Frequency of Apoptosis of Tumor-infiltrating Lymphocytes Induced by Fas Counterattack in Human Colorectal Carcinoma and Its Correlation with Prognosis

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ABSTRACT

We investigated apoptosis in tumor-infiltrating lymphocytes (TILs) obtained from 41 colorectal carcinomas by in situ nick translation (ISNT). When the ISNT labeling index (LI) was determined as the number of positive nuclei per 1000 nuclei of TIL in tissue sections, the median LI was 12.0 (range, 2–30). The ISNT LI of colorectal carcinoma with lymph node metastasis was higher than that of colorectal carcinoma without metastasis. The cases with a high LI of ≥12.0 had a significantly poorer prognosis than those with a low LI. We also confirmed immunohistochemically that a part of the TILs expressed Fas using the sections adjacent to what contained abundant ISNT-positive TILs. Moreover, Fas ligand (FasL) expression was detected on the cell surface as well as the cytoplasm of colorectal cancer cells in 61% of cases. Apoptosis in TILs was consistently seen more frequently in FasL-positive cases than in FasL-negative ones. These findings indicate that the FasL expressed in colorectal carcinoma cells may kill the Fas-positive immune effective TILs by means of a Fas-FasL system termed Fas counterattack. This tumor immune evasion induced by FasL may therefore affect the malignant potential of human colorectal carcinoma.

INTRODUCTION

In most tumors, the malignant potential increases due to the accumulation of various genetic changes that enable them to acquire either invasive or metastatic abilities (1–4). Besides the intrinsic changes in cancer cells, cell-to-cell interaction between cancer cells and surrounding noncancerous cells at the border area has recently been shown to play an important role in the malignant potential of such tumors (5). Ropponen et al. (6) reported that several human neoplasms contain many lymphocytes that infiltrate the center and periphery of the tumor and defined these lymphocytes as TILs. Moreover, the density of TILs was found to correlate with survival in colorectal carcinoma patients (6). In addition, in melanomas that express FasL, apoptotic cell death of TILs has recently been reported to be induced through Fas and FasL interaction (7). This apoptotic depletion of TILs by tumor cells has thus been suggested to cause tumor immune escape (8–11).

Fas is a Mr, 45,000 transmembranous glycoprotein that is a member of the tumor necrosis factor/nerve growth factor receptor family and is known to be expressed in a variety of cells, including activated T cells and tumor cells (12–14). FasL is a Mr, 31,000 type II transmembranous protein that is known to initiate apoptosis in activated T lymphocytes by binding to the Fas transmembranous glycoprotein (14). In addition to the immune system, the apoptotic cell death of hepatocytes, intestinal epithelial cells, kidney cells, and ovarian cells has been known to be mediated through this Fas-FasL system. Moreover, expression of FasL has also been demonstrated in various types of tumor cells (15, 16) such as human melanoma (7), hepatocellular carcinoma (17), esophageal carcinoma (18), and colonic carcinoma (19, 20). These findings thus suggest that the Fas-FasL system is involved in the immune privilege of cancerous tissues due to a Fas counterattack against Fas-expressing TILs (18, 20). However, thus far, only a few detailed studies on the relationship between the expression of FasL in tumor cells and the apoptotic depletion of TILs in vivo have been performed.

In this study, we focused on the apoptosis of TILs around the border zone between the normal and malignant region in colorectal cancer tissue specimens. We used the ISNT method to identify cells with DNA strand breaks as apoptotic cells, based on the usefulness of this method in our previous study (21, 22). Using our specific antisera against Fas and FasL, we immunohistochemically investigated the expression of Fas and FasL in various tissue specimens. Finally, correlations between the apoptosis of TILs and the FasL expression of colorectal cancer cells were investigated to evaluate their potential prognostic value.

