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Increased in situ expression of nitric oxide synthase in human colorectal cancer

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Abstract There is growing evidence that nitric oxide (NO) has an important role in tumor growth. However, information on the expression of NO synthase (NOS) in colorectal cancers is scanty. We therefore investigated the distribution and expression of NOS in human colorectal cancers. The expression of three types of NOS, inducible (iNOS), endothelial (eNOS) and neuronal (nNOS), was examined by immunohistochemistry in 25 cases of colorectal cancer. The expression of iNOS was also investigated at the mRNA level using the reverse transcriptase polymerase chain reaction (RT-PCR) in 6 cases. Correlations were made between iNOS expression and the histopathological findings. Immunoreactive iNOS was detected in the tumor cells in 22 cases (88%) with diffuse cytoplasmic reactions. Expression of iNOS-mRNA detected by RT-PCR in three tumor tissues was over five-fold that in normal mucosa. Intensified immunoreactivity of iNOS was associated with vascular invasion. iNOS expression did not correlate with pathological staging, tumor size, lymph node metastasis, p53 expression or tumor vessel density. Immunoreactive eNOS stained more strongly in the endothelial cells of microvessels within and around the tumor than in the areas remote from the tumor. There is enhanced expression of iNOS and eNOS in human colorectal cancers, which may correlate with tumor growth and vascular invasion.

Key words Nitric oxide · Nitric oxide synthase · Colorectal cancer · Immunohistochemistry

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Introduction

Nitric oxide (NO) is a free radical gas, which is synthesized from L-arginine by three isoforms of nitric oxide synthase (NOS). Two of these, the neuronal type (nNOS) and the endothelial type (eNOS) are constitutively expressed, and their activities depend on elevated intracytoplasmic Ca^{2+} levels. The inducible type (iNOS) is independent of Ca^{2+} and is induced by lipopolysaccharide, tumor necrosis factor-α, interleukin-1β and γ-interferon [8]. NO can regulate diverse functions, including vascular relaxation, neurotransmission, and immune responses [17].

NO has also been implicated in tumor biology. Recent studies have demonstrated elevated NOS expression and activities in ovarian cancer, breast cancer and central nervous system tumors [3, 20, 21]. Levels of NOS activities correlated with the histological grade of malignancy in these tumors [20, 21]. By contrast, the expression and localization of NOS in colorectal cancers have not been extensively investigated in vivo. An experimental study demonstrated increased expression of iNOS and eNOS in rat colon tumors [18], while studies on human colorectal cancers found diminished expression of iNOS [10]. The significance of these results and the localization of different isoforms of NOS in colorectal cancers are still unknown. In the present study, we therefore examined the immunohistochemical expression of eNOS, iNOS and nNOS and the transcript levels of iNOS in human colorectal cancers. The expression levels of iNOS were also examined to see whether they correlated with the histopathological findings.

Materials and methods

The specimens were surgically obtained from 25 patients (11 male, 14 female) with colorectal cancer. Ages ranged from 43 to 88 (mean 67.2, median 69) years). Thirteen tumors were located in the colon (ascending 5, transverse 2, descending 1, sigmoid 6), and 11 were in the rectum. Tumor size ranged from 1.5 to 8.0 cm (mean 4.4 cm, median 4.0 cm). The stage of the tumors was assigned to Dukes' A in 5 cases, Dukes' B in 9, Dukes' C in 7 and Dukes' D in 4.

Tissue processing

For conventional light microscopic observation, tumor samples were fixed in 4% formaldehyde solution and embedded in paraffin. Both hematoxylin and eosin and elastica–van Gieson stains were used to examine vascular invasion.

Tumor tissues with adjacent nonneoplastic areas of intestinal wall were used for immunohistochemical analysis. Remote intestinal wall that was free of inflammatory reactions was also sampled for comparison. A portion of each sample was fixed in acetone at –20°C overnight, followed by paraffin embedding, based on the AMeX method [16]. The other portion of the specimens was fixed in Zamboni solution overnight at –4°C and washed in 0.1 M phosphate buffer. The specimens were then immersed in 0.01 M phosphate-buffered saline containing 20% sucrose for 12 h at 4°C and embedded in Optimal Cutting Compound (Miles, Naperville, Calif.), followed by freezing in liquid nitrogen. For the reverse transcriptase polymerase chain reaction (RT-PCR) the rest of the tumor and the rest of the nonneoplastic tissue were stored at –80°C until analysis.

