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Reappraisal of the Significance of Inducible Nitric Oxide Synthase in Colorectal Cancer

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Abstract

Backgrounds: Inducible nitric oxide synthase (iNOS), which produces high levels of nitric oxide (NO), is overexpressed in activated macrophages and some cancer cells. Although iNOS was thought to be involved in promoting colorectal cancer, contradictory reports supporting its tumoricidal effect exist.

Methods: We first examined the iNOS enzyme activity in colorectal cancer tissue and immunohistochemical expression of iNOS in cancer cells and tumor-infiltrating macrophages. Then, association of iNOS activity or its protein expression was analyzed in relation to various clinicopathological covariates.

Results: Four groups of patients were classified based on their iNOS expression status. Univariate and multivariate analyses showed that group 1 patients (low iNOS in both types of cells) and group 4 patients (high iNOS in both types of cells) had a shorter disease-free survival. Patients with extremely high or low iNOS enzyme activity tended to have a lower disease-free survival rate ($p = 0.059$).

Conclusion: Macrophage/stroma-derived NO negatively regulates colorectal cancer development when cancer cells express low levels of iNOS, but might synergistically contribute to tumor progression in the presence of high levels of cancer-cell derived NO. The dual effects of NO should be considered in the design of anti-iNOS/NO therapy for colorectal cancer patients.

Keywords: Colorectal neoplasm; Nitric oxide synthase; Macrophage; Prognosis

Introduction

Nitric oxide (NO) is a short-lived ubiquitous molecule that regulates numerous physiological processes in the cardiovascular, nervous, and immune systems [1]. NO is synthesized from L-arginine through the L-arginine-NO pathway by NO synthase (NOS). Three isoforms of NOS have been identified: neuronal NOS (nNOS); endothelial NOS (eNOS); and inducible NOS (iNOS) [2]. nNOS and eNOS are constitutively expressed in neuronal cells and vascular endothelial cells and are calcium dependent. Conversely, iNOS is not usually expressed in healthy quiescent cells, but is rapidly transcriptionally induced in activated cells (particularly macrophages) in response to stimulation with bacterial products and inflammation-associated cytokines. Once expressed, iNOS produces higher levels of NO than the other two isoforms [2].

Pathophysiologically, long-term exposure to elevated NO and NO metabolites in cells could have potential genotoxic effects on hosts. The mechanisms include damaging DNA, inhibiting DNA repair, and modulating transcriptional factors [3-5]. Excess NO is involved in many pathological conditions, including inflammation, pain, atherosclerosis, and cancer [4-6]. Increased iNOS expression with sustained NO levels has been reported in a number of human cancers: breast, prostate, bladder, esophagus, and colon [7-14]. In human colorectal cancer, the clinical importance of iNOS expression remains controversial. Expression of iNOS was positively correlated with increased tumor angiogenesis, vascular invasion, decreased lymphocyte response, metastasis, and a poor prognosis. There is, however, a contradictory report that decreased expression of iNOS was associated with increasing tumor stage and decreased patient survival [11-15].

iNOS is also expressed in many host cells, such as macrophages, endothelial cells, gastrointestinal epithelium, and platelets [2,11,13,15]. When produced in activated macrophages as participants of a host response, iNOS is instrumental in killing microorganisms and tumor cells [16,17]. Several investigators have reported that macrophages can participate in the antitumor immune response and selectively destroy a variety of tumors both *in vitro* and *in vivo*, primarily through the production of NO [18,19].

