

Originals

Plasma Endostatin Levels are Elevated in Patients with Gastric Carcinoma

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SUMMARY

Angiogenesis is a key process in tumor growth and metastasis. In 27 patients with advanced gastric carcinoma before operation and 9 healthy controls, plasma levels of endostatin (ES), cathepsin L (Cat-L) and soluble VEGF R1/Flt-1 (sVEGF R1), which are known to suppress angiogenesis, tumor growth and metastasis, were measured by ELISA in order to examine the clinical importance of the agents. In addition, expression of CD34, ES and Cat-L in tissues of the gastric carcinoma was investigated immunohistochemically in order to elucidate the correlation with the above plasma levels.

Plasma ES levels in gastric carcinoma patients were significantly higher than those in controls ($p = 0.001$). Plasma Cat-L levels in controls were slightly lower, though not significantly ($p = 0.065$), than those in carcinoma patients. There was no significant difference in sVEGF R1 levels between gastric carcinoma patients and controls. Expression of ES and Cat-L in tumor tissues was positively associated with plasma sVEGF R1 levels. Expression of CD34 in tumor tissues was not found to be associated with any plasma markers.

Our results suggested that plasma ES levels are elevated in gastric carcinoma and may be useful markers for gastric carcinoma.

Key Words : Angiogenesis, Endostatin, Cathepsin L, Soluble VEGF R1/Flt-1

INTRODUCTION

In the process of tumor growth and metastasis, angiogenesis is indispensable, in addition to the proliferation potency of tumors, for tumors to grow and evolve exceeding a certain size¹⁾. Tumor cells induce angiogenesis¹⁾ by producing angiogenesis stimulators. Angiogenesis stimulators include vascular endothelial growth factor (VEGF)^{2, 3)}, basic fibroblast growth factor (bFGF)^{4, 5)}, thymidine phosphorylase (TP)/platelet-derived endothelial cell (PD-ECGF)⁶⁾ and interleu-

kin (IL)-8⁷⁾. These angiogenesis stimulators are thought to be produced not only in tumor cells but also in tumor-associated macrophages (TAMs) that infiltrate around tumors. The production plays a role in angiogenesis in the manner of paracrine, or in accordance with changes in microenvironment around tumors such as hypoxia⁸⁾. Conversely, tumors are known to produce, by way of protease such as cathepsin, endogenous angiogenesis inhibitors which suppress tumor growth and metastasis.

Endogenous angiogenesis inhibitors include angiostatin (AS)⁹⁾, endostatin (ES)¹⁰⁾, and restin¹¹⁾. AS identified by O'Reilly et al. in 1994 is a polypeptide with molecular weight 38-kDa and produced by decomposition of plasminogen. The decomposition takes place in the presence of proteases such as plasmin^{12, 13)},

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urokinase^{14, 15)}, matrix metalloproteinase (MMP) 2, 3, 7, 9, 12¹⁶⁻¹⁹⁾ and cathepsin D^{20, 21)}. AS suppresses markedly tumor growth and metastasis^{22, 23)}.

In 1997, O'Reilly et al. isolated initially ES from the murine hemangioendothelioma cell line, EOMA. ES is a 20-kDa C-terminal fragment of collagen XVIII, which specifically inhibits endothelial cell proliferation. Collagen XVIII is present predominantly in the basement membranes particularly around blood vessels. The decomposition takes place in the presence of protease such as elastase²⁴⁾, MMP-7²⁵⁾–9²⁶⁾ and Cat-L²⁷⁾. A study reported that cathepsin E is the responsible enzyme in human²⁸⁾. The details of the production process of ES including physical protease and molecular targets remain unclear.

While the balance between stimulators and inhibitors of angiogenesis is strictly controlled under normal circumstances in normal angiogenesis, the balance in tumors leans toward the positive, supporting tumor growth and metastasis and affecting possibly the degree of malignancy and prognosis²⁹⁾.

