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The localization of thrombospondin-1 (TSP-1), cysteine-serine-valine-threonine-cysteine-glycine (CSVTCG) TSP receptor, and matrix metalloproteinase-9 (MMP-9) in colorectal cancer

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Summary. Thrombospondin-1 (TSP-1) is a 450 kDa matrix bound glycoprotein involved in tumor invasion, metastasis, and angiogenesis. One of the receptors involved in TSP-1 mediated tumor cell adhesion and metastasis is the cysteine-serine-valine-threonine-cysteine-glycine (CSVTCG) receptor. One mechanism of TSP-1 in promoting tumor cell metastasis involves the up-regulation of matrix metalloproteinase-9 (MMP-9) expression, specifically through the CSVTCG TSP-1 receptor. TSP-1 and its CSVTCG receptor has been implicated in tumor progression in a variety of cancers including breast adenocarcinomas, head and neck squamous cell carcinomas, and pancreatic carcinomas.

In this study, we examined 99 cases of colorectal cancer by immunohistochemical analysis to investigate 1) the localization of TSP-1 and CSVTCG TSP-1 receptor, 2) the relationship with MMP-9, and 3) the correlation of expression with clinical staging.

Strong expression of TSP-1 was observed in the submucosa or the serosa adjacent to the tumor. Positive staining for CSVTCG TSP-1 receptor was observed in tumor cells and microvessels. MMP-9 was also expressed in tumor cells. In addition, staining intensity of CSVTCG TSP-1 receptor was higher in poorly differentiated adenocarcinoma than well or moderately differentiated adenocarcinoma. Tumors in which inflammatory cells stained strongly for CSVTCG TSP-1 receptor correlated with decreased incidence of distant metastasis and angiogenesis.

These data were consistent with our previous studies for breast, pancreatic, and head and neck carcinoma. They suggest an important role for TSP-1 and CSVTCG TSP-1 receptor in tumor progression in colorectal cancer.

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Introduction

TSP-1 is a 450kd glycoprotein, originally identified as a component of alpha granules of platelets (Lawler et al., 1978). Recent studies have shown various cells including endothelial cells (McPherson et al., 1981; Mosher et al., 1982), smooth muscle cells (Raugi et al., 1982), fibroblasts (Raugi et al., 1982; Jaffe et al., 1983), pneumocytes (Sage et al., 1983), macrophages (Jaffe et al., 1985), monocytes (Jaffe et al., 1985), and tumor cells (Riser et al., 1988; Varani et al., 1989; Mosher, 1990) secrete and synthesize TSP-1. While numerous investigators report TSP-1 inhibits tumor progression with its anti angiogenic character, our laboratory and several other groups have shown TSP-1 promotes tumor cell invasion and angiogenesis. TSP-1 promotes tumor cell adhesion, migration in breast cancer cells (Wang et al., 1996a), and stimulates bovine aortic endothelial cell tube formation in vitro (Qian et al., 1997). TSP-1 also increased lung metastasis of B16F10 cells in murine model (Tuszynski et al., 1992a).

CSVTCG specific TSP-1 receptor is a tumor cell adhesion receptor, isolated from the total cell extract of a lung carcinoma cell line (Tuszynski et al., 1993). This receptor mediates the actions of TSP-1 which are mentioned above, since tumor cell invasion (Wang et al., 1996b) was blocked by anti-CSVTCG TSP-1 receptor antibody. CSVTCG peptide as well as anti-CSVTCG TSP-1 receptor antibody remarkably reduced TSP-1 stimulated lung metastasis in the B16F10 melanoma cell model (Tuszynski et al., 1992a).

Furthermore, we discovered TSP-1 upregulates MMP-9 expression in breast cancer cells and endothelial cells (Qian et al., 1997). MMP-9 belongs to a family of zinc containing enzymes strongly implicated in tumor

invasion and metastasis because of its capacity of degrading type IV collagen, a predominant component of basement membrane. Regulation of MMP-9 by TSP-1 is also believed to be mediated by the CSVTCG specific receptor (Qian et al., 1997).

