Original Paper

Prognostic Significance of CD83 Positive Tumor–Infiltrating Dendritic Cells and Expression of TGF–beta 1 in Human Gastric Cancer

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ABSTRACT

Background/Aims: In this study we analyzed the significance of CD1a and CD83 positive tumor infiltrating dendritic cells (TIDCs) and the expression of TGF-ß1 in gastric cancer tissue, and their relationship with disease progression and prognosis of patients.
Methodology: The immunohistochemical expression of CD1a, CD83 and TGF-ß1, was evaluated in 55 patients with gastric cancer and followed-up for five years.
Results: We found tumor infiltration with CD1a and CD83 positive DCs in all 55 cases and cytoplasmic TGF-ß1 immunoreactivity in tumor cells in 76.4% of cases. TGF-ß1 expression correlated to low CD83 positive DCs in 100% of the samples (χ^2=7.66; p=0.022). Low CD83 positive DCs in tumor border (χ^2=15.38; p<0.001) was also observed in 100% of tumors with TGF-ß1 expression. The number of CD1a and CD83 positive TIDCs in the tumor border was inversely correlated with positive lymph node metastases (χ^2=6.64; p=0.036 and χ^2=6.44; p<0.04, respectively). Patients with a low number of tumor infiltrating CD83 positive DCs had shorter survival rates (p=0.022) and patients with TGF-ß1 expression had a worse prognosis after surgical therapy (p=0.017).
Conclusions: Our results suggest that tumor infiltration with DCs may be of great importance in initiating the primary anti-tumor immune response. In patients with resectable gastric cancer, the grade of TIDCs and TGF-ß1 expression could be a useful predictor of prognosis.

KEY WORDS: CD1a; CD83; Dendritic cell; TGF–1; Immunohistochemistry; Prognosis; Gastric cancer.

INTRODUCTION

Gastric cancer is still the most prevalent neoplasia in many countries. Many specialists including oncologists, surgeries and pathology investigators have great interests of molecular markers who can predict the relapses after gastrectomy and relations between these molecules and clinico-morphological data. Despite the fact that the majority of patients at an early stage of gastric cancer can be cured by surgery, more than one-half of the patients at an advanced stage of the disease die of cancer recurrence, even when they undergo curative gastrectomy (1). Lymph node metastases are the predominant routes of recurrence for gastric cancer and, when present, significantly worsen the prognosis. The survival rate in patients with gastric carcinomas is rather low, even in developed countries. Therefore, besides the clinicopathological factors known to be prognostic markers, new independent parameters such as immunological variables are being investigated.

Dendritic cells (DCs) are the most potent antigen-presenting cells that play a major role in initiating the antitumor immune response. DCs are cells in the pathway of antigen capture and presentation to T cells. They possess the ability to efficiently uptake, process and present captured antigens to CD4+ T cells loaded on major histocompatibility complex (MHC) class I and II molecules together with co-stimulatory molecules. Activation and migration of DCs from the tumor site to local lymph nodes is believed to be essential for stimulating tumor-specific CD8+ cytotoxic T cells and non-specific effectors such as NK, thereby inducing the protective and therapeutic anti-tumor immunity against cancer cells. Given the pivotal role of DCs in the induction of anti-tumor immunity, the infiltration of human tumors with DCs and their subpopulations have been investigated using a variety of markers and methods. Principally, CD1a, CD83 and S-100 protein have been used for tissue section investigations (2), but more recently, CD86, CD208 and other markers have been utilized (3).

The clinical significance of tumor infiltrating dendritic cells (TIDCs) has been reported in colon (3), breast (2) and lung cancer (4). In general, the presence of DCs in tumors has been associated with better prognosis, reduced tumor recurrence and fewer

metastases (5-9); however, there has been little information on gastric cancer. Patients with a high level of TIDCs within gastric carcinoma had a lower positivity of lymph node metastases and lymphatic invasion than patients with a lower level of TIDCs (10). Also, it was shown that the 5-year survival rates of gastric cancer patients with many TIDCs were better than that of patients with fewer TIDCs (11).