There are many reports on angiogenesis that is associated with carcinoma growth and metastasis as well as prognosis of many cases of carcinoma. In gastric carcinoma, VEGF has been reported to play closely in the neogenesis of blood and lymphatic vessels and become a prognostic factor³⁰⁻³⁵⁾. Few studies though have been conducted on the angiogenesis inhibitors.

In the present study, the plasma expression of an endogenous ES, cathepsin L (Cat-L), which is reported to play a role in ES production, and soluble VEGF R1/Flt-1 (sVEGF R1)³⁶⁻³⁸⁾, which inhibits VEGF functions by binding with circulating VEGF, was compared between patients with gastric carcinoma and non-carcinoma subjects. In addition, the correlation was investigated between these plasma markers and clinicopathologic factors, intratumoral microvessel density. We also explored the expression of ES and Cat-L in gastric carcinoma tissues immunohistologically and investigated the relationship with grades of malignancy of gastric carcinoma.

PATIENTS AND METHODS

Enrolled in the study were 27 patients (20 males and 7 females, mean age 67.8 years) with advanced gastric carcinoma in the depth of tumor invasion T2 to T4,

Table 1 Clinicopathological characteristics of 27 patients with gastric cancer

Factors		No of patients
Histological Grade	Differentiated	12
	Undifferentiated	15
Depth of Tumor Invasion	T2(mp, ss)	18
	T3(se) T4(si)	9
Lymph Node Metastasis	N0	5
	N1	4
	N2	9
	N3	9
Liver Metastasis	H0	24
	H1	3
Peritoneal Metastasis	PO	24
	P1	3
Cytological Examination	CY0	19
	CY1	8
Stage	Stage I	5
	Stage II	2
	Stage III	5
	Stage IV	15

who were treated with gastrectomy at our department during the period between April and November 2004. Controls were 9 non-carcinoma subjects (4 males and 5 females, mean age 44.3 years) consisted of generally healthy hospital personnel.

Clinicopathologic factors were followed by the Japanese Classification of Gastric Carcinoma³⁹⁾. Table 1 shows clinicopathologic factors of the subjects.

1) Measurement of plasma endostatin, cathepsin L and sVEGF R1

Plasma samples were obtained from preoperative carcinoma patients and non-carcinoma subjects.

a) Measurement of plasma endostatin

Quantikine Human Endostatin ELISA Kit (R & D Systems, Minneapolis, USA) was used for measurement, in which VERSA max microplate reader (Molecular Devices Co., Sunnyvale, USA) at wavelength 450 nm (correction 570 nm) was used, and the endostatin concentration of samples was read from standard curves.

b) Measurement of plasma cathepsin L

Calbiochem Human Cathepsin L ELISA Kit (Oncogene Research Products, San Diego, USA) was used for measurement, in which VERSA max microplate reader (Molecular Devices Co., Sunnyvale, USA) at

wavelength 450 nm (correction 620 nm) was used, and the cathepsin L concentration of samples was read from standard curves.

c) Measurement of plasma sVEGF R1

Quantikine Human Soluble VEGF R1/Flt-1 ELISA Kit (R & D Systems, Minneapolis, USA) was used for measurement, in which VERSA max microplate reader (Molecular Devices Co., Sunnyvale, USA) at wavelength 450 nm (correction 570 nm) was used, and the sVEGF R1 concentration of samples was read from standard curves.

Values obtained by the above-mentioned methods were compared between carcinoma and non-carcinoma subjects. In carcinoma patients, the correlations were investigated between results of measurement and clinicopathologic factors: degrees of histodifferentiation, depth of tumor invasion, lymph node metastasis, hepatic metastasis and peritoneal cytodiagnosis.

2) Immunohistochemical study

Samples of resected gastric carcinoma were fixed with 10% formalin, embedded in paraffin to make 4- μ m serial sections. Streptavidin-biotin-peroxidase complex (SAB) method was used for immunohistochemical staining.

a) CD34

The above tissue samples were deparaffinized and microwave-treated for 40 min. CD34 antibody (Dako Cytomation, Copenhagen, Denmark) was used for the primary antibody reaction at 4°C for 24 hrs. After washing with PBS, it was reacted with biotinized secondary antibody. Colors were developed using 3,3'-Diaminobenzidine (DAB).