Immunohistochemical procedures

We used the streptavidin–biotin complex method (Histofine SAB-PO Kit, Nichirei, Tokyo, Japan) for immunohistochemical detection of NOS antigens and other related peptides. Staining for eNOS, nNOS, p53 and CD31 was performed on serial sections using rabbit antiserum against nNOS (provided by Dr. Taniguchi, Osaka University, diluted 1:1500), monoclonal antibody to human eNOS (Transduction Laboratories, Lexington, Ky., diluted 1:200), p53 protein (Clone DO-1, Oncogene Science, Uniondale, N.Y., diluted 1:100), and human CD31 (clone JC/70A, Dako Japan, Kyoto, Japan, diluted 1:40), respectively. Deparaffinized sections were pretreated in 3% hydrogen peroxide in methanol for 30 min to eliminate endogenous peroxidase activity. To detect iNOS expression, immunostaining was performed on cryostat sections using anti-macNOS mouse monoclonal antibody (Transduction Laboratories, diluted 1:200). The sections were first incubated with primary antibody at 4°C overnight. After being rinsed in phosphate-buffered saline (PBS), sections were incubated sequentially in secondary biotinylated goat anti-rabbit antibody or biotinylated rabbit anti-mouse antibody for 20 min and peroxidase-conjugated streptavidin for 10 min at room temperature. 3,3'-Diaminobenzidine tetrahydrochloride was used as the chromogen. The sections were then counterstained with hematoxylin. The specificity of immunostaining was verified by replacing the primary antibody with PBS, absorption test or internal control. Absorption tests were done using NOS antibodies in the excess of each NOS protein. Neural tissue and endothelial cells were used as internal controls of nNOS and eNOS, respectively. For the positive control of iNOS staining sections of tuberculous granuloma were used. The antibodies were specific for their given NOS type and did not cross-react with the other subtypes.

Analysis of iNOS mRNA expression by RT-PCR

Total RNA was extracted from 6 cases of fresh frozen tumor tissues and nonneoplastic tissues with Isogen (Nippon Gene, Osaka, Japan). Complementary DNA (cDNA) was synthesized from 5 µg of total RNA in 25 µl of reactive solution using MMLV reverse transcriptase (RT) (Stratagene, Stratascript™ RT-PCR kit, Cambridge, Calif.). After heat inactivation of RT, 2.5 U of DNA polymerase (Takara Shuzo, Kyoto, Japan) and specific primers were added to the above reactive solution, and cDNA was amplified by PCR using a DNA thermal cycler (RTC-100, Funakoshi, Tokyo, Japan). The following primer sequences were used for iNOS: the sense 5'-ACAAGCTGGCCTCGCTCTGGAAAGA-3' and the antisense 5'-TCCATGCAGACAACCTTGGGGTTGAAG-3' with a final PCR product size of 507 base pairs (bp) [2]. β-Actin cDNA was coamplified as an internal control using the following primer sequences: the sense 5'-ATGGATGATGATATCGCCGCGCT-3' and the antisense 5'-GACTCCATGCCCAGGAAGGA-3' with a final PCR product size of 822 bp [12]. PCR assays for both iNOS and β-actin were performed in 20 µl of reaction solution using 34 cycles with steps of 94°C for 1 min for denaturation, 72°C for 2 min for primer extension, 63°C for 1 min for annealing. The PCR products (10 µl) were electrophoresed on 2% agarose gel containing 0.5 µl ethidium bromide and visualized with ultraviolet light. The iNOS PCR products of message bands were densitometrically quantified using the NIH Image software (National Institutes of Health, Bethesda, Md.), and values were normalized by β-actin levels.