DCs have been found in tumor lesions in cancer patients; however the tumor microenvironment can induce immune tolerance by inhibiting DC differentiation and maturation via secretion of immunosuppressive factors (12). Transforming growth factor-ß1 (TGF-ß1), secreted by a variety of cell types is a multifunctional cytokine with unique and potent properties in the maintenance of normal immunological homeostasis (13) and modulation of cell growth, apoptosis and differentiation of intestinal epithelial cells (14,15). TGF-ß1 is involved in the regulation of the activation, proliferation and activity of DCs in the tumor microenvironment (16). TGF-ß1 plays a complex role in carcinogenesis: in the early stage of cancer it acts as a tumor suppressor by inhibiting cellular proliferation or by promoting cellular differentiation and apoptosis (17). In the later stages of cancer, the role of TGF-ß1 switches to a growth stimulator which enhances tumor invasion and metastasis (18,19).

To date, few studies have addressed the maturation status of TIDCs associated with cancer tissue expression of TGF-ß1 and their impact on survival in patients with gastric cancer. We undertook this study with the aim of investigating the distribution and density of CD1a-, (immature) and CD83-positive (mature) TIDCs in relation to TGF-ß1 expression by tumor cells. In addition, we evaluated whether these factors had a relationship with some clinical and pathologic parameters of disease progression and patient survival after surgery.

TABLE 1 The Main Clinical and Histological Parameters of the Patients with Gastric Cancers (n=55)

Parameter	Number (%)
Clinical data	(n=55)
Gender	
Male	34 (61.8)
Female	11 (38.2)
Age (years)	
median	67
(range)	(22-83)
T stage	
T1-2	11 (20.0)
T3-4	44 (80.0)
N stage	
N0	15 (27.3)
N1-3	40 (72.7)
Metastases	
No	46 (83.6)
Yes	9 (16.4)
Clinical stage	
I	6 (10.9)
II	11 (20.0)
III	23 (41.8)
IV	15 (27.3)
Follow-up after surgery	
median (months)	62.76
(range)	(1.68-120.42)
Histological data	
Differentiation grade of tumor	
low	37 (67.3)
moderate	16 (29.1)
high	2 (3.6)
Hystological type	
intestinal	45 (81.8)
diffuse	10 (18.2)

METHODOLOGY

Case selection

Specimens were obtained from 55 patients who underwent curative resection of gastric cancer at the Department of Surgery, University Hospital, Medical Faculty, Trakia University, Stara Zagora, between 1999 and 2009. The patients comprised 34 males and 21 females, aged between 22-83 years (mean, 64.58 years). The surgical procedures carried out included either a radical subtotal or total gastrectomy with a systematic lymph node dissection (D1 or D2 lymphadenectomy. No patient received anti-cancer treatment prior to surgery. Most patients (n=45) had the intestinal histological type tumor and a few (n=10) had the diffuse type. Tumor staging was defined as 10.9% for the stage I, 20.0% for the stage II, 41.8% for the stage III and 27.3% for the stage IV. Tumor grading and staging was performed according to the TNM classification by UICC 2002 and Lauren histological classification (20,21). Tumor specimens were fixed in 10% buffered formalin and embedded in paraffin. Histological grading was performed on hematoxyllin and eosin-stained sections according to Kioshima *et al.* (22). The main clinical and histological data are given in **Table 1**. Informed consent was obtained from all patients.

Immunohistochemistry

Immunohistochemical staining was performed using avidin-biotin-peroxidase complex technique

TABLE 2 Density (cells/mm2) of CD1a Positive and CD83 Positive DCs in Gastric Cancer Tumor and Border Tissue