We hypothesized that TSP-1 promotes tumor cell invasion and angiogenesis by regulation of MMP-9 activity. We postulate that the CSVTCG TSP-1 receptor plays a key role for modulation of MMP-9. Immunohistochemical procedures for human tissue samples were performed to examine *in vivo* expression of these factors and to support the data obtained in our *in vitro* studies.

In previous studies, we have shown localization of TSP-1 and CSVTCG TSP-1 receptor in breast (Roth et al., 1997), pancreatic, and head and neck cancer (Arnoletti et al., 1994). Strong positive staining was observed in the extracellular matrix surrounding the malignant tumor, whereas in normal or benign tissues TSP-1 staining was less. The CSVTCG-receptor, however, localized in malignant tumor cells and microvessels around the malignant tumor. Staining intensity of CSVTCG TSP-1 receptor in tumor cells was positively correlated with malignant histological type and poor prognosis.

In this study, we examined 99 cases of colorectal cancer specimen for MMP-9 expression in addition to TSP-1 and CSVTCG TSP-1 receptor. Statistical analysis for correlation between staining and clinical presentation was also performed.

Materials and methods

Tissue samples were selected in a blind fashion from colorectal cancer, which were resected in the second department of surgery, Fukuoka University Hospital, between 1994 and 1996. After resection, the tissue samples were fixed with 10% formaldehyde and paraffin sections were made. A total of ninety-nine samples were obtained from varying stages of colorectal carcinoma (see Table 1). Tissue samples were immunostained with the avidin-biotin-peroxidase procedure (ABC Elite, Vector Laboratories, Burlingame, CA) and 3-amino-9-ethylcarbazole (AEC) was used as a chromogen.

Slides were deparaffinized and rehydrated by sequential incubation in graded xylene-ethanol solution, and then trypsinized with 0.1% trypsin to expose antigenic determinants masked by formalin and paraffin. Endogenous peroxidase affinity was quenched by treatment with 3% H_2O_2 and blocking solution was applied. Slides were treated with 5-20 μg/ml solution of primary IgG in phosphate buffered saline (PBS) containing 0.1% bovine serum albumin (BSA). Secondary biotinylated and third antibodies were applied. Slides were developed with immunoperoxidase kit from Vector, and then counter-stained with hematoxylin, and mounted with cover slips.

Slides were examined by bright field microscopy. Statistical analysis was performed using the Sigma Stat (Jandel Scientific). Staining was graded from - to ++ with - representing no detectable staining and ++ representing the strongest staining pattern.

Results

TSP-1 staining

Positive staining was observed in the submucosa and serosa adjacent to the tumor in 85 out of 99 cases (see Fig. 1A,C). Tumor cells showed positive staining in 36 out of 99 cases (see Table 2). Of these cells, tumor cells invading into the local vein expressed strong positive staining (see Fig. 1E). Inflammatory cells also showed strong positive staining.

CSVTCG receptor

Positive staining was observed in tumor cells in 72 out of 99 cases (see Table 2 and Fig. 1B). Inflammatory cells around the tumor showed positive staining in 44 cases (see Table 2). Microvessels expressed positive staining in 31 cases (see Table 2 and Fig. 1D). This receptor was also strongly expressed in poorly differentiated adenocarcinoma (see Table 3). These results were statistically significant as assessed through Kruskal-Wallis one way analysis.

Table 1. A total of 99 samples of colorectal carcinoma were used for this study. Tables 1 defines the histology, depth, metastasis, and TNM stage of these samples. Subsequent staining analysis involved correlating staining intensity with these staging parameters. Depth represents the tumor invasion into mucosa (m), submucosa (sm), muscularis propria (mp), subserosa (ss), serosa (se), invasion to other organs (si), rectal cancer invasion further than mp (a1), rectal cancer invasion further than mp, but not other organs (a2), rectal cancer invasion to other organs (ai).

