Differential expression of nitric oxide synthase in human stomach cancer

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Received 19 April 1999; received in revised form 11 June 1999; accepted 5 July 1999

Abstract

The level of expression and cellular localization of isoenzymes of nitric oxide synthase (NOS) was detected in human stomach tumor tissues. Tumor tissues showed 70% higher activity of NOS than that of normal tissues (P < 0.01). Poorly differentiated adenocarcinoma tend to have higher activity (P < 0.05) than well differentiated and moderately differentiated tumor tissues. Aminoguanidine (AG), 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine (AMT), N\textsuperscript{G}-monomethyl-l-arginine (l-NMMA), and N\textsuperscript{v}-nitro-l-arginine (l-NNA) inhibited NOS activity in tumor tissues by 18, 14, 11 and 13%, respectively. The TNF-α mRNA expression was correlated with the inducible NOS (iNOS) level, which was high in adenocarcinomas and low in normal tissues. Tumor tissues showed higher expression of iNOS in gland epithelial cells but the level of eNOS was significantly decreased with an exception of concentrated localization in the proliferating capillary endothelium. These results revealed that isoforms of NOS might contribute differentially to growth and progression of human stomach tumor.

Keywords: Nitric oxide synthase isozyme; Human stomach cancer; Neovascularization; Differential expression; Immunolocalization

1. Introduction

It has been reported that nitric oxide synthase (NOS) isoforms are present in human tumor cell lines and solid tumor tissues [1–10]. Since a Ca\textsuperscript{2+}/calmodulin-dependent constitutive NO synthase (cNOS) activity in human epithelial type tumor cells was characterized [4], increased NOS expression was observed in lung [8,9] and colon cancer tissues [1,3] when compared with normal tissues. The increased expression was inversely correlated with the degree of differentiation in human gynecological cancer [7]. Recent reports have shown that NO induces endothelial cell growth [11], mediates neovascularization and reduces blood flow in tumor tissues [1,12].

There has been mounting evidence that NO acts as a carcinogen. For example, a high concentration of NO and its metabolites such as peroxynitrite and NO\textsubscript{2} causes DNA damage in the course of nitration, nitrosation and deamination [13–15]. Nitric oxide
inhibits DNA ligase activity resulting in the accumulation of DNA breaks [16]. It has been suggested that elevated NO production enhances the growth of some tumors through the suppression of anti-tumor immune responses [18–21]. Nitric oxide as a mediator of tumor vascularization might enhance tumor growth [11]. These observations suggest that NO may have some roles in growth, progression, maintenance and/or metastasis of tumors.

In human stomach tissues, immunohistochemical analysis has been documented [6]. In contrast with the results obtained with other types of tumors, the expression of all types of NOS isoforms in human stomach tumor tissues was significantly lower than in the human normal stomach mucosa. In tumor tissue, the expression of inducible NOS was significantly lower than the expression of constitutive NOS. But, according to the report, there was a tendency of higher expression of both constitutive forms of NOS in earlier stages of the tumor compared to advanced cases. The level of expression of iNOS was markedly higher in the advanced tumor than in the earlier stages of tumor.

The purpose of this work is to investigate the correlation between the expression of NOS and gastric tumor growth, and to reveal the role of each isoform of NOS for gastric tumor growth. We determined the level of NOS activity in normal, peritumoral and tumor tissues by measuring the enzyme activities with radiolabeled arginine and the mRNA expression level using reverse transcription–polymerase chain reaction (RT–PCR). Immunohistochemistry was also performed to observe detailed cellular localization of iNOS and eNOS and to assume the contributions of each isozyme in the growth of stomach cancer.

2. Materials and methods

2.1. Patients and tissues

Tumor (30 adenocarcinoma, one miscellaneous carcinoma), peritumoral (2 cm away from the tumor) and corresponding remote normal tissues of stomach were collected from 31 surgically treated patients (26 males and five females with a median age of 57 years ranging from 29 to 78 years old) at Yonsei University Medical Center. The grade of tumor in 19 cases was classified as poorly differentiated adenocarcinoma, in seven cases as well differentiated and in five cases as moderately differentiated.