Cell types and location	CD1a	CD83
Tumor		
mean±SD	4.5135±9.6222	2.2165±3.3422
Median	2.1800	1.0900
(range)	0.14-68.70	0.14-21.37
Border		
mean±SD	6.0540±7.1341	2.7113±3.8545
median	3.6700	1.2200
(range)	0.01-32.24	0.14-19.18
Mann-Whitney U test	p=0.174	p=0.86

as described earlier (23). In brief, the paraffin blocks were prepared using tumor tissues from the periphery of tumor adjacent to the normal tissues and from the deepest point of the tumor. Paraffin sections, 5µm thick, were dewaxed in xylene at 56°C for 1h and rehydrated in ethanol. Antigen retrieval was done by boiling the sections in citrate buffer pH6 in a water bath for 20min. Later, they were washed in 0.1M phosphate buffered saline (PBS), pH7.4, incubated in 1.2% hydrogen peroxide in methanol for 30min, and rinsed in PBS for 15min. The sections were then blocked for 30min with normal mouse serum (DAKO). After incubating with the primary mouse/rabbit anti-human antibodies overnight, they were washed in PBS and incubated with a secondary anti-mouse biotinylated antibody (DAKO ready-to-use LSAB®2 System, HRP K0675) for 4h, and subsequently with the streptavidin-HRP complex (DAKO ready-to-use LSAB®2 System, HRP K0675) for 4h, rinsed in PBS and then in 0.05M Tris-HCl buffer, pH7.5 for 10min. The reaction was made visible by using a mixture of 3mg 3,3'-diaminobenzidine (DAB) (Sigma, St. Louis MO, USA) in 15mL 0.05M Tris-HCL buffer pH7.5 and 36µL 1% hydrogene peroxide for 10-20min, and rinsed in PBS. The sections were counterstained with Mayer's hematoxylin. Sections incubated with non-immune sera instead of the primary antibodies were used as negative controls. The antibodies used were monoclonal mouse anti-human CD1a antibody (M3571, DAKO A/S, Denmark), monoclonal mouse anti-human CD83 antibody (N1573, Serotec, Oxford, UK) and rabbit anti-human TGF-ß1 antibody (sc-146) all of them in a dilution 1:50. The detection system immunostaining kit used was DAKO LSAB®2 System, HRP (K0675, DAKO) and DAKO®DAB chromogen tablets (S3000, DAKO).

Cell counting

CD1a- and CD83-positive cells were counted in the tumor stroma and at the tumor border, on five fields of vision in the areas with most intensive cell recruitment (hot spots) at a magnification (x320, 0.74mm^2 area). The number of the positive cells was calculated at 1mm^2 area.

Statistical analysis

Statistical analyses were performed using the statistical software SPSS for Windows Version 16 (SPSS, Inc., Chicago, IL, USA). The descriptive statistical tests, including the mean, standard deviation, and median, were calculated according to the standard methods. The non-parametric Mann-Whitney U test and Wilcoxon signed rank test were used to evaluate the significance of the differences of the mean ranks. The frequency of distribution in 2x2 contingency tables was analyzed by χ^2-test. Survival was calculated from the date of operation to the date of death or of the last follow-up. Cumulative survival curves were drawn by the Kaplan-Meier method ant the difference between curves was analyzed by the logrank test. For all statistical analysis, $p<0.05$ was considered to be statistically significant.

