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

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 Ca²⁺ levels. The inducible type (iNOS) is independent of Ca²⁺ 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’-ACAAGCTGCTGCTCCTGGAAGAAGA-3’ and the antisense 5’-TCCATGCAACATCTTGGTGGAAG-3’ with a final PCR product size of 507 base pairs (bp) [2]. β-Actin cDNA was amplified as an internal control using the following primer sequences: the sense 5’-ATGGATGATGATCGCGGCGCT-3’ and the antisense 5’-GACTCCATGCCCCAGGAAAGG-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-quantitatively 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 neo-vascularization was used for quantitative analysis with the aid of the images (0.348 mm² 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