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Expression of costimulatory molecules, B7-1 and B7-2 on human gastric carcinoma

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Abstract Costimulation of T cells via B7-1 and B7-2 molecules on a tumor has been shown to be important for eliciting cell-mediated antitumor immunity. We studied the surface expression of B7-1 and B7-2 in 24 cases of gastric carcinoma from the primary locus, 20 cases of metastatic carcinoma from malignant ascites, 20 cases of benign gastric mucosa and 7 gastric carcinoma cell lines by two-color flow cytometry with mAb CD80 and CD86. The B7-1 and B7-2 molecules were expressed by 6 cell lines, and 1 cell line showed the predominant expression of B7-2 but not B7-1. Almost all patients with primary gastric carcinoma and benign gastric mucosa showed high levels of expression of the B7-1 and B7-2, revealing approximately 40%–60% positive cells. However, the percentage of B7-1-positive cells of poorly differentiated primary carcinomas was significantly lower than that of well-differentiated carcinoma and normal mucosa ($P < 0.01$). Furthermore, all of the metastatic carcinoma cells revealed consistently very low or undetectable levels of expression of the B7-1 molecule, only 8% (mean) of cells being positive, despite showing higher levels of B7-2 expression. Thus, it seems likely that decreased or deleted expression of B7-1 correlates with the grade of tumor differentiation, tumor progression and metastasis. These results suggest that the B7-1 molecule on the gastric carcinoma bearing $CD80^+CD86^+$ is abrogated during tumor invasion and/or metastasis, and the tumor finally acquires the $CD80^-CD86^+$ phenotype. Consequently, inadequate B7-1 costimulation may contribute to the escape of tumors from destruction by the host's immune system.

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Introduction

The induction of efficient T-cell-mediated immunity against tumor cells is believed to involve interaction of the T cell receptor with antigen peptides presented on major histocompatibility complex (MHC) antigens and the receipt of second or costimulatory signals, due to interaction of ligands, such as B7-1 and B7-2, with the CD28 or cytotoxic T lymphocyte antigen-4 (CTLA-4) receptors (Muller et al. 1989). These molecules can be expressed by multiple cell types, including B cells, T cells, macrophages, and dendritic cells, all of which are therefore candidate populations for delivering costimulatory signals mediated by these molecules (Azuma et al. 1993). It has been reported that most tumor tissues, particularly those of nonhematopoietic origin, do not express costimulatory molecules and this would render T cells anergic or unresponsive for the specific antigens (Chen et al. 1993; Schwarz 1990). To date, therefore, many experiments in vitro in animals (Basker et al. 1993; Townsend and Allison 1993; Chen et al. 1994) and human (Yang et al. 1997) have explored whether or not tumor immunogenicity is increased and restored by B7 transfection, and these gene-modified tumors are able to elicit specific antitumor responses. As a prerequisite to such gene-transfer studies, we wished to determine the in vivo expression patterns of B7-1 and B7-2 in human primary and metastatic adenocarcinoma of the stomach, and in benign gastric mucosal cells, because little is indeed known about the frequency of expression of these costimulatory molecules on human solid carcinoma tissues. We prepared single cells, which could be mechanically isolated from gastric tissues, as described previously (Koyama et al. 1992) and evaluated them by two-color flow cytometry. The results show that the B7-1 and B7-2 were constitutively expressed on primary gastric carcinoma cells, representing approximately

40%–60% of the mean number of positive cells, and on most gastric carcinoma cell lines examined. However, the expression of B7-1 was dramatically reduced in poorly differentiated primary carcinomas and in metastatic carcinomas from malignant ascites. The functional implications of the existence, and abrogation or loss of the costimulatory molecules on gastric carcinoma are discussed.