2.2. NOS activity

Activity of NOS was determined by a method based on the conversion of L-[3H]arginine to L-[3H]citrulline [19]. Frozen tissues were homogenized in 4 volumes of 50 mM Tris buffer (pH 7.4) containing 1 mM dithiothreitol (DTT), 1 mM EDTA, 0.1 mM EGTA, 100 mg/l PMSF, 2.5 mg/l antipain and 5 mg/l leupeptin. Whole homogenates of the tissues were incubated for 30 min at 37°C in the presence of 1 mM β-nicotinamide adenine dinucleotide phosphate (NADPH), 100 μM H2B, 10 μM FAD, 10 μM FMN, 1.5 mM CaCl2, 2 μg calmodulin and 500 μM L-[3H]arginine (approximately 200 000 dpm of L-[2,3,4,5-3H]arginine HCl). For inhibition of NOS activity, tumor tissues were analyzed. Aminoguanidine (AG), 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine (AMT), NG-monomethyl-L-arginine (l-NMMA) and N\textsubscript{V}-nitro-L-arginine (l-NNA) were added at a concentration of 1 mM. The reaction was terminated by adding 1 ml ice-cold Dowex-50W (Na\textsuperscript{+} form) equilibrated in 20 mM sodium acetate buffer (pH 5.5) containing 1 mM citrulline, 2 mM EDTA and 0.2 mM EGTA. The reaction mixtures were centrifuged at 12 000 × g for 5 min. The supernatant was collected into a water miscible scintillate and the radioactivity was counted in a Beckman LS 2400 liquid scintillation counter.

2.3. Total RNA preparation

The total RNA from tissues was isolated by the guanidine thiocyanate extraction method [22]. Each RNA sample was quantitated spectrophotometrically and was verified by agarose/formaldehyde gel electrophoresis.

2.4. RT–PCR, PCR and sequencing

Reverse transcription was performed following the BRL protocol for Superscript II reverse transcriptase (Gibco BRL, Grand Island, NY). Briefly, 5 μg of total RNA was reverse transcribed by incubating with 0.1 mM oligo-dT primer for 10 min at 70°C followed by incubation with 5 mM DTT, 0.5 mM deoxynucleotide
triphosphate mix (dNTPs, Promega, Madison, WI), and 200 units of Superscript II reverse transcriptase for 50 min at 42°C. The reverse transcription was terminated by heating for 15 min at 70°C. To remove RNA complementary to the cDNA, RNase H (Gibco BRL) was added and incubated at 37°C for 20 min. The complementary DNA (cDNA) was used for the polymerase chain reaction.

2.5. Immunohistochemistry

Tissues were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Immunohistochemical staining was performed by avidin–biotin complex (ABC) method. Deparaffinized sections were treated with 0.3% hydrogen peroxide for 10 min, and then incubated in 5% normal goat serum for 30 min to prevent the endogenous peroxidase activity and nonspecific binding of antibody, respectively. Incubation in primary antibody was performed at 4°C overnight. Rabbit anti-eNOS and anti-iNOS antibodies (Transduction Laboratories, Lexington, KY), both diluted to 1:500 with phosphate-buffered saline containing 5% normal goat serum, were used as primary antibodies. After rinsing, the sections were incubated in secondary antibody solution, 1:200 diluted goat biotinylated anti-rabbit IgG antibody (Vector Laboratories, Burlingame, CA) at room temperature for 1 h and followed by treatment of ABC reagent (Vector Laboratories) for 30 min. The final coloring agents used were 0.05% 3,3′-diaminobenzidine and 0.003% hydrogen peroxide. Negative controls were made by using primary antibody coincubated with macrophage cell lysate or human endothelial cell lysate (Transduction Laboratories).

2.6. Statistical analysis

The results were evaluated statistically by using paired t-test. A P-value of 0.05 or less was considered to indicate statistically significant difference.

