>
</tbody>
</table>
For example, in gastric cancer, iNOS expression was reduced in the malignant epithelial cells compared with the adjacent normal mucosa in one study (20) and was increased primarily in the stromal cells in another study (7). In breast cancer, high iNOS activity was associated with low proliferation and low-grade disease in one study (9) and with high-grade disease in another study (7). Whether nitric oxide and iNOS activity stimulate or retard tumor growth appears to be highly variable and likely regulated by a number of factors, including the concentration of nitric oxide in the tumor microenvironment. For example, high concentrations of nitric oxide are cytotoxic and induce apoptosis, whereas low concentrations induce angiogenesis and enhance the growth rate of some tumors (7, 21).

The mechanisms leading to the down-regulation of iNOS in the development of colorectal cancer are not clear. Forrester et al. (22) postulate that high concentrations of endogenously generated nitric oxide trigger the accumulation of wild type p53 that in turn down-regulates the expression of iNOS by inhibiting its promoter. Although this mechanism is attractive for the regulation of iNOS in premalignant lesions of the colon before p53 mutations are common, the high incidence of p53 mutations in colorectal cancers (12, 23) makes this less plausible. Whether iNOS expression in colonic epithelial cells is an active participant in the neoplastic process or an indirect indicator of the process awaits a better understanding of iNOS and the factors that control it under physiological and pathological conditions in the human colonic epithelium.

References
Inducible Nitric Oxide Synthase (iNOS) Is Expressed Similarly in Multiple Aberrant Crypt Foci and Colorectal Tumors from the Same Patients

Xing Pei Hao, Thomas G. Pretlow, J. Sunil Rao, et al.

_Cancer Res_ 2001;61:419-422.