Evaluation of immunostaining and vessel counting

After thorough qualitative examination of stained slides, the intensity of vascular endothelial staining for eNOS was evaluated semi-quantitively as negative, weakly positive, or positive. The positivity of tumor cells for iNOS proteins was evaluated as follows: – = negative; + = positive; ++ = strongly positive. p53 was considered positive if more than 5% of the tumor cells showed strong nuclear staining. All semi-quantitative analyses were performed by two pathologists and the grading of positivity was highly concordant (>95%).

CD31 was expressed in the endothelial cells of vessels. On the CD31-stained slides, tumor vessel density was assessed both within and around the tumor. To this end, the area of the highest neovascularization was used for quantitative analysis with the aid of the pictures (0.348 mm^2 per field) captured from the light microscope (×20 objective, Olympus BX50, Tokyo, Japan) using the criteria of Weidner et al. [22]. The area for the measurement of vascular density was chosen from at least ten selected frames.

Statistical comparison of iNOS immunoreactivity with clinicopathological findings and p53 overexpression was performed using a Chi-square test. Statistical significance was reached when *P*-values were <0.05.

Results

Immunohistochemical findings

Inducible-type NOS

Tumor cells in 22 out of 25 cases (88%) were positive for iNOS. Among these, strongly positive reactions were detected in 10 cases, showing diffuse dark brown reaction products in the cytoplasm in most tumor cell areas (Fig. 1a, b). The other 12 cases showed less strong but definitely positive reactions in half of the tumor cell areas, where similar diffuse cytoplasmic staining was identified in the tumor cells. There was no apparent relationship between iNOS positivity and the invasive edge of the tumor, necrosis, inflammatory infiltrates, or fibrosis. Only small numbers of nonneoplastic epithelial cells