Histology	Well differentiated adenocarcinoma	72
	Moderately differentiated adenocarcinoma	18
	Poorly differentiated adenocarcinoma	3
	Mucinous carcinoma	5
	Undifferentiated adenocarcinoma	1
Depth	si ai	2
	se a2	43
	ss a1	25
	mp	19
	sm	9
	m	1
Lymph node metastasis	Positive (+)	41
	Negative (-)	58
Distant metastasis	Positive (+)	11
	Negative (-)	88
TNM Stage	0	1
	1	24
	2	31
	3	32
	4	11

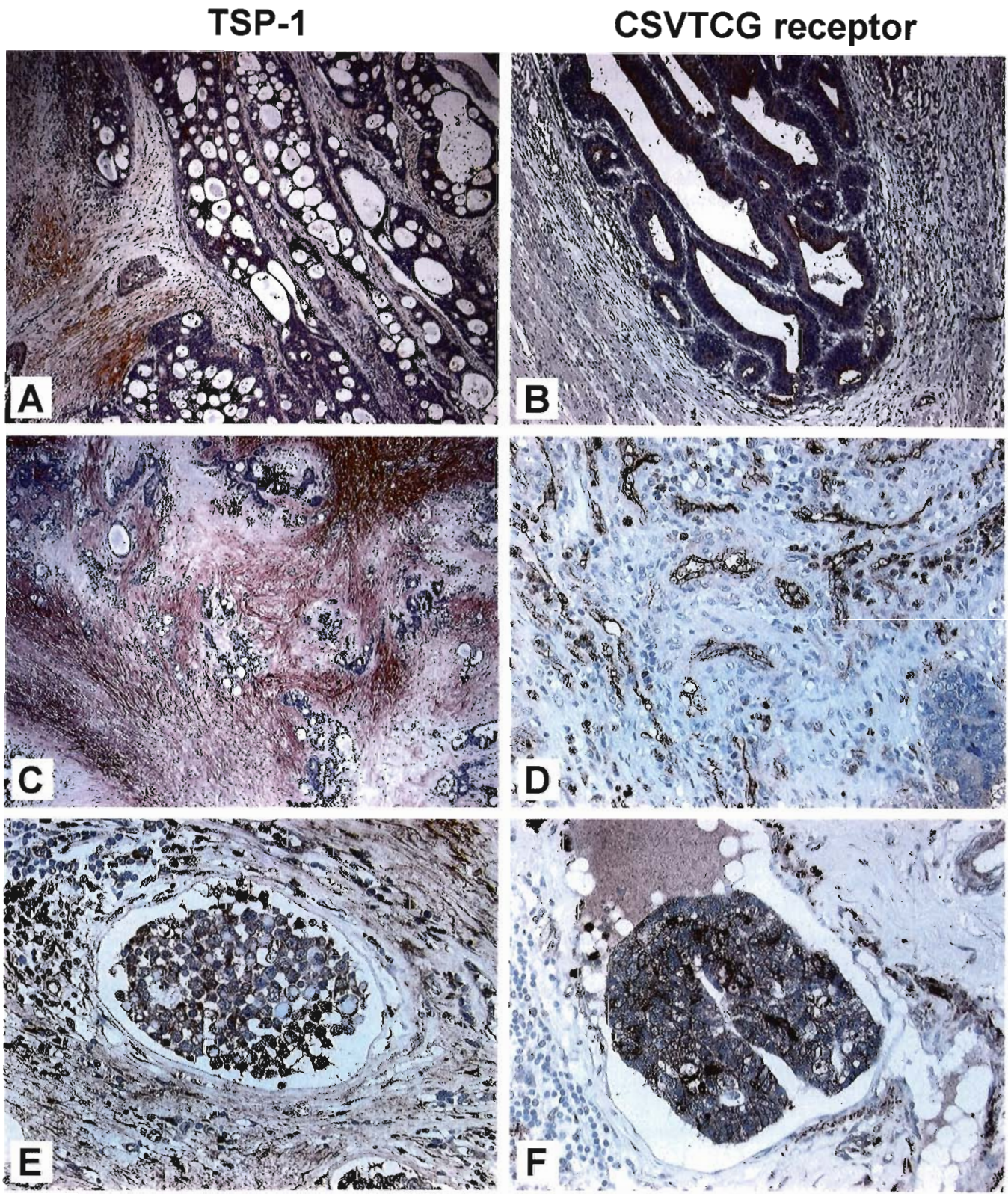

Fig. 1A-F. Colon adenocarcinoma stained for either TSP-1 or the CSVTCG receptor. **A** shows stromal staining of TSP-1 adjacent to tumor tissue, while **B** shows receptor expression within the tumor cells themselves. **C** shows again the expression of TSP-1 in the tumor stroma. **D** reveals extensive staining of microvessels for the CSVCTG receptor. **E, F** show tumor cells with microvessels staining positive for either TSP-1 or the CSVTCG receptor. A, C, x 40; B, x 100; D, E, F, x 200

MMP-9

Positive staining was observed in tumor cells in 41 out of 99 cases (see Table 2). Of these tumor cells staining positive for MMP-9, 36 cases also showed a high expression of the CSVTCG receptor as well (see Table 3). Inflammatory cells around the tumor showed positive staining in 36 cases (see Table 2). Similar to the tumor cells, inflammatory cells expressing MMP-9 also express the CSVTCG receptor (see Table 3).

Table 2. Distribution and intensity of TSP-1, TSP-1 receptor and MMP-9, respectively, in various cell types including tumor cells, tumor stroma, inflammatory cells, and microvessels. Staining intensity was graded on a scale of - to ++ with - representing no staining and ++ representing the strongest staining.

STAINING		-	+	++
TSP-1	Tumor cell	64	18	17
	Stroma (adjacent tumor)	13	27	59
	Stroma (intra-tumor)	63	21	15
	Inflammatory cell	60	27	12