After reactions, samples were observed under a 200-X light microscope. The number of positively stained microvessels was counted in 3 fields showing the most vascularization and mean values were calculated to obtain the microvascular density.

b) Endostatin

The above tissue samples were deparaffinized and microwave-treated for 10 min. Endostatin polyclonal antibody (Lab Vision Co., Fremont, USA) was used for the primary antibody reaction at 4°C for 24 hrs. After washing with PBS, it was reacted with biotinized secondary antibody. Colors were developed using 3,3'-Diaminobenzidine (DAB). Methyl green was used for

nuclear staining.

With non-carcinoma tissues, the number of stained cytoplasm per glandular duct was counted and positive rates were calculated. With cancerous tissues, cancerous cells 500 or more were observed and positive cell rates were calculated. Positive rates were compared between cancerous and non-cancerous tissues.

c) Cathepsin L

The above tissue samples were deparaffinized and microwave-treated for 10 min. Cathepsin L antibody (Transduction Laboratories, Lexington, USA) was used for the primary antibody reaction at 4°C for 24 hrs. After washing with PBS, it was reacted with biotinized secondary antibody. Colors were developed using 3,3'-Diaminobenzidine (DAB). Methyl green was used for nuclear staining.

With non-cancerous tissues, the number of stained cytoplasm per glandular duct was counted and positive rates were calculated. With cancerous tissues, cancerous cells 500 or more were observed and positive cell rates were calculated. Positive rates were compared between cancerous and non-cancerous tissues.

3) Statistical analysis

Plasma levels of ES, Cat-L and sVEGF R1 as well as CD34 positive counts were expressed in mean \pm SD. Statistically significant differences were tested with Mann-Whitney U test. $P < 0.05$ were considered to indicate significant difference.

RESULTS

1) Comparisons of plasma markers between patients with gastric carcinoma and control (Table 2)

The mean value of ES was 2.07 ± 0.8 ng/ml in pre-operative carcinoma patients and 0.98 ± 0.3 ng/ml in control. The value in carcinoma patients was significantly higher than that in control ($p = 0.001$).

The mean value of Cat-L was 12.1 ± 4.8 ng/ml in preoperative carcinoma patients and 15.3 ± 4.8 ng/ml in control. The value was lower, although not significantly, in carcinoma patients than that in control ($p = 0.065$).

The mean value of sVEGF R1 was 64.4 ± 48.7 pg/ml in pre-operative carcinoma patients and 64.8 ± 17.2 pg/ml in control. There was no significant difference between the two groups.

Table 2 Association of plasma endostatin, cathepsin L and sVEGF R1 levels with clinicopathologic factors in gastric cancer patients

Factors		ES (ng/ml) (mean ± SD) P value		Cat-L (ng/ml) (mean ± SD) P value		sVEGF R1 (pg/ml) (mean ± SD) P value	
Cancer		2.07 ± 0.8	0.001	12.1 ± 4.8	NS	64.4 ± 48.7	NS
Normal		0.96 ± 0.3		15.3 ± 4.8		64.8 ± 17.2	
Histological Classification	differentiated	2.22 ± 1.0	NS	12.1 ± 3.3	NS	71.8 ± 62.9	NS
	undifferentiated	1.95 ± 0.7		12.1 ± 5.8		58.4 ± 34.8	
Depth of invasion	T2	2.16 ± 0.9	NS	11.1 ± 3.5	NS	64.6 ± 53.7	NS
	T3, 4	1.90 ± 0.6		14.0 ± 6.5		63.9 ± 39.8	
Lymph node metastasis	positive	1.97 ± 0.7	NS	12.4 ± 5.3	NS	65.7 ± 53.5	NS
	negative	2.53 ± 1.3		10.6 ± 0.6		58.5 ± 18.2	
Hepatic metastasis	positive	2.4 ± 0.6	NS	11.2 ± 1.5	NS	73.2 ± 6.6	NS
	negative	2.03 ± 0.9		12.2 ± 5.1		63.3 ± 51.6	
Intraperitoneal Cytological Exa	positive	1.82 ± 0.5	NS	11.6 ± 7.6	NS	61.3 ± 43.6	NS
	negative	2.18 ± 1.0		12.3 ± 3.3		65.7 ± 51.8	