Accordingly, it brings into question that whether iNOS and NO are pro-tumor or anti-tumor. Several explanations have been proposed to explain for these dual effects of NO, including its cellular source, local NO concentrations, and NO sensitivity among various types of tumor cells. Low levels of NO appear to favor tumor progression, whereas high levels of NO are tumoricidal [20-22]. Most studies that demonstrated an inhibitory effect of NO/iNOS on tumorigenesis utilized biological systems that produced high levels of NO or exogenous NO donors. Thus, continuous high levels of NO synthesized by macrophages are cytotoxic/cytostatic toward tumor cells [17]. By means of stimulation of

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iNOS expression or transfection with iNOS genes, high NO produced by tumor cells is associated with decreased tumor growth and metastasis *in vivo* [23]. There were studies suggesting that high NO is required to induce apoptosis in some mammalian cells, while low NO may protect from apoptotic cell death [24,25].

iNOS-expressing tumor-infiltrating macrophages have been demonstrated in many tumor types, including colorectal cancers [11,13,26], but their clinical significance remains unknown. To address the issue of iNOS in the development of human colorectal cancer, we measured the iNOS enzyme activity and assessed its cellular localization in primary tumors by immunohistochemistry. The results were correlated with tumor stage, histologic grading, disease-free survival, and overall survival. We demonstrated that activated macrophages usually expressed higher levels of iNOS than cancer cells, and the homeostasis between lower iNOS in tumor cells (pro-tumor characteristics) and high iNOS in macrophages (anti-tumor capability) might determine the destiny of tumor progression.

Materials and Methods

Patients and materials

We prospectively collected 51 patients and retrospectively collected 111 patients who had undergone surgery for primary colorectal cancer between 1993 and 2000 at National Cheng Kung University Hospital, Tainan, Taiwan (Table 1). Fifty-one surgical fresh colon tumor specimens were obtained from these prospectively collected patients. Tumors were frozen immediately with liquid nitrogen and stored at -80°C until further analysis. All colon tumors investigated were histologically diagnosed as adenocarcinomas. Ninety-four tumors were located in the colon (ascending 19, transverse 8, descending 6, sigmoid 61), and 68 were in the rectum. The status of the tumors was recorded according to the tumor-node-metastasis (TNM) staging system and the modified Astler-Coller (MAC) staging system [27]. The stage of the tumors was determined from the histopathologic reports obtained at the time of resection. The clinicopathological characteristics were listed in Table 1. Patients who had received neoadjuvant chemotherapy or radiotherapy before their initial resection were excluded from this study. Postoperative 5-fluorouracil-based adjuvant chemotherapy was routinely administered to patients with MAC stage C or D tumors. The observation time in this unselected cohort was the interval between diagnosis and last contact (death or last follow-up). Data was censored at the last follow-up for patients who had not relapsed and for those who had died. All living patients in this study were followed up for more than 5 years. The median follow-up time was 93.5 months (range: 3.7 to 153.0 months). The study protocol was approved by our institutional review board.

Immunohistochemistry (IHC)

A representative block containing a transmural, full-thickness section of adenocarcinoma, including the deepest pericolonic extension, was cut from each patient. IHC staining was carried out using a commercial kit (EnVision; DAKO, Glostrup, Denmark). Briefly, 4-μm-thick sections were prepared and endogenous peroxidase activity was blocked with H_2O_2. Microwave antigen retrieval was done in 10 mmol/L citrate buffer (pH 6.0) at 750 W. The sections were incubated at 4°C overnight with anti-iNOS antibodies (diluted 1:100) (Thermo Fisher Scientific, Fremont, CA, USA) or anti-CD68 antibodies (diluted 1:50) (DAKO) against human iNOS or CD68 protein. The reaction complexes were detected using a kit (EnVision; DAKO) and visualized using an aminoethyl carbazole substrate kit (AEC; DAKO). Finally, the sections were lightly counterstained with hematoxylin. Both positive and negative controls were included in all runs. For negative controls, we omitted the primary antibodies. Positive controls consisted of cells known to express iNOS or CD68.

Assessing IHC

Two different score systems were used to assess iNOS expression, one for colorectal cancer cells and one for infiltrating macrophages. The staining intensity of iNOS expressed in colorectal cancer cells was assessed using a modified three-tier system [12]:

0: No reactivity.