Materials and methods

Specimens

Endoscopical biopsies of cancerous and noncancerous tissues were taken from 24 patients (age range, 26–80 years; average, 63 years) with untreated advanced gastric carcinoma, and from 20 patients (age range, 25–80 years; average, 60 years) with benign gastric mucosa. These specimens were gently pressed and ground in a coarse glass grinder with chilled RPMI-1640 medium (Gibco, Grand Island, N.Y., USA) supplemented with 10% fetal calf serum (M.A. Bioproducts, Walkerville, Md, USA) as described previously (Koyama et al. 1992). The carcinoma or normal gastric mucosal cells were counted in a hemocytometer by the trypan-blue-dye-exclusion method. Metastatic free tumor cells were collected by paracentesis from 20 patients (age range, 46–73 years; average 61 years) with advanced gastric carcinoma with malignant ascites. The carcinoma cells were judged by morphological examination of Papanicolaou's staining and/or by Wright-Giemsa-stained smears. Histological characteristics of the tumor samples of the primary lesion made on standard hematoxylin and eosin sections were evaluated according to the guidelines of the Japanese Research Society for Gastric Cancer (1993). Of the 24 patients having a primary tumor that were examined in this study, 12 had well-differentiated papillary or tubular adenocarcinoma and 12 poorly differentiated adenocarcinoma or signet-ring cell carcinoma. Benign gastric mucosal specimens, which included normal mucosa as well as specimens showing characteristics indicating that the patients had superficial gastritis, atrophic gastritis or intestinal metaplasia, were also investigated for the expression of B7-1 and B7-2 for the control.

Gastric carcinoma cell lines SC-1, SC-2, SC-3 and GCIY used for this study have been established and cultured continuously in our laboratory (Koyama et al. 1987; Koyama 1994; Nozue et al. 1995). The SC-1 cell line was from a poorly differentiated adenocarcinoma and the other three carcinoma cell lines were derived from a signet-ring cell carcinoma. Other gastric carcinoma cell lines, MKN-28, MKN-45 and MKN-74, were obtained from the Human Science Research Resources Bank (Osaka). The MKN-74 and MKN-28 cell lines were both derived from moderately differentiated tubular adenocarcinoma, and the MKN45 cell line was derived from poorly differentiated adenocarcinoma (Motoyama et al. 1986). These cell lines were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum and 100 μg/ml kanamycin in 5% CO_2 in air at 37°C.

Monoclonal antibodies (mAb) and two-color flow-cytometric analysis

Fluorescein-isothiocyanate(FITC)-conjugated anti-B7-1 (CD80) and purified anti-B7-2 (CD86) were provided by the Ancell Corporation (Bayport, Minn., USA). The normal gastric mucosal or carcinoma cells were incubated with heat-treated human aggregate IgG (GII 2388, Cohn fraction II and III; Sigma, St. Louis, Mo., USA) for 15 min to prevent antibody binding to FcR and modulation of cell-surface molecules. The cells were then stained with a saturating concentration of primary mAb, CD86, followed by secondary antibody [phycoerythrin-conjugated goat anti-(mouse IgG) antibody; Biomeds, Foster City, Calif., USA] for 30 min on ice and washed twice. The cells were further treated with mouse IgG (Inter-cell Technologies Inc., Hopewell, N.J., USA) and stained with a saturating concentration of FITC-conjugated CD80 for 30 min on ice, as described previously (Koyama et al. 1992; Koyama and Fukao 1994; Koyama 1997). As a negative control, an aliquot of the cells from each cell line and patient was stained with an irrelevant mAb of the same phenotype and secondary antibody. Flow cytometry was performed on a FACScan analyzer (Becton Dickinson, Mountain View, Calif., U.S.A.). A gated area was created around the live tumor or normal gastric mucosal cells populations using forward-angle versus light-scattering parameters, in order to eliminate the mixed lymphoid cells, as described previously (Koyama et al. 1992). The data were collected on 10, 000 cells per sample. The percentage of fluorescence-positive events within this area was determined after gates were set to contain 99% or more of the negative-control samples. When two-color analysis was performed, appropriate compensation was initially determined by using stained samples. The percentage of cells positive for B7-1 or B7-2 in carcinoma cell lines and in normal mucosal and carcinoma cells from each patient was shown by the sum total of $CD80^+CD86^-$ and $CD80^+CD86^+$ cells, or by $CD80^+CD86^+$ and $CD80^-CD80^+$ cells respectively. The statistical significance of changes in mean levels of B7-1 and B7-2 expression was determined by Student's *t*-test and expressed by *P* values.