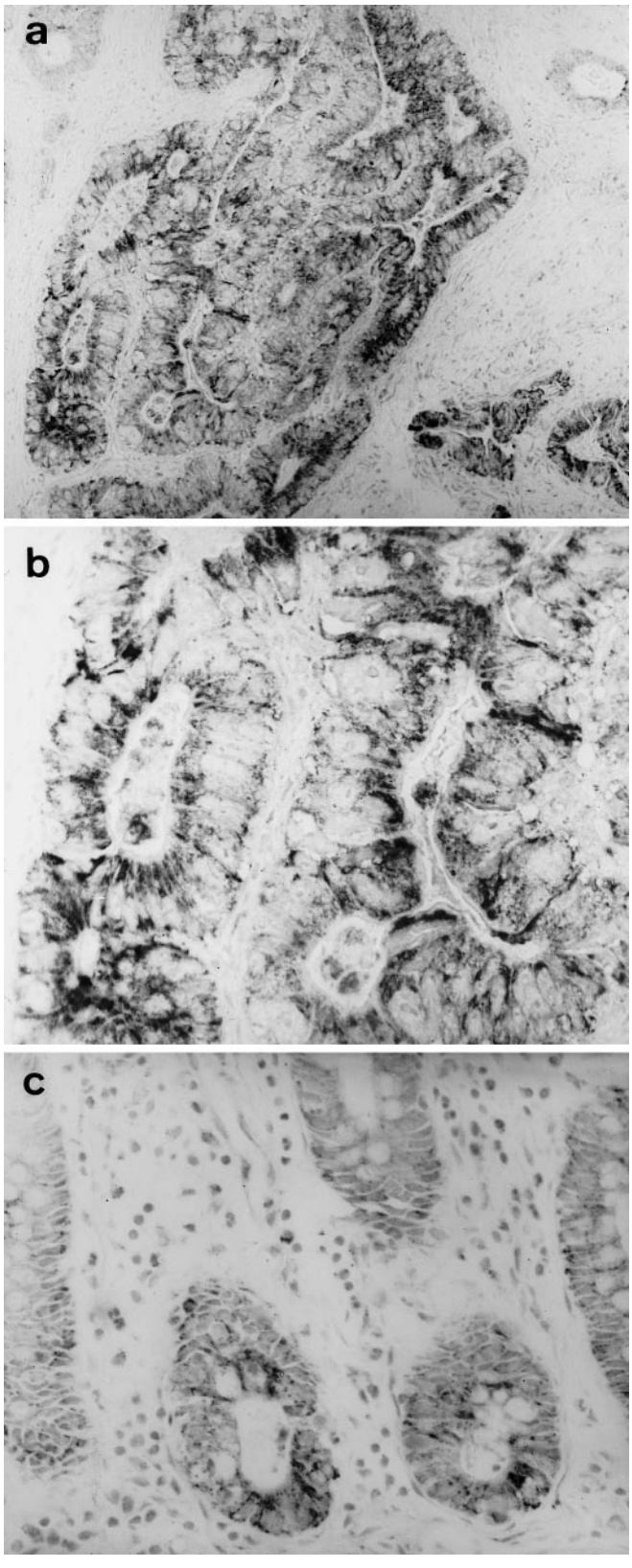

Fig. 1a, b Ten cases showed strong positive reactions for inducible nitric oxide synthase (iNOS) in many tumor cells. Immunoperoxidase, **a** ×40, **b** ×200 **c** Nonneoplastic glands remote from tumor were negative or weakly positive for iNOS. Immunoperoxidase, ×100

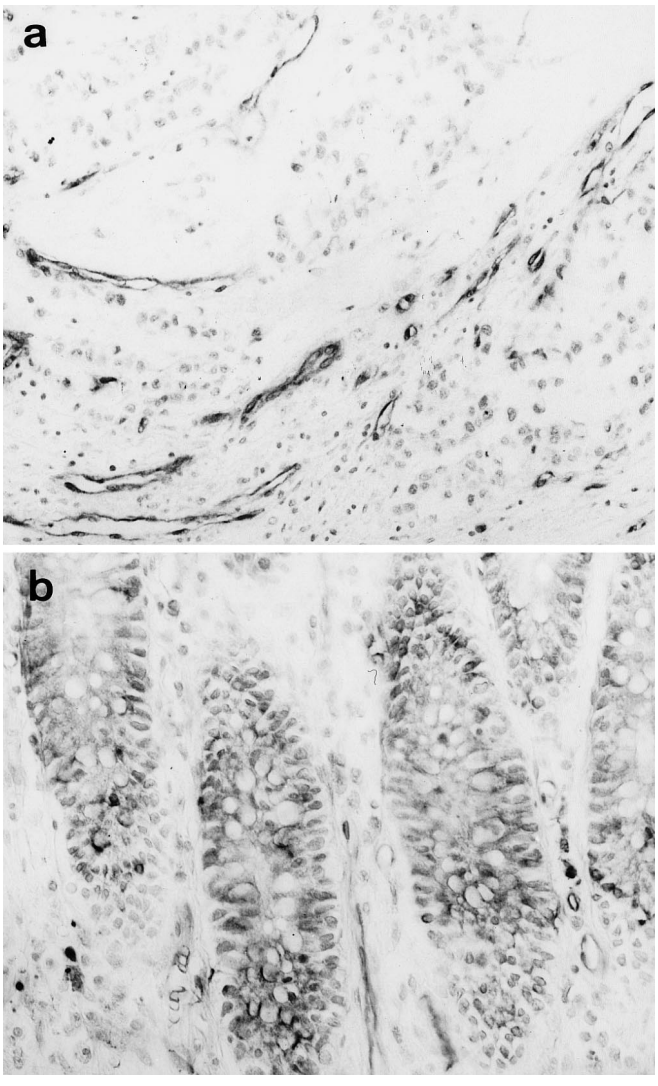

Fig. 2 a Tumor vessels showed intense reactions for endothelial nitric oxide synthase (eNOS). Immunoperoxidase, ×50 **b** Mucosal vessels in distant wall from the tumor were negative or weakly positive for eNOS. Nonneoplastic epithelial cells contained some scattered eNOS-positive cells. Immunoperoxidase, ×75

were weakly positive (Fig. 1c). Inflammatory cells were negative for iNOS expression.