1+: Weak reactivity. Faint or light brown staining in the cytoplasm.

2+: Strong reactivity. Dark brown staining in the cytoplasm.

The entire area of each slide was analyzed, and the score was determined according to the area showing the strongest reactivity.

Both hematoxylin and eosin (H&E) and CD68 stains were used to highlight the macrophages. The density of iNOS-expressing macrophages infiltrating around the tumor front was scored using a modified four-tier system [28]:

0: No infiltration. No iNOS-expressing macrophages around the tumor front.

1+: Weak infiltration. Some scattered iNOS-expressing macrophages around tumor front.

2+: Moderate infiltration. "iNOS-expressing macrophages were continuously around the tumor front but had not extended from the tumor front by more than one cell layer on average" or "at least 45 iNOS-expressing macrophages in three x400 magnification fields".

3+: Strong infiltration. "iNOS-expressing macrophages extended more than two cell layers from the tumor margin" or "at least 75 iNOS-expressing macrophages in three x400 magnification fields".

All slides were assessed by two independent observers blinded to the clinical outcomes.

In addition, patients were divided into four groups based on the iNOS expression patterns in their cancer cells and infiltrating macrophages (Figure 1):

Group 1 (low in cancer cells and macrophages): iNOS grades 0 to 1 cancer cells and iNOS grades 0 to 1 macrophages;

Group 2 (low in cancer cells and high in macrophages): iNOS grades 0 to 1 cancer cells and iNOS grades 2 to 3 macrophages;

Group 3 (high in cancer cells and low in macrophages): iNOS grade 2 cancer cells and iNOS grades 0 to 1 macrophages;

Group 4 (high in cancer cells and macrophages): iNOS grade 2 cancer cells and iNOS grades 2 to 3 macrophages.

Measuring iNOS enzymatic activity

A modified method was used to measure iNOS activity in fresh frozen tumors (n = 51). Two of the tumors were in MAC stage A, 29 in B, and 20 in C. iNOS activity was measured using a modified method [28]. Briefly, tissue fragments (<500 mg) frozen with liquid nitrogen were crushed with a pestle and mortar and then homogenized in a buffer containing 50 mM of Tris/HCL, 250 mM of sucrose, 0.1 mM of EDTA, 0.1 mM of EGTA, and 10 μg/ml of leupeptin. After the tissue had been centrifuged, the supernatant was stored on ice for 2 hours. The 18 μl of supernatant was then incubated at 37°C for 30 minutes

with 10 µl of assay buffer containing 50 mM of potassium, 120 µM of NADPH, 1.2 mM of L-citrulline, and 1 mM of DL-dithiothreitol. Then 100 µl, 10 µM [3H]-L-arginine was added to the tissue homogenates. To determine how iNOS affected L-arginine metabolism, each sample was assayed with/without 1 mM of NG-mono-methyl-L-arginine (L-NMMA). The reaction was terminated by adding 1.5 ml of 1:1 H_2O/Dowex-50 w (200-400, 8% cross-link; Na+-form). The mixture was left to settle for 10 minutes, and then the newly formed [3H]-L-citrulline in the supernatant (1 ml) was measured with a Beckman scintillation counter. The iNOS activity was determined from the difference between samples containing 1 mM of EGTA and samples containing both 1 mM of EGTA and 1 mM of L-NMMA. The iNOS activity was expressed as pmol of [3H] citrulline formed per minute per mg of protein.

Statistical analysis

Kaplan-Meier curves were used to assess the IHC expression or enzyme activity of iNOS on disease-free and overall survival. The significance of various clinicopathological covariates was measured in univariate analysis using the log-rank test. The multivariate Cox proportional-hazards model was used to study the effects on survival of several covariates found significant in the univariate analysis. The relation between the IHC expression of iNOS and other covariates was analyzed using a chi-square test. Comparisons between enzyme activity of iNOS and other covariates were done using one-way analysis of variance. Statistical significance was set at $p<0.05$.