3. Results

The total NOS activity (pmol/min per mg protein) was measured in human stomach tissues from surgically treated patients. The activity was determined as the conversion of L-[^3]H]arginine to L-[^3]H]citrulline. The activity in stomach tumor tissues was about 70% higher than those in normal tissues (P < 0.01) and peritumoral tissues (P < 0.05) (Fig. 1). Analysis of adenocarcinoma specimens by histological classification showed poorly differentiated adenocarcinoma marked to have 1.8 and 2.1 times higher activity (P < 0.05) than those of well differentiated and moderately differentiated adenocarcinoma, respectively (Fig. 2). The NOS activity of stomach tumor tissue was inhibited by various NOS inhibitors to different degrees. The relative inhibitory effects of 1 mM each of AG, AMT, L-NMMA and L-NNA showed 18, 14, 11 and 13%, respectively (Fig. 3).

The increased NOS activity in tumor tissues was supported by the results of RT–PCR analysis. The mRNA concentrations of iNOS, eNOS, and tumor necrosis factor (TNF)-α were determined by RT–PCR in pairs of stomach adenocarcinoma and corresponding remote normal tissues. As shown in Fig. 4A, the tumor tissues contained higher levels of iNOS, and eNOS mRNA than the normal tissues. The TNF-α mRNA expression was correlated with the iNOS expression level, which was high in adenocarcinomas and poorly expressed in the normal tissues. The brain-type NOS (bNOS) was not detected by RT–PCR in normal stomach and tumor tissues. Densitometric units were used to quantify the levels of mRNA expression (Fig. 4B). The differential expression of
iNOS between tumor and normal tissues was more apparent than that of eNOS.

To determine the source of NOS activity we performed immunohistochemistry with polyclonal anti-eNOS and anti-iNOS antibodies. In normal tissue, eNOS was highly expressed in epithelial cells throughout the gastric glands. However, iNOS was not detected in the mucosa (Fig. 5A,B) of normal tissue. Expression of eNOS in normal glandular cells was more prominent in basal portion of gastric glands. There was a significant difference in the expression of individual isoform in tumor samples. Whereas the immunoreactivity for eNOS was greatly decreased in tumor epithelial cells, it was confined predominantly to the proliferating endothelial cells of neovasculature of tumor tissue (Fig. 5C,E). In contrast to eNOS expression, immunoreactivity for iNOS was mainly localized in transformed epithelial cells but not distinct in endothelial cells (Fig. 5D). The localization of iNOS was mostly in the apical cytoplasm of the tumor epithelial cells (Fig. 5F).

4. Discussion

Stomach cancer tissues showed about a 70% increase (P < 0.01) of overall NOS activity when
compared with the normal surrounding tissues in our studies. The increased activity of the tumor was well reflected in the elevated level of NOS mRNA expression in the cancer tissues. The results coincide with previous data obtained with colon [1], gynecological [4,7], lung [8] and breast [10] tumors. However, our results differ from a recent report which showed a marked reduction of all NOS isoforms expression in gastric cancer tissues than in normal gastric mucosa [6]. We assume that the difference in expression levels is due to different methods of determination employed by the authors, immunostaining vs. activity assay and RT–PCR. However, we understand the activity assay and RT–PCR data could be more direct evidence of...
NOS expression level. Our new finding of the increased expression of NOS in stomach tumor supports the generalized hypothesis that excessive NO production may contribute to the pathogenesis of cancer progression and maintenance [1], although the beneficial roles of NO including the apoptotic killing of tumor cells and cytotoxic effect of macrophage against transformed cells have been proposed.

In our studies, there was no correlation between total NOS activity and tumor grade of stomach cancer as observed by others in lung [8], although it has been reported that NOS activity correlated with tumor grade in colon, breast and brain tumors [1,7,10]. However, poorly differentiated adenocarcinoma of the stomach demonstrated a statistically higher activity of NOS ($P < 0.05$) than those of well differentiated and moderately differentiated adenocarcinoma. Analysis of adenocarcinoma specimens by TNM classification revealed no significant difference in NOS activity (data not shown), although it has been suggested that NO may play a role in the suppression of lymph node metastasis by inhibiting platelet aggregation [2–4,8].