TSP-1 receptor	Tumor cell	27	55	17
	Inflammatory cell	55	28	16
	Microvessel	68	31	
MMP-9	Tumor cell	58	32	9
	Inflammatory cell	63	28	8

Statistical analysis

TSP-1 receptor staining of cancer cells was positively correlated with MMP-9 staining of cancer cells through the Spearman rank order correlation, a method chosen to describe the correlation between groups with non-normal distribution. The intensity of CSVTCG TSP-1 receptor staining was higher in poorly differentiated adenocarcinoma than well or moderately differentiated adenocarcinoma as determined through the Kruskal-Wallis one-way analysis to find differences among five groups with unequal variances. TSP-1 staining in tumor stroma was positively correlated with depth, lymph node metastasis, and TNM stage through the Mann-Whitney rank sum test, the method to compare two groups with non-normal distribution, and the Spearman rank order correlation.

Discussion

As previously mentioned in the introduction, our hypothesis is that TSP-1 is a promoter of tumor invasion and angiogenesis. The mechanism of this activity involves regulation of MMP-9 specifically through its

Table 3. This table shows the presence of CSVTCG staining in the various grades of colorectal carcinomas. Although the sample number of poorly differentiated adenocarcinomas is low, the results are statistically significant in that poorly differentiated adenocarcinoma show a high percentage of positive staining for the CSVTCG receptor ($p<0.05$). This table also demonstrates a statistically significant correlation between MMP-9 and CSVTCG receptor expression, adding credibility to the hypothesis that TSP-1 modulates MMP-9 expression through the presence of this receptor. TSP-1 expression in tumor stroma is correlated with depth, lymph node metastasis, and TNM stage, significantly.

