Jpn. J. Cancer Res. **93**, 320–328, March 2002

Close Association between Fas Ligand (FasL; CD95L)-positive Tumor-associated Macrophages and Apoptotic Cancer Cells along Invasive Margin of Colorectal Carcinoma: A Proposal on Tumor-Host Interactions

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Anti-tumor immune responses are considered to be one of the key host reactions in human colorectal cancer, with T cells as important effector cells. For the induction of tumor-specific immunity, processing of cancer cells and priming of T cells by antigen-presenting cells are important. The present study was designed to clarify the relationship between Fas ligand (FasL; CD95L) expression and apoptotic cancer cells. Immunohistochemistry using frozen sections taken from 58 patients with colorectal cancer revealed that stromal cells composed mainly of tumor-associated macrophages expressed FasL, leaving cancer cells negative for FasL. These macrophages were abundantly distributed along the invasive margin. *In situ* hybridization revealed that these macrophages as well as cancer cells expressed FasL mRNA, whereas macrophages in the normal colon mucosa rarely expressed FasL. Apoptotic cancer cells recognized by monoclonal antibody M30 CytoDEATH were localized not only in cancer cell nests, but also in the stroma along the invasive margin showing a dissociated pattern, which was particularly evident in the areas of FasL$^+$ macrophages. Furthermore, these macrophages, phenotypically similar to dendritic cells, occasionally contained M30$^+$ apoptotic cancer cells in the cytoplasm. Clinicopathologic analyses in 123 cases revealed 1) a positive correlation between the degree of dissociated M30$^+$ apoptotic cancer cells and the number of macrophages along the invasive margin and 2) an inverse association between the degree of dissociated M30$^+$ apoptotic cancer cells and the occurrence of hematogenous metastasis after surgical resection of the primary tumor. In conclusion, the present study shows the importance of FasL$^+$ activated macrophages as one of the host defense mechanisms against cancer cell spread in human colorectal cancer.

Key words: Colorectal cancer — Fas ligand — Tumor-associated macrophages — Anti-tumor immunity

The biological malignancy of human tumors is determined by the total effect of tumor cell aggressiveness and various host reactions modifying it. In human colorectal cancer, host immune and/or inflammatory infiltrate is observed in the tumor tissue, being particularly evident along the invasive margin (tumor-host interface).[1)] Previous analyses by us in human colorectal cancer revealed that a) macrophages distributed along the invasive margin are phenotypically similar to dendritic cells,[2,3)] b) these macrophages may be inhibitory to hematogenous spread of cancer cells[4)] and c) the degree of CD8$^+$ T cell infiltration into cancer cell nests could be an independent prognostic factor.[5)] These results underscore the importance of host immune responses within cancer tissue. To initiate effective immune responses, however, uptake and subsequent processing of antigenic molecules by antigen-presenting cells are key factors. Recent studies have clarified the importance of dendritic cells in priming T cell-mediated immune responses,[6)] and further suggested the significance of phagocytosis of apoptotic cells by immature (precursor) dendritic cells to induce cytotoxic effector T cells.[7)]

Several pathways have been proposed as effector mechanisms of activated cytotoxic T lymphocytes against target cells, including the perforin-granzyme system and the Fas/Fas ligand (Fas/CD95L) system.[8–10)] FasL is a tumor necrosis factor (TNF)-related type II transmembrane molecule, which induces apoptosis in Fas-expressing cells.[11)] FasL is expressed in cytotoxic T-lymphocytes and natural killer (NK) cells.[11)] FasL is involved not only in the elimination of target cells, but also in the negative selection of lymphocytes during the development and maintenance of homeostasis of the immune system.

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FasL is detected in various cancer cells *in vitro* and *in vivo*, and this has been interpreted as a protective mechanism against cytotoxic T cells. This concept is designated as the "counter-attack theory," which has attracted much attention to explain ineffective tumor immunity.[12] However, this theory is rather problematic because of the lack of direct evidence for killing effects against cytotoxic T cells and the unreliability of anti-FasL antibodies used in previous studies,[13–15] and because tumor grafts transfected with FasL underwent accelerated rejection by neutrophil infiltration.[16] In response to these considerations, O'Connell *et al.* reiterated the counterattack theory, taking account of both anti- and pro-inflammatory functions of FasL.[17] Therefore, it seems important to re-evaluate the cellular distribution of FasL in human cancer tissue. In the present paper, we newly identify activated macrophages as one of the sources of FasL in human colorectal cancer tissue, and found that apoptotic cancer cells were frequently associated with these cells. We propose a new role of macrophages as anti-tumor effector cells.