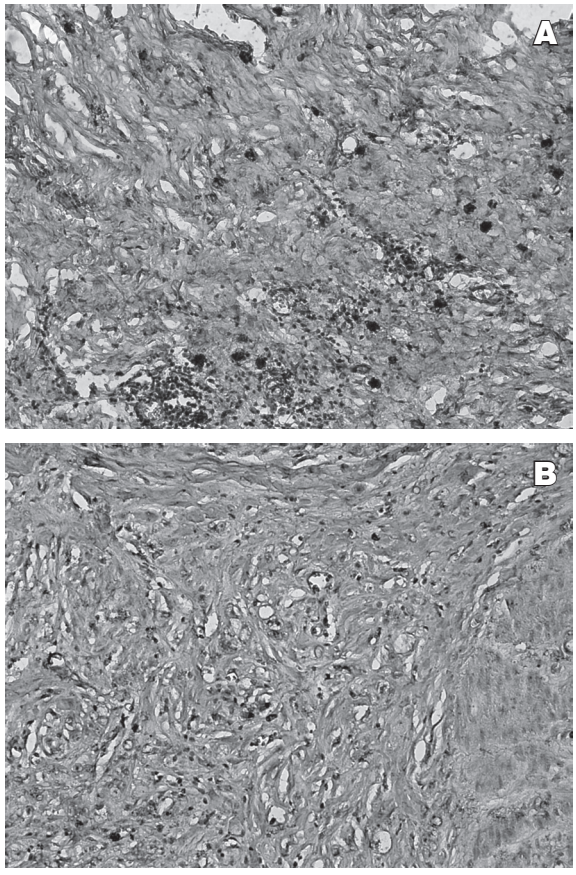

FIGURE 1 Infiltration of gastric cancer stroma with: **A**. CD1a–positive DCs; **B**. CD83–positive DCs. (Magnification, x200).

RESULTS

Infiltration of gastric cancer tissues by TIDCs expressing CD1a or CD83

We found an infiltration of tumor tissue by CD1a- and CD83-positive cells in all studied specimens (**Figure 1A** and **B**). **Table 2** shows the density of cells, immunostained for CD1a and CD83 in tumor stroma and the tumor border. CD1a-positive TIDCs could be found in all 55 cases, with a median number of 2.18 (range, 0.14-68.7) for the tumor stroma, and median number of 3.67 (range, 0.01-32.24) for the tumor border. DCs labeled with CD83, were located as single cells in the stroma and border. We identified mature CD83-positive TIDCs in cancer stroma sections, with median number of 1.09 (range, 0.14-21.37). We also found CD83-positive TIDCs located in the cancer border with a median number of 1.22 (range 0.14-19.18).

When comparing the density of cells expressing CD1a and CD83, CD1a-positive cells were more than CD83-positive cells in tumor stroma ($p=0.004$) and in tumor border ($p<0.001$, Wilcoxon Signed rank test). Statistically significant differences were not observed between CD1a- and CD83-positive DCs in tumor stroma and tumor border ($p=0.174$ and $p=0.86$, respectively, Mann-Whitney U test).

TABLE 3 Correlation of TIDCs in Border and Clinicopathological Factors

	CD1a+				CD83+			
	Low	Moderate	High	*p*-value	Low	Moderate	High	*p*-value
Age	67.6±8.97	62.7±14.2	64.4±11.2	NS	65.1±10.9	62.8±13.6	68.0±9.4	NS
Tumor size								
T1-2	3	6	2		2	6	3	
T3-4	13	20	11	NS	13	21	10	NS
Nodal involvmen								
No	1	11	3		2	6	7	
Yes	15	15	10	0.036	13	21	6	0.04
Metastases								
No	12	22	12		11	23	12	
Yes	4	4	1	NS	4	4	1	NS
Clinical stage								
I-II	3	10	3		2	8	6	
III-IV	13	16	10	NS	13	19	7	NS
Differentiation								
Low	12	16	9		8	21	8	
Moderate/High	4	10	4	NS	7	6	5	NS
Histologic type								
Intestinal	16	18	11		14	22	9	
Diffuse	0	8	2	0.041	1	5	4	NS

TABLE 4 Correlation between Expression of TGF–1 and Number of Tumor–Infiltrating Dendritic Cells (Fisher's exact test, two tailed)

(A) Correlation between expression of TGF-β1 and dendritic cells in tumor stroma

	CD1a+			CD83+		
	Low	Moderate	High	Low	Moderate	High
TGF-ß1 in tumor cytoplasm						
no	1	7	5	0	7	6
yes	17	12	8	15	19	8
		p=0.072			*p=0.022*	

(B) Correlation between expression of TGF-β1 and dendritic cells in tumor border

	CD1a+			CD83+		
	Low	Moderate	High	Low	Moderate	High
TGF-ß1 in tumor cytoplasm						
no	1	8	4	0	5	8
yes	15	18	9	15	22	5
		p=0.151			*p<0.001*	