Results

Two-color flow-cytometric analysis of the expression of B7-1 and B7-2 with mAb CD80 and CD86 in gastric cancer cell lines and gastric carcinoma tissues is shown in Figs. 1 and 2. Significant levels of B7-1 and B7-2 antigens were constitutively expressed by cancer cell lines SC-1, SC-3, GCIY, MKN-28, MKN-45 and MKN-74 (Fig. 1a, c, d–f, g). SC-2 cells (Fig. 1b), however, failed to express detectable amounts of B7-1, although B7-2 was predominantly detected on their surface. Results in Fig. 2 show the representative FACS dot-plots of adenocarcinoma cells prepared from actual solid tumor tissue and from malignant peritoneal effusion. B7-1 and B7-2 were commonly coexpressed by normal mucosa and primary carcinoma of the stomach, while B7-1 alone was deleted in the metastatic carcinoma from malignant ascites.

The proportions of $CD80^+CD86^-$, $CD80^+CD86^+$ and $CD80^-CD86^+$ cells in the primary and metastatic lesions of patients with advanced gastric carcinoma are summarized in Table 1. The $CD80^+CD86^-$ tumor cells appeared to be absent from each group. Although the $CD80^+CD86^+$ cells were certainly noted in normal mucosa and primary gastric carcinoma, the percentage of doubly positive metastatic carcinoma cells from malignant ascites was significantly lower than the value for cells from normal gastric mucosa and primary carcinoma tissues from the stomach ($P < 0.001$). The metastatic carcinoma cells characteristically also comprised a significantly higher percentage of $CD80^-CD86^+$ cells than did normal mucosa and primary carcinomas ($P < 0.001$). In normal gastric mucosa and primary carcinomas, a lower percentage of $CD80^-CD86^+$ expression was found. When primary carcinomas of the stomach were classified by histological type, the percentage of $CD80^+CD86^+$ cells in poorly differentiated