Table I. Patient Profiles

		Frozen sections	Paraffin-embedded sections	
			Metastasis group	Control group
Total number		58	67	56
Age	(average)	65.7	64.8	66.5
Sex	male	32	34	31
	female	26	33	25
Location	colon (right)	24	21	19
	colon (left)	11	13	19
	rectum	23	22	29
Histologic type	well	21	18	22
	moderate	34	47	33
	poor	2	2	1
	mucinous	1	0	0
Stage	A	5	0	0
	B	20	27	46
	C	17	29	21
	D	16	0	0

MATERIALS AND METHODS

Patient profiles and tissue fixation Fifty-eight patients with colorectal cancer were immunohistochemically analyzed by using frozen sections (Table I). For this purpose, fresh specimens 5×5×2 mm in size were fixed immediately after surgical resection in periodate-lysine-4% paraformaldehyde (4% PLP) for 6–16 h at 4°C. After washing, specimens were frozen as described previously.[18] For clinicopathological analyses, we analyzed 123 patients with colorectal cancer using routinely processed, formalin-fixed, paraffin-embedded sections. These patients consisted of a group with metachronous hematogenous metastasis and a control group, which were used in our previous study (Table I).[4] The control group was composed of colorectal cancer patients with penetration of cancer beyond the muscularis propria, who showed no metastasis or local recurrence in more than 5-year follow-up. There were no patients overlapping between the groups analyzed with frozen and paraffin-embedded sections. For *in situ* hybridization, we adopted fixation in 4% paraformaldehyde + 0.5% glutaraldehyde, which significantly enhances the signal detection sensitivity.[18] After overnight fixation, the specimens were embedded in paraffin. For the control, normal-appearing colonic mucosal tissues were obtained in surgical operation and processed by the same method in 6 cases.

Immunohistochemistry (Table II)

Frozen sections: PLP-prefixed frozen sections were mounted on silane-coated glass slides (Muto Pure Chemicals, Ltd., Tokyo). First, the sections were immersed in normal goat + human serum (10–20%) for 30 min to block the non-specific binding of immunoglobulins, particularly of IgG2a isotype. The sources of the primary antibodies used are listed in Table II. The incubation time of these antibodies was 12–16 h at 4°C. Envision plus kit (DAKO Japan, Kyoto) was used as the secondary antibody. 3,3′-Diaminobenzidine tetrahydrochloride (DAB; Dojin, Kumamoto) was used as the chromogen. The endogenous peroxidase activity was blocked by treating the specimens with 0.3% hydrogen peroxide in methanol for 15 min after the incubation with the primary antibodies, and by adding 0.5% sodium azide in DAB solution. For the negative control, the primary antibodies were replaced with the same concentration of isotype-matched control antibodies (DAKO) or mouse monoclonal antibodies with irrelevant specificity.

Paraffin-embedded sections: CD68 staining with a monoclonal antibody, clone PG-M1 (DAKO), was also performed on paraffin-embedded sections prepared for *in situ* hybridization as described above. The pretreatment condition for this immunostaining was 0.1% pronase for 15 min at room temperature. Detection of apoptotic epithelial cells with monoclonal antibody M30 CytoDeath was also performed on routinely processed paraffin-embedded sections after antigen retrieval by microwave-heating in 0.01 *M* citrate buffer (pH 6.0) for 15 min.