MATERIALS AND METHODS

Tissues. Human primary colorectal carcinoma specimens were obtained from 41 patients who had been operated on in the...
Second Department of Surgery, Nagasaki University Hospital (Nagasaki, Japan) between 1991 and 1994. None of the patients had received chemo- or radiotherapy before tissue collection. The diagnosis of all specimens was made by a histopathological examination. The mean age of the patients was 63.2 years (range, 37–87 years). According to the Dukes’ stage classification, 7, 13, and 21 patients were Dukes’ stage A, B, and C, respectively.

**ISNT.** To identify nuclei with DNA strand breaks at a cellular level, ISNT was performed (21, 22). The tissue sections described above were deparaffinized with toluene and rehydrated in a serial ethanol solution. After repeated washing with PBS, the sections were treated with proteinase K (Sigma Chemical Co.) in PBS (1 µg/ml, 37°C, 15 min). After several washings with PBS, they were immersed in 50 mM Tris-HCl (pH 7.5). The ISNT reaction was conducted for 3 h at 37°C in a medium containing 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 0.1 mM DTT, 50 µg/ml BSA, 200 units/ml DNA polymerase I (Takara Co.), and 20 µM each of dATP, dGTP, dCTP, and biotin-11-dUTP (Enzo Diagnostics, Inc.). As a negative control, TTP was used in place of biotin-11-dUTP. After repeated washing with the 50 mM Tris-HCl buffer, the sections were reacted with HRP-labeled anti-biotin antibody (Vector Laboratories, Inc.) dissolved in 5% BSA in PBS for 3 h at room temperature. The HRP sites were visualized by using 3,3'-diaminobenzidine, H₂O₂, CoCl₂, and NiSO₄(NH₄)₂SO₄ (23).

**Immunohistochemical Detection of Fas and FasL Protein.** The localization of Fas and FasL was examined using the rabbit anti-Fas and FasL sera, respectively, which were raised against a synthetic peptide of human Fas or rat FasL and have been described previously (12, 24). To confirm the specificity of the reaction, both normal rabbit serum and an excess amount of the synthetic peptide (FasD or P5) were used. Deparaffinized and rehydrated sections were immersed in 50 mM Tris-HCl in methanol and then preincubated with 0.5 mg/ml normal goat IgG and 1% BSA in PBS for 1 h at room temperature to block any nonspecific binding of the antibodies. Next, the sections were reacted with the primary antibodies (1:200) diluted with 1% BSA/PBS instead of the specific primary antibody. As a negative control, normal mouse IgG was used in place of UCHL-1 at the same concentration.

**Identification of TILs.** For the following histochemical experiments, the specimens were fixed in 10% neutral buffered formalin and embedded in paraffin, and then 4-µm-thick sections were cut and placed onto aminopropyltriethoxysilane-coated glass slides. Some sections were stained with H&E for histological examination. In addition to H&E-stained sections, lymphocyte infiltration into tumors was examined immunohistochemically using the anti-human T lymphocyte antibody (UCHL-1; DAKO, Carpinteria, CA) in serial sections. The immunostaining was performed as described above, except that HRP-conjugated goat anti-rabbit IgG (1:200) was diluted with 1% BSA in PBS for 2 h at room temperature and washed as described above. The HRP sites were visualized by H₂O₂ and 3,3'-diaminobenzidine for 5 min. As a negative control, some of the sections were incubated with normal rabbit serum instead of the specific primary antiserum.

**Identification of apoptotic TILs by ISNT Staining.** In the conventional H&E-stained sections, the TILs were found in the boundary area between normal glands and colorectal carcinomas. The ISNT-positive lymphocytes were observed predominantly in the border fields (Fig. 1). We counted the number of apoptotic cells under each experimental condition. Actuarial survival was calculated by using the Kaplan–Meier method. Multivariate regression analysis was performed using a stepwise multiple regression test including different clinical pathological factors. *P* < 0.05 was considered statistically significant.