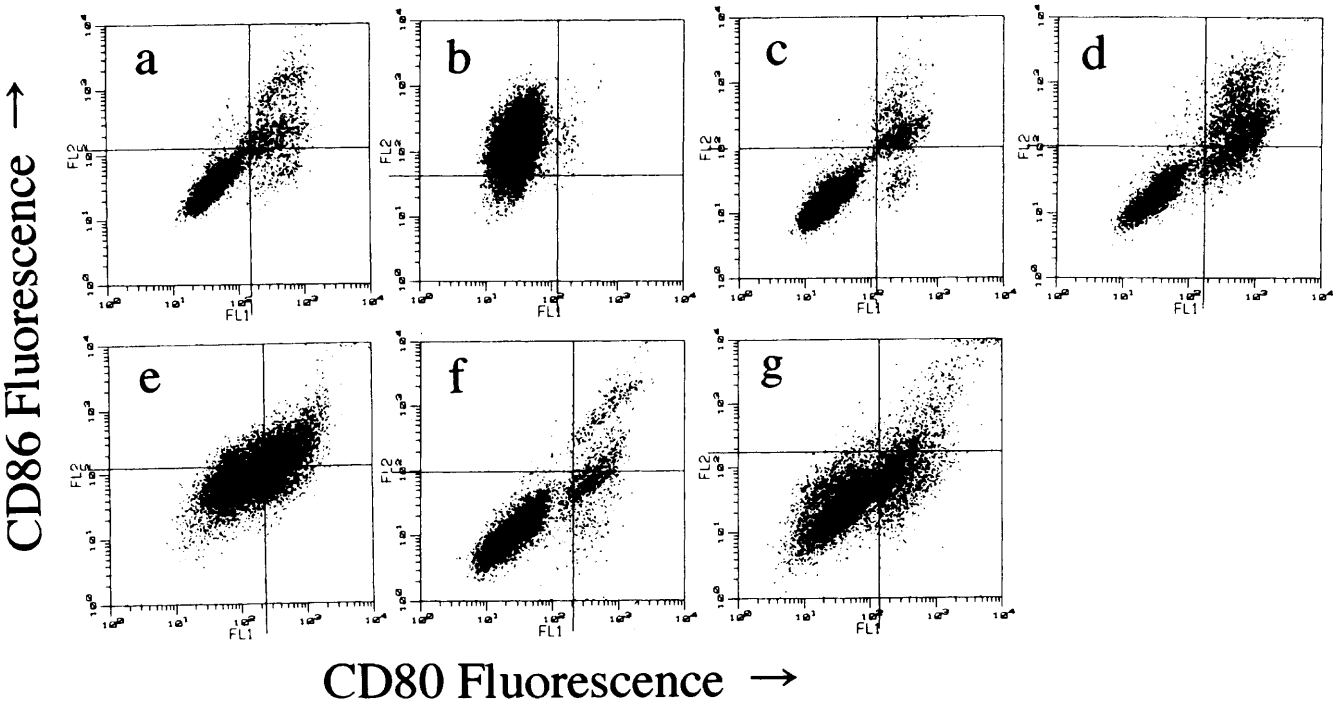

Fig. 1a–g Two-color fluorescence-activated cell sorting (FACS) profiles of cell-surface B7-1 and B7-2 expression on gastric carcinoma cell lines. The horizontal and vertical cursors distinguish positive cells, defined as those with a fluorescence intensity. The background for the negative control of each cell line was slightly different. Integration of four subsets in each tumor cell. **a** SC-1 cells: $CD80^+CD86^-$ 7.2%, $CD80^+CD86^+$ 20.4%, $CD80^-CD86^+$ 2.9%. **b** SC-2 cells: $CD80^+CD86^-$ 0.1%, $CD80^+CD86^+$ 3.1%, $CD80^-CD86^+$ 85.9%. **c** SC-3 cells: $CD80^+CD86^-$ 5.0%, $CD80^+CD86^+$ 15.8%, $CD80^-CD86^+$ 1.4%. **d** GCIY cells: $CD80^+CD86^-$ 12.1%, $CD80^+CD86^+$ 33.5%, $CD80^-CD86^+$ 3.3%. **e** MKN-28 cells: $CD80^+CD86^-$ 15.1%, $CD80^+CD86^+$ 29.2%, $CD80^-CD86^+$ 12.2%. **f** MKN-45 cells: $CD80^+CD86^-$ 10.4%, $CD80^+CD86^+$ 12.2%, $CD80^-CD86^+$ 1.3%. **g** MKN-74 cells: $CD80^+CD86^-$ 24.8%, $CD80^+CD86^+$ 15.8%, $CD80^-CD86^+$ 2.4%

adenocarcinoma was significantly lower than that of well-differentiated adenocarcinoma and normal gastric mucosa ($P < 0.05$ and $P < 0.01$ respectively).

Figures 3 and 4 summarize B7-1 and B7-2 expression on the cells in three experimental groups. Immunopositivity for CD80 was noted in almost all of 20 normal control subjects and in 24 cases of primary advanced carcinoma, with the percentage of positive cells ranging from 14.1% to 89.5% (mean ± SE, 43.8 ± 3.2%) and from 12.1% to 75.1% (37.3 ± 3.5%) respectively. While, 20 cases of metastatic tumor cells from the peritoneal effusions revealed very low CD80 immunoreac-

Fig. 2a–c Two-color FACS profiles of cell-surface B7-1 and B7-2 expression on cells of primary and metastatic carcinoma of the stomach. Integration for subsets in each cell. **a** Benign gastric mucosa: $CD80^+CD86^-$ 1.8%, $CD80^+CD86^+$ 37.6%, $CD80^-CD86^+$ 18.0%. **b** Primary carcinoma (well-differentiated): $CD80^+CD86^-$ 1.8%, $CD80^+CD86^+$ 72.0%, $CD80^-CD86^+$ 10.8%. **c** Metastatic carcinoma: $CD80^+CD86^-$ 0.1%, $CD80^+CD86^+$ 3.4%, $CD80^-CD86^+$ 73.7%