Results

Expression of iNOS in colorectal cancer cells and tumor-infiltrating macrophages

iNOS was diffusely expressed predominantly in the cytoplasm of cancer cells (Figure 1) with different intensity levels. Therefore, iNOS expression was scored by its staining intensity. Strong iNOS expression (grade 2) in cancer cells was found in 56 cases (35 percent) (Figure 1 and Table 1).

Characteristic	No. of patients	Disease-free survival		Overall survival	
		5 years (%)	P-value*	5 years (%)	*P-value**
iNOS expression in cancer cells			0.484		0.258
0	11	72.7		90.9	
1	95	66.3		64.2	
2	56	58.9		64.3	
iNOS expression in macrophages			0.869		0.384
0	26	61.5		53.8	
1	54	61.1		63	
2	56	67.9		73.2	
3	26	65.4		69.2	
iNOS expression in cancer cells and macrophages#			0.003		0.01
Group 1	59	55.9		54.2	
Group 2	47	80.9		83	
Group 3	21	76.2		76.2	
Group 4	35	48.6		57.1	
T status			0.001		0.066
pT1	8	87.5		87.5	
pT2	25	96		84	
pT3	121	59.9		62	
pT4	8	37.5		50	
N status			< 0.001		< 0.001
pN0	85	80		78.8	
pN1	47	63.8		70.2	
pN2	30	20		23.3	
Distant metastasis			< 0.001		< 0.001
None detected	145	71		71	
Present	17	5.9		23.5	
Astler-Coller stage			< 0.001		< 0.001
A	6	100		100	
B	76	81.6		78.9	
C	63	55.6		58.7	
D	17	5.9		23.5	
Sex			0.67		0.535
Male	87	62.1		67.8	
Female	75	66.7		64	
Differentiation			0.016		0.005
Well	23	69.6		73.9	
Moderate	124	66.1		67.7	
Poor	15	40		40	

*Log-rank test. # Group 1 (low in cancer cells and macrophages), group 2 (low in cancer cells and high in macrophages), group 3 (high in cancer cells and low in macrophages), group 4 (high in cancer cells and macrophages).

Table 1: Disease-free and overall survival in 162 colorectal cancer patients.

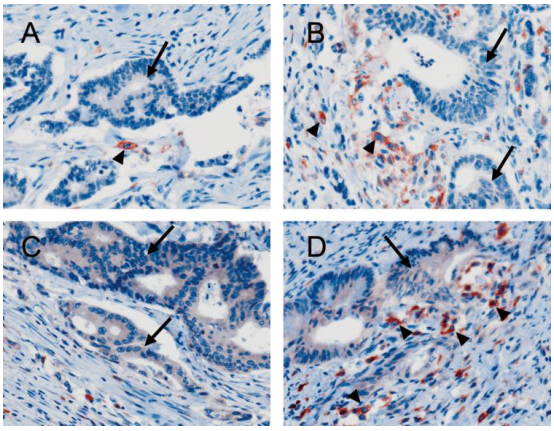


Figure 1: Representative iNOS staining of **(a)** group 1 (low in cancer cells and macrophages: iNOS grades 0 to 1 cancer cells and iNOS grades 0 to 1 macrophages) (×100), **(b)** group 2 (low in cancer cells and high in macrophages: iNOS grades 0 to 1 cancer cells and iNOS grades 2 to 3 macrophages) (×100), **(c)** group 3 (high in cancer cells and low in macrophages: iNOS grade 2 cancer cells and iNOS grades 0 to 1 macrophages) (×40), and **(d)** group 4 (high in cancer cells and macrophages: iNOS grade 2 cancer cells and iNOS grades 2 to 3 macrophages) (×40) tumors. The intensity of iNOS staining was usually higher in infiltrating macrophages (arrowheads) than in cancer cells (arrows).