Correlation between TIDCs and clinicopathological factors

In the next set of analyses, we examined the correlation between the infiltration of tumors by DCs and clinicopathologic factors. Patients were divided into groups with low, moderate, and high infiltration based on the 25th and 75th percentiles of the average numbers of CD1a+ and CD83-positive cells in tumor tissue. There were no associations between the number of TIDCs in tumor stroma and all studied clinicopathological parameters (data not down). Also, there were no significant correlations between the presence of CD1a- and CD83-positive TIDCs in tumor border and patient age, tumor size, metastases, clinical stage and differentiation (**Table 3**). However, the number of CD1a- and CD83 positive TIDCs in tumor border was inversely correlated with positive lymph node metastases (χ^2=6,64; p=0.036 and χ^2=6,44; p=0.04, respectively). Finally, the high infiltration of CD1a positive DCs in the tumor border was found in all tumor specimens with diffuse histologic type and in 64% of tumors with intestinal type of cancer (χ^2=6,39; p=0.04).

Relationship between expression of TGF-β1 and TIDCs in tumor stroma and in the tumor border

TGF-ß1 expression was observed in tumor cell cytoplasm in 42 (76.4%) out of 55 gastric cancers (**Figure 2**). TGF-ß1 expression was correlated with a low number of CD83 positive DCs in tumor stroma in 100% of the samples (χ^2=7,66; p=0.022) (**Table 4A**). In 100% of tumors with TGF-ß1, a low number of CD83-positive DCs in tumor border (χ^2=15,38;

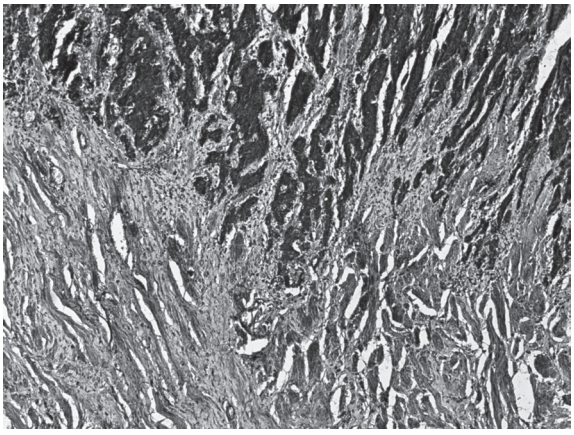

FIGURE 2 Intense TGF–ß1 expresion in the cytoplasm of tumor glands in gastric cancer (Magnification, x100).

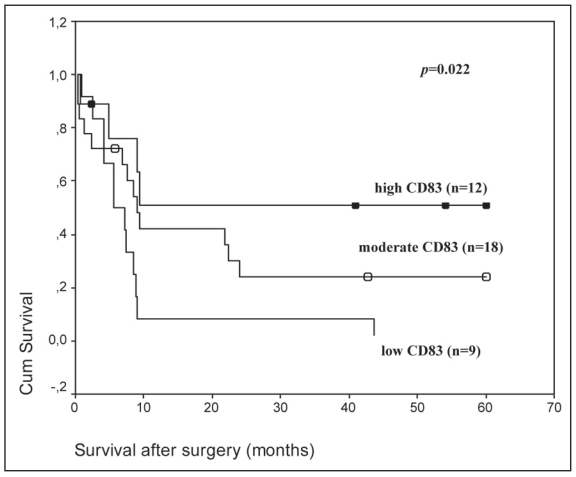

FIGURE 3 Kaplan–Meier survivil curve for CD83 expression in tumor border. Mean survival of 8.97 months of patients with low expression of CD83 (low CD83, n=12) *vs.* 21.34 months of patients with moder–ate expression of CD83 (moderate CD83, n=18) *vs.* 33.57 months of patients with high expression of CD83 (high CD83, n=9), *p*=0,022; logrank test.