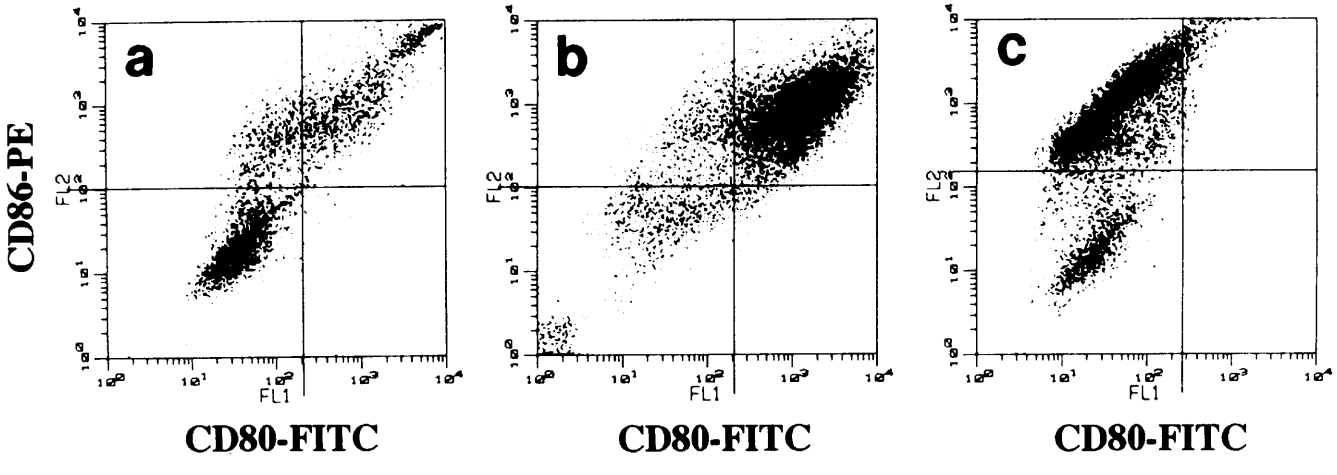

Table 1 Two-color flow-cytometric analysis of B7-1 and B7-2 on gastric carcinoma cells. Each value represents the mean percentage of positive cells ± SE

Cells from	Patient no.	$CD80^+CD86^-$ (%)	$CD80^+CD86^+$ (%)	$CD80^-CD86^+$ (%)
1. Normal mucosa	20	1.9 ± 0.4	41.9 ± 3.1	14.4 ± 2.0
2. Carcinoma (primary locus)	24	3.3 ± 0.8	34.0 ± 1.2	21.3 ± 2.2
Well-differentiated	12	4.8 ± 1.4	39.3 ± 2.5	18.6 ± 1.8
Poorly differentiated	12	1.7 ± 0.5	28.7 ± 2.4	24.0 ± 2.2
3. Carcinoma (malignant ascites)	20	0.5 ± 0.1	7.8 ± 1.5	64.1 ± 5.2

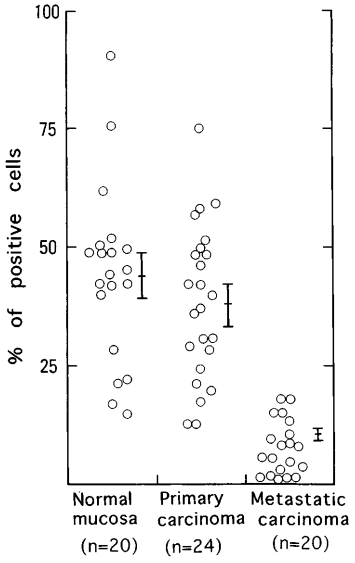

Fig. 3 Flow-cytometric analysis for expression of B7-1 on gastric carcinoma. *Bar* average (mean ± SE) of percentage positive cells

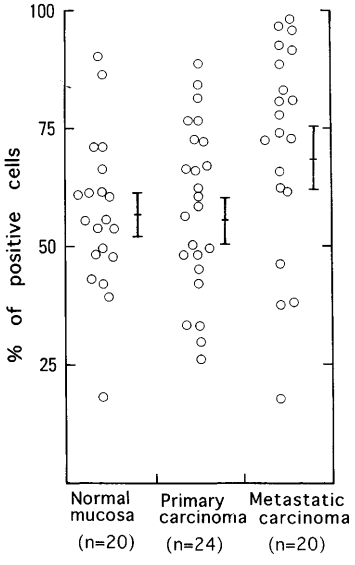

Fig. 4 Flow-cytometric analysis for expression of B7-2 on gastric carcinoma. *Bar* average (mean ± SE) of percentage positive cells