		RECEPTOR +	RECEPTOR -
Histology & Receptor tumor	Undifferentiated	0	1
	Mucinous	3	2
	Poorly differentiated	3	0
	Moderately differentiated	11	7
	Well differentiated	55	17
		MMP-9 +	MMP-9 -
Receptor tumor & MMP-9 tumor	Receptor +	36	36
	Receptor -	5	22
		MMP-9 inf +	MMP-9 inf -
Receptor inflammation & MMP-9 inflammation	Receptor inf +	24	20
	Receptor inf -	12	43
		TSP-1 +	TSP-1 -
Depth & TSP-1 stroma (intra-tumor)	Depth m	0	1
	sm	0	9
	pm	6	13
	ss a1	11	14
	se a2	17	26
	si ai	2	0
		TSP-1 +	TSP-1 -
Lymph node metastasis & TSP-1 stroma (intra-tumor)	Lymph node metastasis +	22	19
	Lymph node metastasis -	14	44
		TSP-1 +	TSP-1 -
TNM stage & TSP-1 stroma (intra-tumor)	TNM stage 0	0	1
	1	4	20
	2	9	22
	3	17	15
	4	6	5

CSVTCG TSP-1 receptor.

However, a considerable number of investigators reported TSP-1 inhibits tumor progression by means of its anti-angiogenic activity. TSP-1 inhibited endothelial cell proliferation (Bagavandoss and Wilks, 1990) and migration *in vitro*, and corneal neovascularization *in vivo* (Good et al., 1990). TSP-1 expression was reduced in bladder cancer, whereas normal epithelial cells produce high levels of TSP-1 (Campbell et al., 1998). The reduced expression of TSP-1 was correlated with increased neovascularization (Grossfeld et al., 1997). Breast cancer cells transfected with TSP-1 c-DNA displayed decreased tumor metastasis as well as decreased angiogenesis in a murine model (Weinstat-Saslow et al., 1994). The effects of TSP-1 on angiogenesis is controversial, however, as many laboratories have also cited that TSP-1 is a promoter of angiogenesis. The exact role of TSP-1 may be mediated by a variety of factors including concentration and interaction with growth factors and cytokines.

In this study, localization of TSP-1, the CSVTCG TSP-1 receptor and MMP-9 were investigated in 99 cases of colorectal cancer to provide support for our hypothesis that TSP-1 through its CSVTCG receptor regulates MMP-9 expression.

Strong expression of TSP-1 was observed in the thick fibrous tissue especially in the submucosa or serosa adjacent to the tumor (see Fig. 1A,C). In contrast, expression of CSVTCG TSP-1 receptor was observed in tumor cells and microvessels around tumors (see Fig. 1B,D). MMP-9 expression was also observed in tumor cells. This result is consistent with our previous studies with pancreatic carcinoma. These studies suggest TSP-1 in stroma stimulates tumor invasion and angiogenesis through CSVTCG TSP-1 receptor in tumor cells and vascular endothelial cells. In addition, the fact that expression of CSVTCG TSP-1 receptor in tumor cells positively correlated with MMP-9 in tumor cells (see Tables 3, 4) supports our hypothesis that activation of MMP-9 is mediated by the TSP-1 receptor.

Tumor cells invading into local vessels express strong positive staining for TSP-1 and TSP-1 receptor (see Fig. 1E,F). Furthermore, invading cells from the tumor margin expressed strong positive staining for TSP-1. These results suggest that cells expressing TSP-1 successfully invade into vessels, or these tumor cells have capacity to express TSP-1 when they are stimulated under certain circumstances. These results support the conclusion that TSP-1 is a promoter of tumor invasion and metastasis.

In the area around tumor expressed TSP-1, tissue destruction and new matrix formation are proceeding with tumor growth and inflammation as observed in the healing wound. However, in the healed wound, TSP-1 is not observed after fibrosis has occurred. In wound healing, TSP-1 was expressed early at the wound edge and microvessels (Raugi et al., 1987). mRNA of TSP-1 was up-regulated in inflammatory cells immediately after injury, and decreased steeply after the first day of injury. Treatment with TSP-1 antisense oligomers delayed wound healing (DiPietro et al., 1996). These results suggest TSP-1 initiates tissue remodeling including activation of proteases or proliferation of fibroblasts. Therefore, TSP-1 expression in cancer stroma may be an indication of new tissue remodeling and fibrosis. These stromal reactions are probably mediated by tumor released cytokines or growth factors. Our result showed TSP-1 expression in tumor stroma was positively correlated with lymph node metastasis, depth, and TNM stage (see Tables 3, 4). These results suggest that stromal TSP-1 may promote progression of advanced cancer. Recently, an elevated plasma level of TSP-1 in cancer patients has been reported (Tuszynski et al., 1992b; Nathan et al., 1994). In colorectal cancer, plasma level of TSP-1 correlated with Dukes staging and vessel invasion (Yamashita et al., 1998). The source of the TSP-1 in the plasma may be either platelets or tumor. It is possible that the plasma level of TSP-1 observed in these studies may have originated from the tumor stroma, since our data show a strong stromal expression of TSP-1 in all of the advanced cancers.