Endothelial type NOS

The endothelial cells of the vessels within or around tumors in all cases were weakly to strongly positive for eNOS regardless of vessel type (Fig. 2a). Their staining reactions appeared to be more intense than those in the tissue remote from the tumors (Fig. 2b). Five cases showed positive reactions to eNOS in tumor cells, although the percentage of positive cells was small, ranging from approximately 5% to 8% of tumor cells. Only a few nonneoplastic epithelial cells were positive in about two thirds of cases.

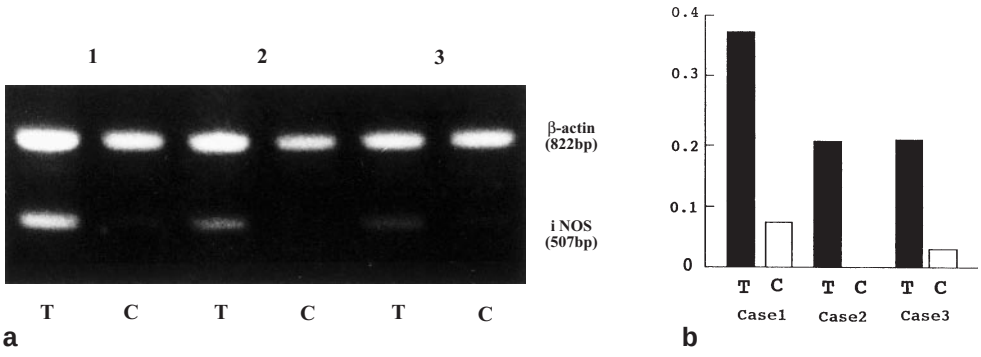

Fig. 3a, b RT-PCR analysis of iNOS mRNA in samples of normal colon mucosa (*C*) and colon cancer (*T*). **a** Higher expression was observed in the tumor tissues of 3 cases out of 6 cases examined. **b** Densitomeric analysis of RT-PCR (*T* tumor tissue, *C* normal mucosa

Neuronal-type NOS

nNOS was located in nerve fibers and some ganglion cells. Cancer cells showed no immunoreactivity, but a very few nonneoplastic epithelial cells were positive.

p53 protein

p53 protein was detected in many tumor cells in 64% (16/25) of cases.

CD31

Tumor vascular density ranged from 112–376/mm^2 (average 235/mm^2). There was no apparent trend toward increased vessel density in the leading front of the tumors.

RT-PCR analysis for iNOS

iNOS mRNA expression was detected in the tumor tissues in 3 of 6 cases (Fig. 3a). The mRNA values for iNOS, normalized to β-actin, were 0.37, 0.20 and 0.20, respectively. These 3 cases were all positive for iNOS according to immunostaining. The expression levels of iNOS mRNA in tumor tissues were more than 5-fold those in normal mucosa in all 3 cases (Fig. 3b). All of 3 cases that were negative on RT-PCR were positive for iNOS on immunohistochemistry. We think that this discrepancy between RT-PCR and immunohistochemistry could be due to mRNA degradation during the tissue processing or to topographic diversity of iNOS expression within the same tumor.

Relations of iNOS expression to clinico-pathological findings

There was no significant correlation between iNOS staining intensity and Dukes' staging, tumor size, lymph node metastasis, tumor vessel density and p53 expression. By contrast, cases with strong iNOS reactions (++) were associated with the presence of vascular invasion (Chi-square test; P=0.0274) (Table 1).

Table 1 Inducible nitric oxide synthase *(iNOS)* immunoreactivity of the tumor cells and pathological parameters (– negative iNOS staining, + positive, ++ strongly positive staining)

Parameter	iNOS positivity (no. of cases)		
	–	+	++
Dukes' staging[a] (P=0.3913)[b]			
A	1	4	0
B	1	4	4
C	1	1	5
D	0	3	1
Tumor size (P=0.4136)[b]			
>4 cm	2	8	5
<4 cm	1	4	5
Lymph node metastasis (P=0.3913)[b]			
Positive	1	4	6
Negative	2	8	4
Vascular invasion (P=0.0274)[b]			
Positive	0	2	7
Negative	3	1	3
Vessel density (P=0.3949)[b]			
<235 (/mm^2)	0	4	6
>235	3	7	4
P53 expression (P=0.3777)[b]			
Positive	2	9	5
Negative	1	3	5