2) Relationship between plasma markers and clinicopathologic factors in gastric carcinoma patients (Table 2)

There were no correlations between clinicopathologic factors (degrees of histodifferentiation, depth of tumor invasion, lymph node metastasis, hepatic metastasis and peritoneal cytodiagnosis) and preoperative plasma levels of ES, Cat-L and sVEGF R1 of gastric carcinoma patients. While plasma Cat-L levels 9.6 ng/ml or higher occurred in either carcinoma patients or non - carcinoma patients, the level lower than 9.6 ng/ml occurred only in carcinoma patients (n = 7).

Subjects were divided into higher level group and lower level group based on the cut-off levels of ES at 1.6 ng/ml (i.e., mean ± SD in control), Cat-L at 10.46 ng/ml (i.e., mean - SD in control) and 47.69 pg/ml (i.e., mean - SD in control). There were no correlations between both groups for clinicopathologic factors.

3) Immunohistological findings of cancerous and non-cancerous tissues (Fig. 1)

The number of CD34 positive blood vessels was 47.9 ± 20.1 in cancerous tissues and 40.2 ± 20.7 in non-cancerous tissues, demonstrating that the number of blood vessels was higher in cancerous tissues than in non-cancerous tissues. However, the difference was not significant.

The rate of ES positive cells in cancerous tissues was 40.8 ± 32.7 and higher, but not significantly, than

that in non-cancerous tissues at 12.5 ± 11.8.

The rate of Cat-L positive cells in cancerous tissues was 19.7 ± 31.1 and higher, but not significantly, than that in non-cancerous tissues at 5.8 ± 6.4

4) Relationship between immunohistological findings and clinicopathologic factors

The number of CD34 positive blood vessels was 64.3 ± 21.1 in patients with positive peritoneal cells and 41.1 ± 15.6 in patients with negative peritoneal cells. The former was significantly higher than the latter (p = 0.039).

However, there were no correlations between the number of CD34 positive blood vessels and degrees of histodifferentiation, depth of tumor invasion, presence or absence of lymph node metastasis and presence or absence of hepatic metastasis.

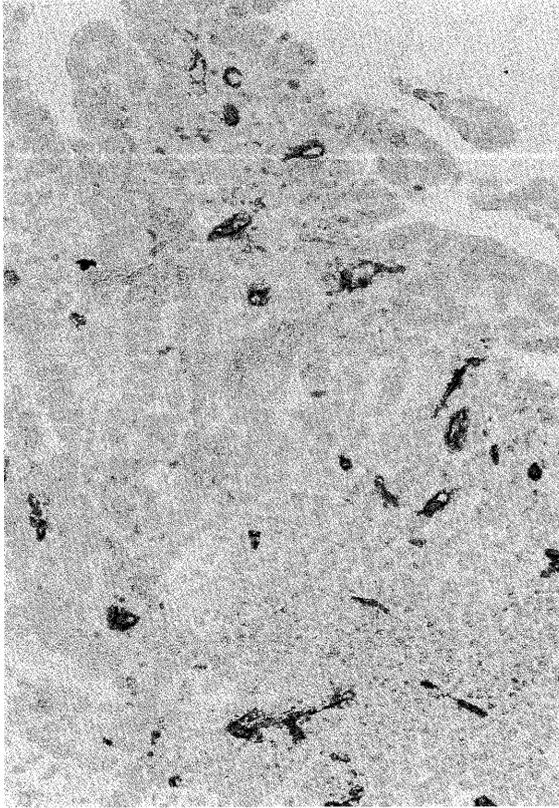
There was no correlation between the ES positive rate and each of clinicopathologic factors.