With the assistance of histology, CD68, and iNOS staining, infiltrating macrophages expressing iNOS could be clearly identified (Figure 1), and most of them were in the invasive front of tumors, as previously described [29]. Not all of the infiltrating macrophages expressed iNOS, however. The intensity of iNOS staining was usually higher in infiltrating macrophages than in cancer cells (Figure 1). The iNOS-expressing macrophages around the tumor front were scored as grades 2 and 3 in 82 cases (51 percent). Besides, iNOS was also expressed in the fibroblasts, endothelial cells, and inflammatory cells as reported before [15].

Correlation between IHC expression of iNOS and other clinicopathological covariates

Neither the iNOS grade of cancer cells nor the grade of infiltrating

macrophages was correlated with other covariates. However, patterns of iNOS expression in both cell types were associated with MAC stage (p = 0.018) (Table 2). Group 4 pattern positively correlated with advanced MAC stages, i.e. a higher frequency of MAC stage D with a lower frequency of the MAC stage A or B. Group 3 cases showed the reverse trend. There was an association between expression patterns of iNOS and the T status (p = 0.005) (Table 2). Group 4 cases have a significantly higher frequency of pT3-pT4 status than pT1-pT2 status. In contrast, group 3 cases more often belong to pT1-pT2 status (Table 2).

IHC expression of iNOS and patient prognosis

Univariate analysis showed that the modified Astler-Coller stage, T status, N status, M status, and tumor differentiation were significantly influenced five-year disease-free and overall survival (p<0.05 for all comparisons; Table 1).

Neither iNOS grade of cancer cells nor the grade of infiltrating macrophages was correlated with disease-free or overall survival. Taken expression patterns in both types of cells together, patients in groups 1 and 4 had a significantly lower five-year disease-free survival and overall survival rate (p = 0.003, 0.010 respectively) (Table 1). Analysis of the Kaplan-Meier curves revealed the same result (Graph 1a). Patients with concurrent high or low iNOS expression in both colorectal cancer cells and tumor-infiltrating macrophages had a poorer prognosis than the other two groups.

When stratifying the cases by the iNOS grade of colorectal cancer cells, in cases with cancer cells of iNOS grades 0 to 1, more densely infiltrating iNOS-expressing macrophages around the tumor invasive front were correlated with a higher disease-free survival rate (group 2 vs. group 1) (p = 0.004, data not shown). For those cancer cells having grade 2 iNOS, more densely infiltrating iNOS-expressing macrophages were correlated with a lower disease-free survival rate (group 4 vs. group 3) (p = 0.041, data not shown).

The influence of all significant covariates on survival was simultaneously tested with the use of a multivariate Cox proportional-hazards model. The modified Astler-Coller stage and T status were not included for multivariate analysis because of their correlation with the iNOS expression patterns in cancer cells and macrophages. After adjusting for N status, M status, and tumor differentiation, multivariate analysis revealed that the risk of tumor recurrence for group 1 patients and group 4 patients was 2.063 (p = 0.039) and 2.738 (p = 0.007) times higher than for group 2 patients (Table 3 and Graph 1a).

iNOS activity

An iNOS enzyme activity assay was performed in 51 frozen tumor tissues which were collected prospectively. The mean iNOS activity was 35.56 (range: 5.98 to 142.02) p mole citrulline/min/mg protein (SD: 27.13). iNOS activity was not correlated with any clinicopathological covariates except N status (data not shown). There was no significant correlation between enzyme activity and the IHC expression patterns

	iNOS expression#					
Characteristic, n (%)	**Group 1**	**Group 2**	**Group 3**	**Group 4**	**Total**	***P*-value***
Study population	59 (36.4%)	47 (29.0%)	21 (13.0%)	35 (21.6%)	162 (100%)	
Astler-Coller stage						0.018
A or B	25 (30.5%)	27 (32.7%)	16 (19.5%)	14 (17.1%)	82 (100%)	
C	29 (46.0%)	17 (27.0%)	4 (6.3%)	13 (20.6%)	63 (100%)	
D	5 (29.4%)	3 (17.6%)	1 (5.9%)	8 (47.1%)	17 (100%)	
T status						0.005
pT1 or pT2	8 (24.2%)	13 (39.4%)	9 (27.3%)	3 (9.1%)	33 (100%)	
pT3 or pT4	51 (39.5%)	34 (26.4%)	12 (9.3%)	32 (24.8%)	129 (100%)	