tivity, with the percentage of positive cells ranging from 1.5% to 20.9% (8.3 ± 1.4%), high levels of CD86 immunoreactivity were seen in almost all patients in the three experimental groups, although there was a great deal of variability case-to-case in each group. Briefly, the percentage of CD86 reactivity of the cells ranged from 18.2% to 92.1% (56.3 ± 3.7%) in normal controls, from 25.7% to 88.3% (55.3 ± 3.5%) in primary lesions and from 17.8% to 96.2% (71.7 ± 5.2%) in metastatic lesions. When the primary lesion of the gastric carcinomas was classified by histological type, the expression of B7-1 on poorly differentiated adenocarcinoma was found to be significantly lower than that on well-differentiated adenocarcinoma 30.4 ± 2.3% versus 42.8 ± 2.5%, $P < 0.01$). However, there was no difference in the incidence of B7-2 expression between poorly differentiated adenocarcinomas and well-differentiated adenocarcinomas in the primary gastric carcinoma (52.9 ± 3.3% versus 57.4 ± 3.9%).

Discussion

We have clearly shown that B7-1 and B7-2 can be constitutively detected in normal gastric mucosa, primary gastric carcinomas and most cancer cell lines of the stomach, by using tow-color FACS analysis. However, poorly differentiated adenocarcinoma at the primary locus showed decreased B7-1 expression and, in the majority of the metastatic carcinoma cells from malignant ascites, the surface expression of B7-1 was very low or at undetectable levels, although high levels of B7-2 expression were observed.

Expression of B7-1 and B7-2 on human carcinoma tissues was studied by Denfeld et al. (1995) in primary malignant melanoma, metastatic malignant melanoma from subcutaneous, regional lymph nodes or a distant organ, and benign melanocytic nevi by immunohistochemistry [the avidin-biotin-peroxidase (ABC) technique] and by reverse transcription/polymerase chain reaction (RT-PCR). Although they could not detect any B7-1 and B7-2 on primary and metastatic melanoma cells or on nevus cells by the ABC technique, mRNA for B7-1 or B7-2 were essentially detected in the primary and metastatic tumor tissues by RT-PCR. However, they finally concluded that the costimulatory molecules, B7-1 and B7-2 were not expressed by the malignant melanoma, since the positive RT-PCR findings are due to contamination of $CD80^+$ and/or $CD86^+$ antigen-presenting cells (APC) and lymphocytes infiltrating into the tumor tissues. Hersey et al. (1994) first analyzed B7

expression in human malignant melanoma cell lines that were devoid of APC and tumor-infiltrating lymphocytes (TIL) by RT-PCR and flow-cytometric analysis. Their results revealed that mRNA for B7 was present in approximately 40%–50% of melanoma cell lines, but that B7 expression at the protein level in the cell lines was low or undetectable by single-color flow cytometry. Their immunohistochemical studies on melanoma cells from tissue sections prepared from primary and metastatic lesions failed to show B7 expression, even though B7 could be detected on adjacent lymphoid cells. On the basis of these reports, RT-PCR analysis may not be appropriate for detecting B7-1 and B7-2 at the mRNA level on tumor tissues, perhaps because the results of PCR analysis can not always be explained for tumor-cell-derived B7-1 and B7-2 mRNA in primary and metastatic lesions. In addition, the ABC technique appears not to be sensitive enough to detect B7-1 and B7-2 on tumor tissue sections. The advantage of flow-cytometric analysis, presented here, is that it is capable of detecting expression of the molecule at the even lower protein level on the cells prepared from solid tumor tissues. FACS analysis, furthermore, could distinguish tumor cells from mixed TIL using the forward-angle versus light-scattering parameter. The analysis of B7-1 and B7-2 expression on the tumor was not influenced by the TIL in the samples. Therefore, it seems likely that tumor-characteristic B7-1 and B7-2 expression was being observed in the present study.