There was no correlation between the Cat-L positive rate and each of clinicopathologic factors.

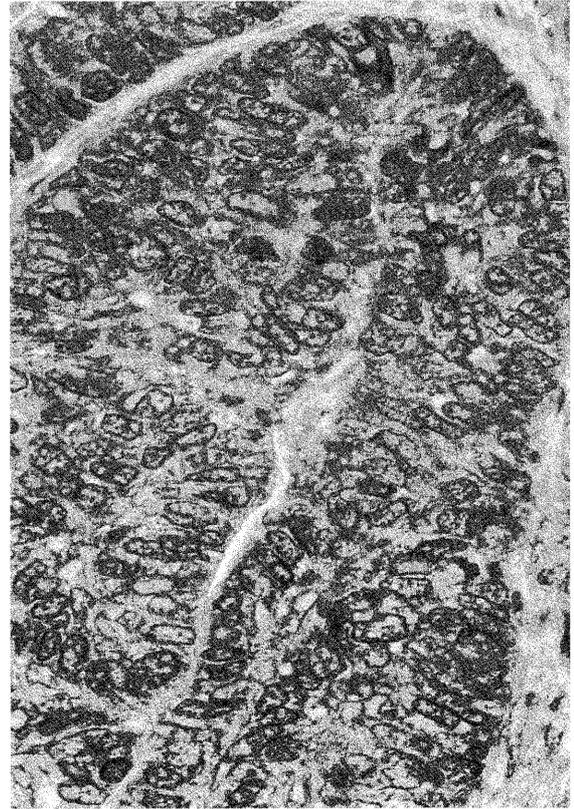
5) Relationship between plasma markers and immunohistological findings

a) Relationship between preoperative plasma ES levels and immunohistological findings in patients with gastric carcinoma

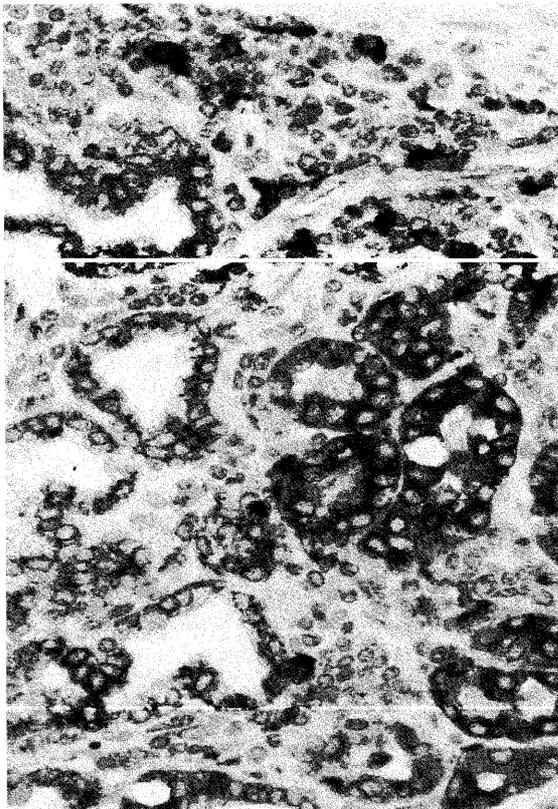
The correlation coefficient r was -0.01 between preoperative plasma ES levels and the number of



A



B



C

Fig. 1 Immunohistochemical stainings of CD34 (A), Endostatin (B), Cathepsin L (C) in gastric cancer tissue.