[a] Dukes' staging was grouped into A+B and C+D for comparison
[b] Chi-square analysis was performed to compare a group of cases with strongly positive staining (++) with a group of cases with positive staining reactions (+)

Discussion

In the present study, we have demonstrated that human colorectal adenocarcinoma in vivo had NOS protein expressed in the tumor cells, and that the major NOS isoform was the inducible type. In addition, we have shown greater iNOS mRNA expression in the tumor tissue than in normal mucosa. These results are at odds with the report of Moochhala et al. [10], who failed to detect immunoreactive iNOS in 13 cases of human colorectal cancers. This discrepancy may be due to the different methods used for the detection of NOS proteins in the study by Moochhala et al. and in our own study. For the

detection of iNOS proteins, we used Zamboni solution-fixed tissue to preserve antigens, while frozen sections were used in their studies. The current results appear to be consistent with those reported earlier by Jenkins et al. [6], who demonstrated iNOS mRNA expression and enzymatic activity of cultured human colonic adenocarcinoma cells. Up-regulated levels of iNOS mRNA detected in this study further indicated enhanced protein synthesis in the tumor tissues. Our results are also in keeping with more recent studies by Ambs et al. [1], who found greater expression of iNOS proteins and their mRNA levels in human colon tumor tissues than in nontumor tissues. In their studies, however, the iNOS localization was mainly restricted to mononuclear inflammatory cells and endothelial cells. The reason for the topographic difference in the iNOS proteins between these two studies is not clear, but the differences in the methods of NOS detection and the antibodies used may be possible explanations. Attempts to depict mRNA expression of iNOS by in situ hybridization are now under way in our laboratory.

Two opposing biological effects of NO on tumors have been proposed [4, 5, 7, 9, 23]. The human colon adenocarcinoma cell line (DLD-1), when engineered to generate NO continuously, showed cytostatic effects in vitro, while these cells grew faster and were more vascularized than wild-type cell lines in vivo [7]. Maeda et al. [9] have demonstrated that NO may be involved in enhanced vascular permeability and increased blood flow, which further promote tumor growth. By contrast, Xie et al. [23] have demonstrated that NO produced by iNOS can induce regression in focal hepatic metastases of mouse reticulum cell sarcoma, which could be attributed to acceleration of apoptotic cell death. It is thus likely that the effects of NO on tumor cells are different in various conditions and may be dependent on the local NO concentrations, tumor cell sensitivity and tumor microenvironments.

The expression of NOS proteins has been reported in human surgical tumor specimens, including tissue from ovarian cancers, breast cancers, central nervous system tumors and squamous cell carcinomas of the head and neck [1, 15, 20, 21]. In the studies cited, the extent of NOS expression correlated with the histological grade of malignancy, but localization and expression of NOS isoforms were found to be variable. In this study, we could not detect significant relationships between iNOS reactions and histological grade or pathological stage. By contrast, tumors with strong iNOS expression were associated with vascular invasion. Degradation of extracellular matrix, including vascular basement membranes, by matrix metalloproteinases (MMPs) is a crucial step for the vascular invasion [13]. It is known that NO can activate metal-dependent proteases in articular chondrocytes and cartilage [11, 19]. Such activation of MMPs may occur in response to NO release in invasive tumors. Rajagopalan et al. [14] recently reported that peroxynitrites, reaction products of NO with superoxide, elicited activation of MMP-2. Further studies will be required to confirm whether NO activates MMPs in colonic cancers.

The enhanced expression of eNOS in tumor microvessels detected in this study may also relate to progression of the tumor growth by way of increasing blood flow and vascular permeability. However, any conclusion on the role of the expression of the three NOS isoforms in the biological behavior of tumors would be premature. Information about this field is still fragmentary and additional cases of colonic cancer are needed.

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