The NOS activities in human stomach tumor tissues were inhibited by various NOS inhibitors. Aminoguanidine, a specific inhibitor of iNOS, showed higher inhibitory effects than AMT, L-NMMA, and L-NNA although the difference was not significant, indicating that the NOS activity was originated by both isoforms. Our results also demonstrate that NOS inhibitors may be used to hamper the carcinogenic activity of NO in stomach cancer.

The expression of iNOS mRNA was much higher in stomach tumor tissues than in normal tissues. The increased expression of iNOS in stomach tumor tissues renders a major contribution to high activity of NOS in stomach tumor tissues. Although the expression of eNOS in tumor tissues was not prominent as in the case of iNOS, the increased level might be due to the concentrated localization of eNOS in proliferating endothelial cells of neovasculature as observed in immunohistochemistry data. In human breast cancer, rat brain-type NOS was observed in vascular endothelial and myoepithelial cells by immunolabeling [23]. In our studies, eNOS was localized in vascular endothelial cells of human stomach cancer but brain-type NOS (bNOS) was not detected in human stomach tissues by RT–PCR. An increased NOS expression level could cause mutations that contribute to human carcinogenesis, and it might augment blood flow to the tumor, stimulation of neovascularization, or promotion of metastasis [8–17]. Concomitant expression of TNF-α and iNOS may indicate that TNF-α might contribute to induction of NOS in human stomach cancer. The induction of NOS in several tumor cell lines by various exogenous cytokines and cytokines released from themselves has been reported [6,24].

Immunolocalization of both isoforms of NOS in normal and tumor tissues of stomach performed by Rajnakove et al. [6] shared common features with our observations. Expression of the eNOS in our studies was observed to be strong in gland epithelium of stomach normal tissues in immunohistochemical localization, but was concentrated only in capillary endothelium of tumor tissues, suggesting that NO produced by eNOS triggers angiogenesis. When rat stomach cells were separated by centrifugal elutriation, constitutive NOS activity was enriched in a fraction containing mucous-epithelial cells. These observations indicate a role of NO in the regulation of epithelial cell integrity or secretion [25]. In addition, the localization of constitutive isoforms of nitric oxide synthase in the gastric glandular mucosa supported the role played by NO in gastric mucus secretion [26]. The concentrated localization of eNOS in endothelial cells of tumor tissue suggests that NO may act as part of a signaling cascade for neovascularization [11]. Further studies are required to reveal the mechanism for the involvement of tumor-associated NO production by eNOS in the process of new blood vessel formation in tumor tissues.

The inducible type of NOS was heavily localized over gland epithelium in tumor tissues, while normal stomach tissue sparingly expressed iNOS. These immunohistochemical results suggest that increased NOS activity in tumor might be closely associated with the expression of the inducible form, rather than the constitutive form, in the tumor epithelium. The localization of iNOS in the apical part of gland epithelium of tumor by Rajnakove et al. [8] was confirmed by our data. Although some authors [10] confirmed iNOS in tumor tissues are localized to migrated macrophages, there is conflicting evidence that nitric oxide production by tumor tissues of epithe-
lial origin was enhanced by culture in the presence of TNF-α [25]. Our data clearly showed that iNOS is localized in gland epithelial tissues and not in macrophages.

While the expression of eNOS is believed to be related with normal functions of epithelial secretion, the meaning of dominant expression of iNOS in tumor tissues is intriguing. It could be interpreted either as toxic weaponry of tumor cells to surrounding normal cells providing better environment for propagation, or the consequence of expression of NOS triggered by immunoreactive cytokines.

In summary, NO produced by iNOS in tumor tissues has an unknown function for cancer cell maintenance and propagation, while eNOS was observed to have roles in neovascularization. Further work must be focused at efforts to reveal the causes and consequences of increased expression of iNOS in stomach tumor tissues.

Acknowledgements

We are grateful to Drs Ki Churl Chang and Chang Shin Park for providing technical information. We also thank Messrs. Hyun-Kyu Kim and Bumjun Kim for excellent technical assistance. This work was supported by Grant #96-04-03-17-01-3 from the Basic Research Program of the Korea Science and Engineering Foundation.

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