Double-labeling immunohistochemistry (performed in 5 representative cases) To identify the type of FasL$^+$ cells, double-labeling immunohistochemistry for FasL/CD68 and FasL/CD3 was performed by an enzyme-linked method using PLP-fixed frozen sections as described previously.[4, 19] Also, to determine the relationship between apoptotic cancer cells and immune cells, double-labeling

Table II. List of Antibodies Used

Antibodies for	Isotypes	Sources	Final dilution
CD95L/FasL	polyclonal	Nichirei, Tokyo	1:1
CD95L/FasL (NOK-2)[a)]	mouse IgG2a	Sumitomo, Yokohama	1:50, 4 μg/ml
CD68 (EBM11)	mouse IgG1	DAKO Japan, Kyoto	1:3000
CD86/B7.2 (BU63)	mouse IgG1	Ancell, Bayport, MN	1:100
CD95/Fas (DX-2)	mouse IgG1	Pharmingen, San Diego, CA	1:200
M30 CytoDEATH	mouse IgG2b	Roche Diagnostics, Mannheim, Germany	1:5
Cytokeratin (AE1/AE3)	mouse IgG1	DAKO Japan	1:300, 0.5 μg/ml
Neutrophil elastase (NP57)	mouse IgG1	DAKO Japan	1:100, 0.65 μg/ml
CD4 (Leu 3a)		Becton Dickinson, San Jose, CA	1:100
CD8 (Leu 2a)	mouse IgG1	Becton Dickinson	1:100

a) Antibody for FasL mainly used in the present paper.

immunohistochemistry for M30/CD68, M30/B7.2 and M30/HLA-DR was performed. The controls for the staining were performed by omitting the first-step primary antibody, second-step primary antibody, or both.

***In situ* nick-end labeling (performed in all 58 cases with frozen sections)** We applied apoTACS In Situ Apoptosis Detection Kit (Trevigen, Inc., Gaithersburg, MD) as recommended by the manufacturer for PLP-prefixed frozen sections. The pretreatment condition was modified from the manufacturer's instruction to 20 μg/ml proteinase K for 15 min at 37°C. In 3 representative cases, CD3-immunostaining with Vector red as a chromogen was preceded by this method to selectively observe apoptotic cells in areas with lymphocytic infiltration.

***In situ* hybridization (performed in 10 cases)** Digoxigenin-labeled cRNAs with antisense or sense orientation were transcribed *in vitro* from the template human FasL cDNA[20)] as recommended by the manufacturer's manual (Boehringer-Mannheim, Mannheim, Germany). The final concentration of the probe used was 1 μg/ml. The pretreatment, probe composition, washing, and immunohistochemical detection of signals were performed as previously described.[18)] The negative control reaction was performed in all experiments using digoxigenin-labeled cRNA in the sense orientation at the same concentration.

Semiquantitative assessment of results

1) FasL, CD68, CD4, neutrophil elastase, CD8 along the invasive margin, and M30: Distribution of the cells immunoreactive for these markers along the invasive margin was scored as 1 (mild), 2 (moderate: discontinuous and no aggregate formation) or 3 (abundant: continuous or aggregate formation).

2) $CD8^+$ T cells within cancer nests: The number of $CD8^+$ cells within cancer cell nests (intratumoral $CD8^+$ cells) was counted per unit area (0.0025 mm^2) in 200× microscopic fields as described elsewhere.[21)]

3) Fas in cancer cells: Immunoreactivity of cancer cells was scored as positive or negative (i.e., presence or absence of immunoreactivity).

4) Quantification of macrophages along the invasive margin (paraffin-embedded sections): Data obtained in our previous study were used.[4)]

5) Method for judgment of semiquantification: Two observers independently performed all the semiquantitative scoring. In cases where the judgments were not in agreement, the two observers reached a consensus after observing the specimens again.

Immunoelectron microscopy We adopted the pre-embedding, immunoperoxidase method for FasL as described previously.[3, 18)]

Statistical analyses

a) Correlation between the degree of dissociated $M30^+$ apoptotic cancer cells and the number of macrophages was tested by Spearman's test (Dr. SPSS, SPSS Japan, Tokyo).

b) Differences in semiquantitative scoring of dissociated $M30^+$ apoptotic cancer cells between the group with hematogenous metastasis and the control group were tested by the Kruskal-Wallis test (Dr. SPSS).