**RESULTS**

**Identification and Quantitative Analysis of Apoptotic TILs by ISNT Staining.** In the conventional H&E-stained sections, the TILs were found in the boundary area between normal glands and colorectal carcinomas. The ISNT-positive lymphocytes were observed predominantly in the border fields (Fig. 1). We counted the number of ISNT-positive TILs in the border area of 41 colorectal carcinomas. The ISNT LI ranged from 2–30, with a mean ± SD of 10.7 ± 6.1 in all tumors. No significant correlation was observed between the ISNT LI and the age of the patients (>65 years, <65 years), the sex of the patients, the size of the carcinomas (>5 cm, <5 cm), or the localization of carcinomas in the colon (proximal and distal to the splenic flexure). In addition, no significant correlation was seen between the average value of the LI and liver metastasis. However, patients with lymph node metastasis had significantly higher LIs than patients without metastasis (Table 1). In these patients, on the other hand, apoptotic lymphocytes in other tissues including noninvolved lymph nodes or Peyer’s patches...
were much less numerous than those in the border fields. In addition, we also confirmed the TIL apoptosis by TUNEL with serial sections. Although the staining intensity tended to vary with TUNEL, the distribution of positive cells was quite similar to that of ISNT (data not shown).

**Prognostic Value of ISNT LI.** To divide all of the cases into high and low LI subgroups, the LI of the TILs was classified at 12.0, which was the median value for all tumors. Among the patients who had undergone curative resection, the 5-year survival rate in the high LI subgroup was significantly poorer than that of the low LI subgroup (74.4% versus 51.3%, respectively; \( P < 0.05 \), Cox-Mantel; Fig. 2). Multivariate analysis was conducted among the different clinicopathological variables by using a stepwise regression test. Although lymph node metastasis, in addition to ISNT LI, was an independent prognostic parameter (\( P < 0.05 \), Cox-Mantel), other variables including patient age, gender, tumor size, location, histological differentiation, and depth of invasion were not found to be independent predictors of prognosis.

**Expression of Fas on TILs.** We immunohistochemically examined the expression of Fas antigen using rabbit antiserum raised against synthetic peptides forming a part of the Fas antigen. As shown in Fig. 3A, a portion of the TIL population was positive for Fas antigen in most of the tumors that contained apoptotic TILs. On the other hand, 35% of colorectal carcinoma cases expressed Fas antigen in colorectal carcinoma cells. In the adjacent sections, ISNT-positive lymphocytes were detected in the same area, where Fas-positive TILs were abundant (Fig. 3B). Unfortunately, however, it was hard to correlate the Fas-positive TILs with ISNT-positive TILs in serial sections. When the sections were incubated with normal rabbit serum instead of the anti-Fas serum, no positive staining was found (data not shown).

**Expression of FasL in Colorectal Cancer.** Using anti-FasL polyclonal antibody, FasL-positive cells were examined immunohistochemically in the surgically resected colorectal adenocarcinoma specimens (\( n = 41 \)). The specificity of immunohistochemical staining was confirmed by elimination of the staining using a normal rabbit serum and an excess amount of peptide as described above. The signal for FasL was detected in the plasma membrane as well as in the cytoplasm of some tumor cells, which were limited to the focal area of the tumor tissue specimens (Fig. 4).

**Relationship between FasL Expression and the Frequency of Apoptosis of TILs.** We finally examined the relationship between the expression of FasL and apoptosis of TILs to confirm the possible involvement of the Fas-FasL system in the induction of TIL apoptosis. As shown in Fig. 5, about 61%
DISCUSSION

In addition to colon carcinoma, many carcinomas, including hepatocellular carcinoma, lung carcinoma, melanoma, and esophageal carcinoma, have also been reported to express FasL. However, thus far, only a few detailed studies have been made concerning the relationship between the apoptosis of TILs and FasL expression in vivo. Bennett et al. (18) reported a possible correlation between apoptosis of TILs and FasL expression in vivo in human esophageal carcinoma. They reported that the number of apoptotic TILs is greater in the FasL-positive regions than in the FasL-negative regions. In our study, some cases showed a similar regional relationship between TIL apoptosis and the FasL-expressing area (data not shown). The fact that apoptotic changes of lymphocytes were more frequent in the border area around the carcinoma than in other distant areas may suggest direct interaction between the tumor and TILs.