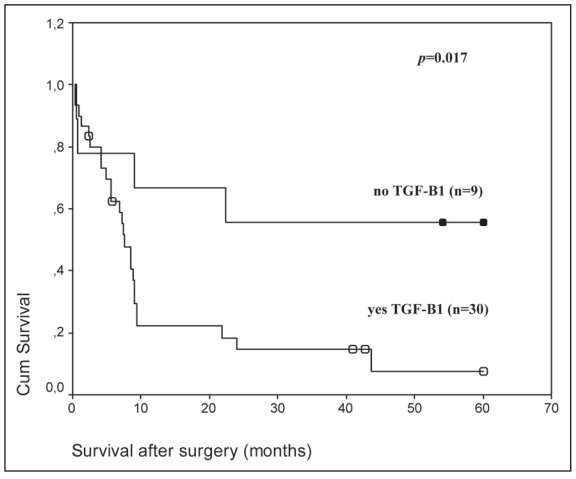

FIGURE 4 Kaplan–Meier survival curve for TGF–ß1 expression in tumor cell cytoplasm. Mean survival of 37.03 months of patients without ex–pression of TGF–ß1 (no TGF–ß1, n=9) *vs.* 13.8 months of patients with expression of TGF–ß1 (yes TGF–ß1, n=30), *p*=0.017; logrank test.

p<0.001) (**Table 4B**). TGF-ß1 expression in tumor cytoplasm tended to correlate with low infiltration with CD1a-positive DCs in tumor stroma (χ^2=5,25; *p*=0.072). There was no correlation between the numbers of CD1a positive DCs in thr tumor border with TGF-ß1 expression in tumor cytoplasm.

Prognostic significance of TIDCs and TGF-β1

In the final set of experiments, we examined whether the infiltration with DCs and TGF-ß1 expression were correlated with prognosis. Clinical data obtained from the archival records were available for 39 patients. All of them were followed-up until the 1st of April 2010. At the end of the follow-up, 10 (25,64%) of the patients survived, with median survival period of 57.1 months (range, 2.4-60.08 months). Twenty-nine patients died with median survival period of 7.2 months (range, 0.3-43.6 months). Patients with a low number of tumor infiltrating CD83-positive DCs had shorter survival rates as shown in **Figure 3** (*p*=0.022; logrank test).

For analyzing the impact of TGF-ß1 on the survival after surgery, we determined survival rates for the patients with and without TGF-ß1 expressing tumors and tumor stage. The patients with TGF-ß1 expression had a worse prognosis after surgical therapy compared to those without TGF-ß1 expression (*p*=0.017, logrank test) (**Figure 4**).

DISCUSSION

Tumor invasion in the gastric wall, lymph node metastases and distant metastases are the most important prognostic factors in the progression of gastric cancer. The more deeply the stomach wall is invaded or the more widely the lymph node and distant metastasis are involved, the poorer the prognosis is (24). Additionally, many factors, like TIDCs and expression of cytokines and growth factors such as TGF-ß1 from tumor cells have an influence upon gastric tumorigenesis (25).

In the present study, we found a relation between the tumor infiltration with DCs, TGF-ß1 expression by tumor cells and the outcome in 55 patients with carcinomas of the stomach. CD1a-positive and CD83-positive tumor-infiltrating DCs were detected in all gastric cancer samples with variable density and prevalence of immature CD1a-positive DCs. The number of both mature and immature DCs was inversely related to lymph node involvement. Our study demonstrated a correlation between TGF-ß1 expression by cancer cells and the low infiltration with mature CD83-positive DCs in tumor tissue. In addition, TGF-ß1 expression as well as the low number of CD83-positive DCs influenced the overall survival in studied patients.