RESULTS

Distribution and cell-type identification of $FasL^+$ cells $FasL^+$ cells identified by monoclonal antibody NOK-2[20)] were mainly large, round, non-neoplastic cells, which were most abundantly distributed along the invasive margin (tumor-host interface) of colorectal cancer. Small lymphocyte-like cells were infrequent among $FasL^+$ cells. Of 58 cases examined by frozen sections, the number of cases of mild, moderate, and abundant expressions was 16, 31 and 11, respectively (for details, see the semiquantification method section). As shown in Fig. 1, a and c, the distribution patterns of FasL were similar to those of CD68, the macrophage marker, and semiquantification analyses confirmed a positive correlation between the degree of immu-

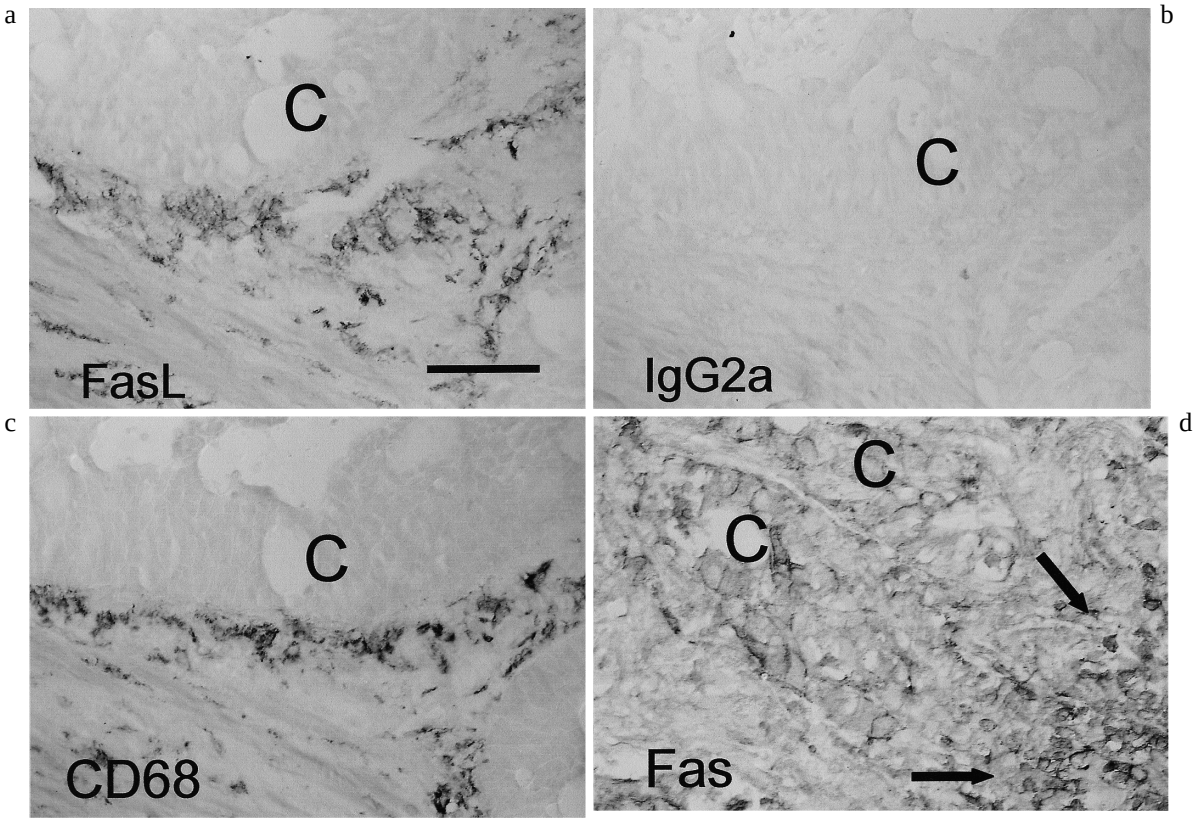

Fig. 1. Immunohistochemistry for FasL (a), isotype-matched control (IgG2a) (b), CD68 (c) and Fas (d) in human colorectal cancer. C, cancer cells. Arrows in d, Fas+ lymphocytes. Scale bar, 50 μm.