Recent studies have shown that interactions between tumor and stromal fibroblasts play a crucial role in cancer progression (Horgan et al., 1987; Camps et al., 1990; Chung, 1991; Gartner et al., 1992). Coculture of tumor cells with fibroblasts induced MMPs expression in several cancer cell lines (Munaut et al., 1995; Himelstein and Muschel, 1996; Kataoka et al., 1997; Sato et al., 1999). This activity may be associated with tumor invasion. In our results, expression of CSVTCG TSP-1 receptor was higher in the invasive margin than in middle of tumor. Since even well differentiated cancer expressed positive staining for the TSP-1 receptor, cancer cells may have the capacity to express TSP-1

Table 4. This table lists the statistical test used for the various correlations in this study. A more detailed description of each test is listed in the results section. LN meta: lymph node metastasis.

	TYPE OF STATISTICAL ANALYSIS	P VALUE
Histology & Receptor tumor	Kruskal-wallis one way analysis	0.0497
Receptor tumor & MMP-9 tumor	Spearman rank order correlation	0.0387
Receptor inflammation & MMP-9 inflammation	Spearman rank order correlation	0.00
Depth & TSP-1 stroma (intra-tumor)	Spearman rank order correlation	0.0368
LN meta & TSP-1 stroma (intra-tumor)	Mann-whitney rank sum test	0.0093
Stage & TSP-1 stroma (intra-tumor)	Spearman rank order correlation	0.00

receptor when they were transformed, or this capacity may be associated with increased proliferation. It may also be possible that contact with stroma, including fibroblasts and inflammatory cells, regulates TSP-1 receptor expression in tumor cells. Furthermore, staining intensity of TSP-1 receptor was higher in poorly differentiated adenocarcinoma than well or moderately differentiated adenocarcinoma (see Tables 3, 4). Poorly differentiated adenocarcinoma is highly proliferative, and tends to grow diffusely with severe fibrosis. This result also supports the importance of cancer-stroma interactions on receptor expression.

In colorectal cancer, inflammation around tumor is commonly observed, and there are some studies reporting inflammation around tumors is associated with tumor progression and angiogenesis (Leek et al., 1996; Ono et al., 1999; Takanami et al., 1999). Inflammatory cells surrounding the tumor express more TSP-1, TSP-1 receptor and MMP-9. However, the localization of these three factors was distinguishable. Inflammatory cells expressing TSP-1 was observed diffusely around tumor. In contrast, the cells expressing TSP-1 receptor and MMP-9 were observed at the interface between tumor and stroma. In addition, there was a significant correlation between TSP-1 receptor and MMP-9 staining in inflammatory cells (see Tables 3, 4). These three molecules may play different roles in host immune reactions against cancer. The cells expressing TSP-1 may be immuno-initiators or modulators. They may form satellites around the tumor and express tumor antigens on their surface and modulate inflammation. The cells expressing TSP-1 receptor and MMP-9 may be cytotoxic for tumor cells. CD36, another TSP-1 receptor which recognize CSVTCG sequence, is one of markers of phagocytic macrophage, derived from monocytes. MMP-9 has been associated with the process of phagocytocis. These facts suggest that the TSP-1 receptor may play an important role in the host immune reaction and angiogenesis.

These data do not only suggest the importance of TSP-1 in tumor invasion or angiogenesis, but also suggest dynamic interactions between cancer and stroma mediated by TSP-1. Our study further underscores the need to further investigate the function of TSP-1 in cancer progression, tissue remodeling and inflammation.

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