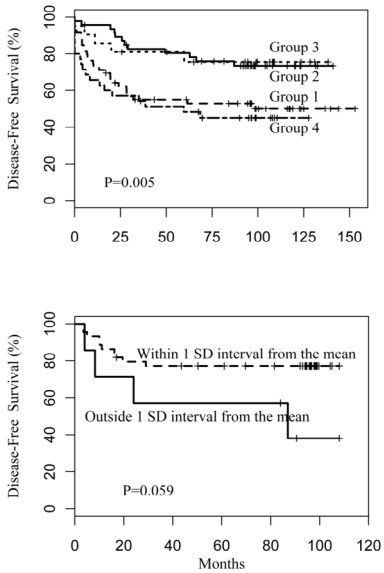
*Chi-square test. # Group 1 (low in cancer cells and macrophages), group 2 (low in cancer cells and high in macrophages), group 3 (high in cancer cells and low in macrophages), group 4 (high in cancer cells and macrophages).

Table 2: iNOS expression patterns and pathological parameters.

Characteristic	Disease-free survival		Overall survival	
	RR (95% C.I.)	*P* value	RR (95% C.I.)	*P* value
iNOS expression*		0.047		0.098
Group 1	2.063 (1.036-4.107)	0.039	2.145 (1.115-4.127)	0.022
Group 2	1	—	1	—
Group 3	1.405 (0.480-4.109)	0.535	1.551 (0.604-3.980)	0.361
Group 4	2.738 (1.320-5.680)	0.007	2.219 (1.095-4.496)	0.027
N status		< 0.001		< 0.001
pN0	1	—	1	—
pN1	1.461 (0.760-2.808)	0.256	1.042 (0.563-1.930)	0.895
pN2	3.722 (1.887-7.341)	< 0.001	3.642 (1.923-6.899)	< 0.001
Distant metastasis		< 0.001		< 0.001
None detected	1	—	1	—
Present	6.734 (3.531-12.845)	< 0.001	3.495 (1.886-6.476)	< 0.001
Differentiation		0.592		0.821
Well	1	—	1	—
Moderate	1.335 (0.587-3.035)	0.491	1.158 (0.562-2.388)	0.691
Poor	1.749 (0.599-5.106)	0.306	1.378 (0.507-3.743)	0.53

*Group 1 (low in cancer cells and macrophages), group 2 (low in cancer cells and high in macrophages), group 3 (high in cancer cells and low in macrophages), group 4 (high in cancer cells and macrophages.

Table 3: Prognostic significance of iNOS expression for colorectal cancer patients (multivariate Cox model).



Graph 1: (a) Patients in groups 1 and 4 (Figure 1 legend for group descriptions) were significantly correlated with a lower disease-free survival rate (p = 0.005). **(b)** Patients with tumors that had iNOS activity outside one standard deviation from the mean tended to have a shorter disease-free survival than patients with tumors that had iNOS activity within one standard deviation from the mean (p = 0.059).

of iNOS. The limited case numbers for iNOS activity assay may be responsible for the lack of correlation between these two methodologies. Furthermore, the immunohistochemistry can be used to discriminate and score the expression according to the cellular localization, respectively, whereas iNOS activity assay measures the total enzyme activity in the tumor tissue. Local iNOS expression of macrophages might not be reflected in the results of enzyme activity assay.