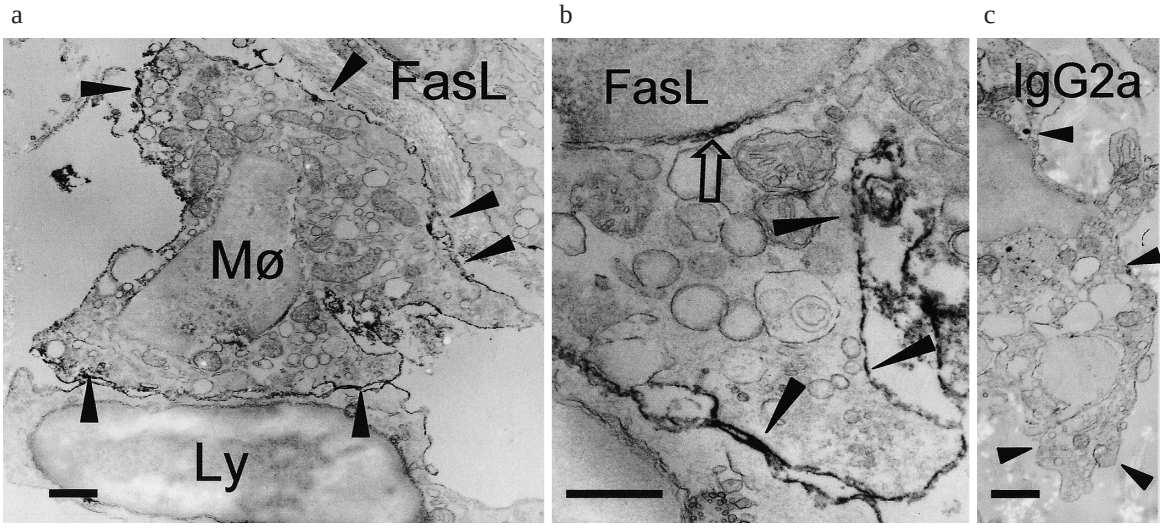

Fig. 2. Immunoelectron microscopy for FasL (a, b) and isotype-matched control (c). FasL is localized along the plasma membrane (a, b, arrowheads), in contrast with the negative control (c, arrowheads). The shape of the cells (Mø in a) is not round, in contrast to neighboring lymphocytes (Ly in a). Higher magnification revealed membrane reactivity (b, arrowheads) and perinuclear space labeling (arrow). Scale bar, 2 μm.