The frequency of B7-1 or B7-2 expression on primary gastric carcinoma cells from 24 patients was similar to that observed on the normal gastric mucosal cells from 20 patients, despite morphological deviations of the tumor tissues from the histology typical for normal mucosa. These findings might be compatible with the idea that the tumor originates from these normal cells and maintains the expression pattern of the cells from which it arises. However, frequency of B7-1 expression in poorly differentiated carcinoma of the primary locus was significantly lower than in well-differentiated carcinoma and normal mucosa ($P < 0.01$). Poorly differentiated adenocarcinoma or signet-ring cell carcinoma is almost compatible with the so-called diffuse-type carcinoma classified by Lauren (1965). The diffuse-type carcinoma especially tends to grow through the gastric wall and reach the serosa, and can directly involve adjacent structures (Nakamura 1982; Antonioli 1990). Thus, this type of cancer cells poses a high risk of seeding and contaminating the peritoneal cavity leading to peritoneal carcinosis. Indeed, the metastatic cancer cells obtained from malignant ascites were almost all derived from poorly differentiated or signet-ring cell carcinomas. The frequency of B7-1 expression on metastatic carcinoma from 20 patients was significantly lower than that on primary carcinoma and normal mucosa ($P < 0.001$). Different expression of the B7-1 molecule in poorly differentiated and well-differentiated adenocarcinoma cell lines was also observed. The mean positive percentage of B7-1 expression on carcinoma cell lines (SC-1, SC-2, SC-3, GCIY and MKN-45 cells) derived from the diffuse-type gastric carcinoma was only 24.0%, while the mean positive percentage of B7-1 expression on moderately differentiated adenocarcinoma cell lines (MKN-28 and MKN-74 cells) derived from the intestinal-type gastric carcinoma (Motoyama et al. 1986) was 41.0%. However, there was no difference in B7-2 expression between the diffuse-type gastric carcinoma cell lines and the intestinal gastric carcinoma cell lines. These findings suggest that the reduced expression of B7-1 on the tumor correlates with the grade of differentiation, tumor progression and metastatic status. The B7-2 molecule is commonly expressed by normal gastric mucosa and primary and metastatic lesions of gastric carcinoma. However, no significant incidental differences between grade of tumor differentiation and invasion were observed, although metastatic carcinomas seemed to express B7-2 more often than primary carcinoma and normal mucosa. On the basis of the above descriptions, $CD80^+CD86^+$ tumor cells on the primary locus might be abrogated in the process of metastasis to the peritoneum, and finally the tumor cells might show the $CD80^-CD86^+$ phenotype.

Tumor-rejection antigens in human gastric carcinoma, which are recognized by autologous cytotoxic T lymphocytes (CTL) have recently been identified (Yasoshima et al. 1995; Hoshino et al. 1997). However, regardless of the demonstration of CTL against gastric carcinoma, metastatic carcinoma of the stomach especially is capable of progressive growth in patients, without rejection by the immune system. The coexistence of tumor-specific immunity with the escape of gastric carcinoma from immunological destruction is certainly a major paradox of tumor immunology. Although precise and/or distinct functions of B7-1 and B7-2 on the cellular and molecular mechanisms involved with tumor rejection have remained obscure, it has been proposed that B7-2 initially interacts with CD28 on most resting T cells to initiate the immune response, and that cytokine production early during the response may be up-regulated, which in turn may amplify the response (Azuma et al. 1993). Unlike CD28, CTLA-4 appears only after T cell stimulation (Linsley et al. 1992). It has also been reported that B7-1 seems to be better than B7-2 at costimulating $CD8^+$ CTL bearing CD28/CTLA-4 molecules (Gajewski 1996). Therefore, if the tumor lacks B7-1 on its surface, the effective T-cell-mediated antitumor immunity against human tumor cells would not be induced. In fact, we previously found an increase in $CD28^+$ TIL from malignant ascites in patients with gastric carcinoma (Koyama et al. 1992; Ebihara and Koyama 1990). These increased TIL in the malignant ascites, however, were not actually able to lyse the tumor, resulting in anergy or unresponsiveness to the tumor. The decrease, abrogation or loss of B7-1 on primary and metastatic tumor, as observed in this study, may have profound implications for the down-regulation of the immune system and for enhancing the metastatic capacity of the tumor in cancer patients.

In conclusion, our findings suggest that B7-1 and B7-2 molecules are constitutively expressed in gastric carcinoma, that decreased or deleted expression of B7-1 might be involved in cancer progression and metastasis and be related to the grade of differentiation of gastric carcinoma, and that inadequate B7-1 expression may contribute to the escape of tumors from immune surveillance. However, detection of B7-2 seems not to be directly related to the grade of differentiation and invasion of gastric carcinoma.

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