The induction of apoptosis in Fas-positive TILs by tumor cells expressing FasL is termed Fas counterattack because the tumors guard against the attack of activated lymphocytes that express Fas by using the Fas-FasL system (18, 19). As a result, the mechanism underlying this phenomenon can be regarded as the same as that attributed to the immune privilege in the eye and testis (8, 10). Hahne et al. (7) reported recently that FasL-expressing melanoma cells could induce apoptosis in Fas-bearing lymphoma cells in vitro. More interestingly, tumor growth was substantially delayed in Fas-deficient lpr mutant mice, whereas the FasL-expressing melanoma grew rapidly in wild-type mice and in gld mutant mice that lacked functional FasL (7). These results may indicate that an apoptotic signal deficiency from tumor cells to activated lymphocytes may enhance the immune response against tumor cells because of a lack of the suicide-triggering mechanism.

In colorectal carcinoma cell lines (25), expression of functional FasL was confirmed, and FasL was also reported to exert a killing effect against activated T cells in vitro. Furthermore, FasL from colorectal carcinoma cells in metastatic regions was known to induce apoptosis of the surrounding hepatocytes, which are Fas positive (26). In our study, the cases with FasL-expressing tumor cells had a larger number of apoptotic TILs around the tumor. Moreover, colorectal carcinomas that included a large amount of apoptotic TILs tended to significantly metastasize to the regional lymph nodes. These findings suggest that colorectal carcinoma cells that express FasL may also induce apoptosis of Fas-positive T lymphocytes in the regional lymph nodes. As a result, the malignant potential of FasL-expressing colorectal carcinoma may increase, thus leading to a poorer prognosis.

On the other hand, it has been suggested that tumor cells may be resistant to Fas-mediated apoptosis by means of a molecular defect concerning Fas signal transduction. In our previous study, we examined the correlation between ISNT LI of colorectal carcinoma and its metastasis to lymph node and liver, and we found that the LI of carcinoma without metastasis was significantly higher than that of carcinoma with metastasis. It is possible that FasL expression in the carcinoma may play a pivotal role in these LIs. However, because the expression of Fas or FasL in colorectal carcinoma did not correlate significantly with the frequency of ISNT-positive carcinoma cells, the LI of carcinoma and the LI of TILs should thus be considered to be independent prognostic parameters. Therefore, in carcinoma cells, FasL expression, but not Fas expression, may be a crucial parameter for characterizing tumor malignancy.

Although Fas-positive lymphocytes seemed to undergo...
apoptosis, we could not show direct evidence that Fas-positive lymphocytes were also positive for ISNT staining in serial sections. ISNT-positive cells were identified as round independent cells accumulating in the stroma. In this context, it should be noted that apoptotic cells may not express Fas antigen because dying cells would not maintain the normal gene expression. Furthermore, it is not clear whether cell surface antigens are maintained in this death process because structural features of the cell surface may vary in apoptotic cells. Simultaneous detection of Fas-positive and ISNT-positive cells might be helpful to demonstrate the keen connection (18). Again, however, intensive study of the loss of Fas by protease treatment included in the ISNT protocol or of the induction of DNA single-strand breaks during the Fas immunostaining procedure would be prerequisite.

Recently, proinflammatory or antitumor properties of FasL were also indicated in murine models (27). FasL-transfected tumor cells recruited neutrophils to the tumor site and led to tumor rejection in vivo (28). Similarly, FasL overproduction in the islets of Langerhans resulted in neutrophilic infiltration and islet destruction (29). However, in our study, massive neutrophilic infiltration was not observed around FasL-expressing colorectal tumors. To clarify the discrepancy, further investigation would be required. In summary, this study strongly demonstrated that the apoptosis of TILs was significantly correlated with lymph node metastasis and poor prognosis in primary colorectal carcinoma. Furthermore, because apoptosis was observed more abundantly in the cases expressing FasL, tumor immune escape through the Fas-FasL pathway may affect the malignant potential of colorectal carcinoma cells.

REFERENCES


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