Kaplan-Meier analysis showed that cases with an outlier of iNOS activity (outside one standard deviation from the mean) tended to have a shorter disease-free survival ($p = 0.059$) (Graph 1b), suggesting that extremely high or low iNOS activity is associated with tumor progression (Graph 1b).

Discussion

Tumor-infiltrating macrophages are major cellular component of tumor stroma. Most of them were demonstrated in the invasive front of tumors [28], and thus are thought to represent host response to cancer development, although their efficiency in the suppression of tumor growth *in vivo* is still debated [30,31]. In this investigation, expression of iNOS was higher in macrophages than in most tumor cells, supporting that high levels of NO synthesized by macrophages are cytotoxic/cytostatic to tumor cells. These iNOS-expressing macrophages (so-called M1 phenotypes) were reported to be pro-inflammatory, microbicidal, and possibly tumoricidal because of their capability in producing inflammatory cytokines and reactive nitrogen intermediates [30,31].

This investigation found that iNOS-expressing macrophages may play distinct role in predicting patient prognosis in relation to iNOS status of tumor cells. The dense iNOS- expressing macrophages around tumor front was a good prognostic factor for those low iNOS tumors (iNOS grade 0 to 1). This observation concurs with that reported by Wei et al. The iNOS-null (iNOS-/-) tumor cells grew much faster and produced more lung metastases in iNOS-/- mice than in iNOS+/+ mice [18]. iNOS expression was only observed in the host infiltration cells in iNOS+/+ mice. Therefore, physiological expression of iNOS in host cells may provide protection from tumor growth and metastasis.

In contrast, in cases of cancer cells with grade 2 iNOS, a higher density of iNOS-expressing macrophages correlated with a lower disease-free survival rate (group 4 vs. group 3). The result seems to suggest that iNOS produced by macrophages may play a positive role in the tumor progression if tumor cells express high levels of iNOS. Two possible reasons may explain for this paradoxical result. The first possibility is because of the association of group 4 cancers with advanced MAC stages. Alternatively, Su et al proposed that NO production could initially act as an autocrine suicide or paracrine killing mechanism in cells undergoing malignant transformation [32]. However, once failed, NO promotes tumor formation by enhancing the selection of cells that can evade immune attack by acquiring apoptosis resistance [32]. Thus, constant exposure to NO may enhance the development of NO resistance, resulting in tumor progression, even in the presence of cytotoxic levels of NO [33]. Consistent with this interpretation comes from report from Shi et al. showing that host stromal-cell-derived NO, which is synthesized by iNOS, suppressed the growth of NO-sensitive tumors, but might accelerate the growth of NO-resistant tumors [19].

Recently, Tatemichi et al. created four combinations of tumor cells and host cells having different iNOS gene status [34]. It showed that the microscopic invasion of tumors was observed only in cases where the iNOS gene was present both in tumor and host cells, suggesting that iNOS gene activity in both tumor cells and host cells may synergistically contribute to tumor progression. This NO-associated tumor progression was attributed to activation of matrix metalloproteinases [34,35]. Although iNOS inhibitors are not the mainstay treatment of the colorectal cancer, these inhibitors have shown anti-cancer effects in preclinical studies [20]. The results of this investigation imply that colorectal cancer having high iNOS in both cancer cells and macrophages may be considered for anti-iNOS/NO therapy. A prospective clinical trial is required to confirm this hypothesis.

Both anti-NO and NO-based anticancer strategies appear effective in several preclinical models, and most of them were in the colon [20,22,36]. This investigation put emphasis on dual effects of NO in the development of targeted therapy. Our study revealed that tumor-infiltrating macrophages usually express higher levels of iNOS than cancer cells. A higher density of iNOS-expressing macrophages is a good prognostic factor for colorectal cancer cells expressing low iNOS. The dual effects of NO should be considered in the design of anti-iNOS/NO therapy for colorectal cancer patients.

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