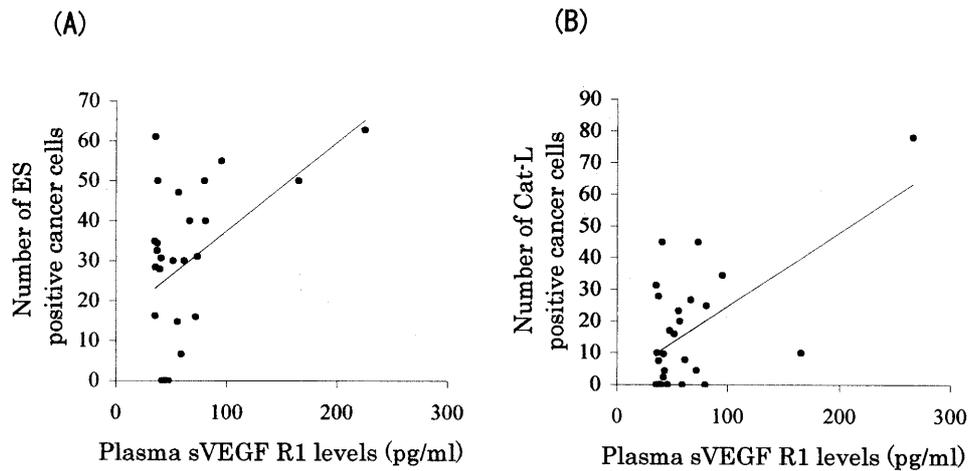


Fig. 2 The correlation between the expression of ES (A), Cat-L (B) and preoperative plasma sVEGF R1 levels

CD34 positive blood vessels in cancerous tissues. Thus, there was no correlation between the two.

The correlation coefficient r was 0.108 between plasma ES levels and the rate of ES positive cells. Thus, there was no correlation between the plasma ES level and expression in tissue cells.

The correlation coefficient r was 0.26 between plasma ES levels and the rate of Cat-L positive cells. Thus, there was no correlation between the two.

b) Relationship between preoperative plasma Cat-L levels and immunohistological findings in patients with gastric carcinoma

The correlation coefficient r was -0.173 between plasma Cat-L levels and the number of CD34 positive blood vessels in cancerous tissues. Thus, there was no correlation between the two.

The correlation coefficient r was 0.009 between plasma Cat-L levels and the rate of ES positive cells. Thus, there was no correlation between the two.

The correlation coefficient r was -0.107 between plasma Cat-L levels and the expression of Cat-L in tissue cells. Thus, there was no correlation between the two.

c) Relationship between preoperative plasma sVEGF R1 levels and immunohistological findings in patients with gastric carcinoma

The correlation coefficient r was -0.114 between plasma sVEGF R1 levels and the number of CD34 positive blood vessels in cancerous tissues. Thus, there was no correlation between the two.

The correlation coefficient r was 0.409 between plas-

ma sVEGF R1 levels and the rate of ES positive cells ($p < 0.05$). Thus, there was a correlation between the two (Fig. 2A).

The correlation coefficient r was 0.605 between plasma sVEGF R1 levels and the rate of Cat-L positive cells ($p < 0.01$). Thus, there was correlation between the two (Fig. 2B).

DISCUSSION

Based on the hypothesis set up by Judah Folkman in 1971 that tumor growth depends on angiogenesis and thus, the control of angiogenesis leads to cure of tumors⁴⁰, many studies have been reported concerning tumor growth and angiogenesis. It is said that in order for tumors to grow beyond a few millimeters in size, angiogenesis is needed as supply routes of nutrition and oxygen⁴¹. Tumor angiogenesis is thought to be controlled in the net balance between various stimulators and inhibitors²⁸. With advancement of molecular biology in recent years, many stimulators and inhibitors have been identified.

ES is one of the most potent inhibitors of angiogenesis and can induce tumor regression in several animal models. Boehm et al. demonstrated using animal models that continuous administration of ES leaves tumor in the dormant state without resistance to ES^{42, 43}. Suppression of tumor growth is also reported in other animal models of renal cell carcinoma, ovarian cancer, non-small cell lung cancer, non-Hodgkin lymphoma, chondrosarcoma and liver carcinoma^{44~53}. Human ES of circulation and tissue forms have also been identi-

fied. Increase levels in circulating ES have been demonstrated previously in patients with clear cell renal cancer, non-Hodgkin lymphoma, soft tissue sarcoma, hepatocellular carcinoma, non-small cell lung cancer, ovarian cancer, breast cancer and colorectal cancer^{54~60)}.