The infiltration of tumors by DCs was related to the immune system activation and was found to be associated with a favorable prognosis for patients (26,27). In 1992, Tsujitani *et al.* first related the grade of S-100-positive DCs infiltration to lymph

node involvement and prognosis in patient with gastric cancer (7). S-100 protein is a well known histological marker for DCs, however, it is not an exclusive DCs marker and stains for several types of DCs (28). It is now apparent that TIDCs are heterogeneous in regard to maturation, differentiation and state of activation (29), and surface marker expression may reflect those differences among DC subpopulations. The availability of novel markers specific to DC subsets has made it possible to evaluate the maturation status of TIDCs. Of those, CD1a is an antigen-presenting molecule classified as a marker of immature DCs (30). Expression of CD83 has been found on mature DCs (2) and more recently CD208 has been introduced as a marker of mature DC (31). Studies have shown that increased infiltration with CD1a- and CD83-positive TIDCs is associated with a better prognosis in patients with gastric cancer. Conversely, a recent study evaluated the clinical impact of CD208-positive cell infiltration in gastric cancer and reported that intratumoral CD208-positive DCs had an inverse correlation with patients' postoperative outcome (32). One speculative explanation of this finding is that the secretion of immunosuppressive cytokines hinders mature DCs on their way to migrate to secondary lymphoid tissue (3).

Our results confirm that, in gastric cancer tissues, the number of TIDCs expressing CD1a and CD83 is associated with lymph node involvement. The patients with low CD1a- and CD83-positive DC infiltration in tumor border had more distant lymph node metastasis than those with high-grade infiltration with DCs. This suggests that infiltration by DCs may prevent widespread nodal involvement in advanced carcinoma (33). Furthermore, the patients with moderate and high number of CD83-positive TIDCs in the tumor border had improved survival rates. Tsukayama *et al.* also reported that the 5-year survival rate was significantly higher in patients with a high density of mature DCs in gastric cancer tissue than those with low density CD83-positive DCs (34). The CD1a-positive subpopulation of DCs was not associated with outcome in our study. This is not surprising because the mere presence of DCs in the tumor microenvironment is not necessarily related to the activation of antitumor immune response, due to the presence of tumor-derived cytokines and growths factors that may suppress DC maturation and activation into powerful antigen-presenting cells.

Recent studies reported that gastric carcinoma cells overexpress TGF-ß1 (35,36,37). The current evidence suggests that TGF-ß1 may have a complex role in gastrointestinal carcinogenesis (38). Experimental models suggest that TGF-ß1 exerts a biphasic influence on carcinogenesis by protecting against the early formation of benign epithelial growths and by promoting malignant cell invasion and metastasis during tumor progression (25). In addition, TGF-ß1 is an immunosuppressive cytokine that may facilitate the development of cancer by supporting tumor escape from the immune surveillance (39). In this study, we found immunohistochemical staining for TGF-ß1 in the tumor cell cytoplasm of the majority of gastric cancers, which is consistent with previous reports (36). TGF-ß1 produced by tumors down regulates the expression of co-stimulatory molecules on DCs and thus prevents the function of antigen presentation (40,41). In the present study, the number of CD83-possitive TIDCs was inversely correlated with tissue expression of TGF-ß1, suggesting that this immunosuppressive cytokine may play an important role in inhibition of the TIDC maturation sequence in the tumor microenvironment.

Most gastric carcinomas are refractory to the suppressive effect of TGF-ß1 with elevated TGF-ß1 expression, suggesting an important role of TGF-ß1 in gastric cancer tumorogenesis (42,43). It has been shown that elevated levels of human TGF-ß1 protein in cancers correlate with an increased metastatic potential (42,43). A study found that the expression of TGF-ß1 was strongly correlated with the presence of tumor-budding, vascular invasion at the front of invasion and presence of lymph node and distant metastases in patients with colorectal cancer (44). There is a significant correlation between tumor expression of TGF-ß1 and a shorter postoperative survival in gastric cancer patients (45). We also found that the expression of TGF-ß1 in investigated tumors was strongly correlated with the post-operative survival. These results suggest that TGF-ß1 may be closely related to the aggressiveness of gastric cancer.

In conclusion, we demonstrated that the presence of increased numbers of TIDCs expressing CD1a and CD83 was inversely correlated to lymph node metastasis. We have also found that TGF-ß1 expression in the tumor cytoplasm was related to low infiltration with both types of studied antigen-presenting cells in tumor tissues and shorter survival time for the patients. In patients with resectable gastric cancer, the grade of infiltrating dendritic cells and TGF-ß1 expression could be a useful predictor of prognosis.

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