In the present study, plasma ES levels in patients with gastric carcinoma were significantly elevated compared to those in healthy control, corresponding with reported findings in other cancers. However, no clinicopathologic factors were significantly associated with plasma ES levels. Therefore, it is suggesting that there were no correlation between increases in plasma ES levels and grades of malignancy of gastric carcinoma.

The rate of ES positive cells in cancerous tissues tended to be higher, but not significantly, than that in non-cancerous tissues. There were no correlations between plasma levels and clinicopathologic factors or expression of ES positive cells in tissues and grades of malignancy. Similarly, there was no correlation between plasma levels and expression rates in cancerous tissues, suggesting that increases in ES expression were changes common to gastric carcinoma in association with cancerization of cells. The findings that ES positive cells were expressed in non-cancerous tissues and present in plasma of healthy control group suggested that ES was secreted not only in cancerous tissues but also in non-cancerous tissues. It may be released by stroma cells of the surrounding tissue, endothelial cells or cells of the immune system. Investigation of tissues in healthy control group may be needed.

The findings that plasma ES levels are elevated in patients with gastric carcinoma, suggesting that biophylactic functions might change in association with the development of carcinoma. However, the mechanism of circulating ES action is unknown at present. Further studies are clearly required to understand the process of ES production, site of interaction, and the mechanism of activity.

Cat-L takes part in ES production and plays an important role in tumor infiltration and metastasis by way of extracellular matrix degradation⁶¹⁾. High expression of Cat-L is observed in tissues in early-stage gastric carcinoma and prostate cancer^{62, 63)}. In the

present study on gastric carcinoma, plasma Cat-L levels were lower, but not significantly, in cancerous tissues than those in non-cancerous tissues. The rate of expression of Cat-L in tissues tended to be higher in cancerous tissues than that in non-cancerous tissues, corresponding with reported other cancers. Although there were no correlations with any clinicopathologic factors, low plasma levels 9.6 ng/ml or lower occurred only in cancer cases (n = 7), 5 of which were of stage VI. Low plasma Cat-L levels appear to be associated with aggressiveness of gastric carcinoma.

sVEGF R1 is an extracellular domain soluble protein and VEGF specific endogenous inhibitor protein. Studies reported that administration or gene transduction of sVEGF R1 suppressed tumor growth^{16~18)}. Expression of sVEGF R1 is often increased in tumors⁶⁴⁾. Therefore, measurement was conducted to find if the expression was increased in gastric carcinoma. There was no difference in plasma levels between cancer patients and control subjects. Similarly, there were no correlations between plasma levels and clinicopathologic factors, suggesting that plasma sVEGF R1 levels could not be a useful marker for malignancy of gastric carcinoma. As there were correlations between VEGF R1 levels and the rates of ES positive cells and Cat-L positive cells in cancerous tissues, it can be said that when serum VEGF is relatively low, expression of tissue ES and Cat-L is high. In tumors in such a state, angiogenesis may not depend on VEGF but on other angiogenesis factors, suggesting that resistance be shown in treatment with anti-VEGF antibody.

The present study focused on the relationship between angiogenesis inhibitors and gastric carcinoma. Tumor angiogenesis is controlled in a balance of stimulators and inhibitors which form a complex network. Therefore, factors taking part in the process of angiogenesis may vary in multi-phases according to tumor growth. This may be the reason why expression of angiogenesis varied in individual patients and was independent from clinicopathologic background factors such as tissue types and depth of cancer growth in the gastric wall.

In conclusion, plasma ES levels are elevated in patients with gastric carcinoma. Our results suggest that there were no correlation between increases in plasma ES levels and grades of malignancy of gastric carcinoma.

ma. However, plasma ES levels may be a useful tumor marker in patients with gastric carcinoma.

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