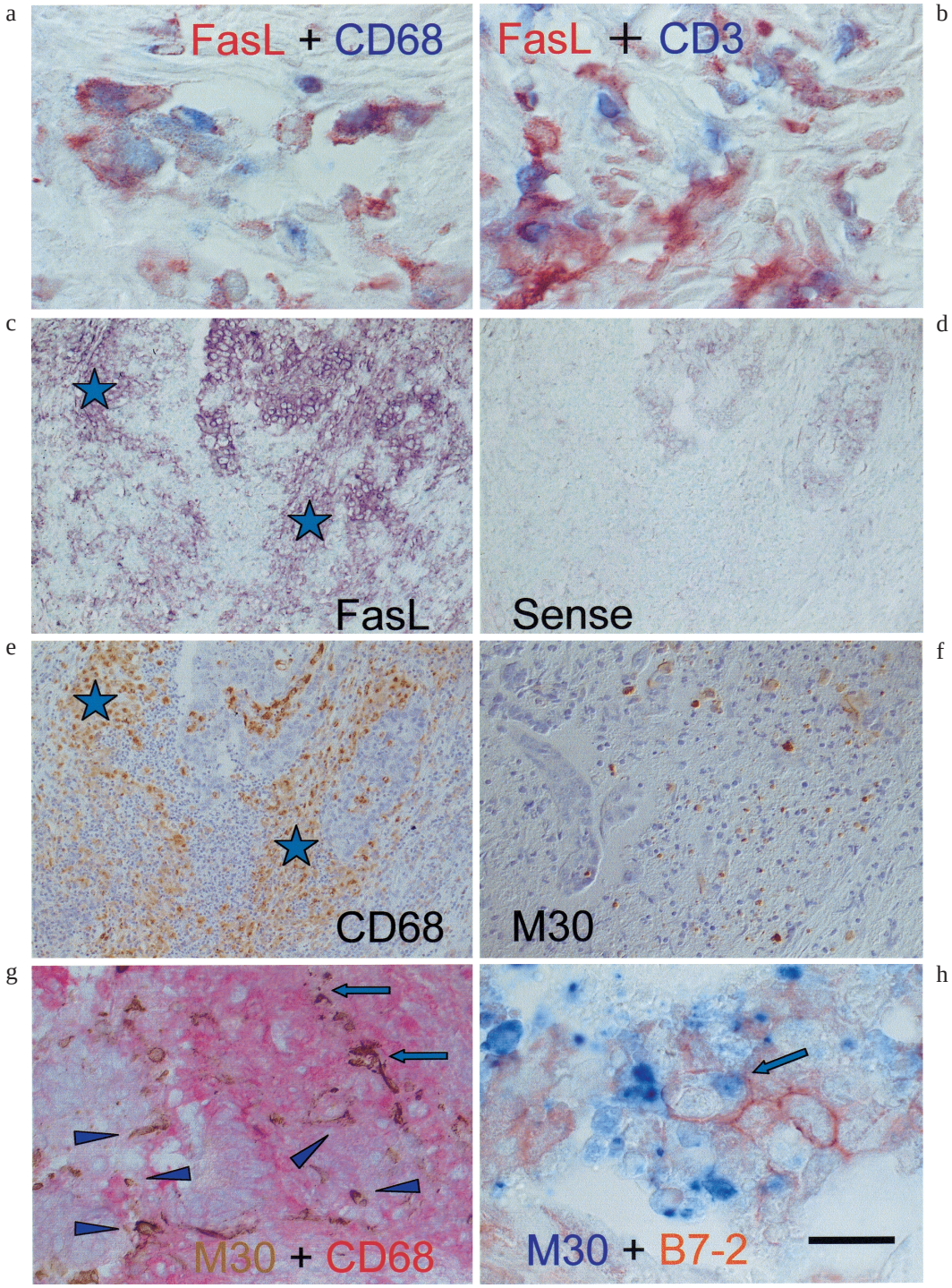

Fig. 3. Double immunohistochemistry for FasL (red)/CD68 (blue) (a) and FasL (red)/CD3 (blue) (b). *In situ* hybridization for FasL (c) and negative control with labeled cRNA with a sense orientation (d). Immunohistochemistry for CD68 in a serial section (e). Asterisks (c and e) indicate the same areas. Immunohistochemistry with M30 (f) showing dissociated $M30^+$ apoptotic cancer cells (score 3, abundant). Double immunohistochemistry for M30 (brown)/CD68 (red) (g). Arrows (g) indicate dissociated $M30^+$ apoptotic cancer cells in the area of macrophages. Blue arrowheads (g) indicate $M30^+$ apoptotic cancer cells in cancer cell nests. Double immunohistochemistry for M30 (blue)/B7-2 (red) (h) with arrows indicating $M30^+$ bodies in the cytoplasm of $B7\text{-}2^+$ cell. Scale bar, 40 μm in a, b, f, h and 200 μm in c, d, e, g.

noreactivity for FasL and that for CD68 (γ=0.57, P<0.01). Immunoelectron microscopy clearly revealed that FasL is localized along the plasma membrane (Fig. 2, shown in black), and that positive cells have cytoplasmic projections and vacuoles in the cytoplasm, suggesting that these cells are macrophages.

To further analyze FasL$^+$ cell types, we performed double labeling immunohistochemistry. Immunolocalization of FasL and that of CD68 were frequently overlapped (Fig. 3a), while most FasL$^+$ cells were distinct from CD3$^+$ T cells (Fig. 3b). These data indicate that FasL$^+$ host cells consisted mainly of macrophages (tumor-associated macrophages), but not lymphocytes. Cancer cells were negative for FasL in all cases examined (Fig. 1a). Results obtained with polyclonal anti-FasL antibody (Nichirei, Tokyo) using frozen sections were essentially the same, with a decreased signal-to-noise ratio (data not shown). Macrophages in the normal colon or rectal tissue were rarely positive for FasL (<5%) (data not shown).

Quantification and semiquantification analyses showed that the expression of FasL was positively correlated with the number of the following cell types distributed along the invasive margin: CD4$^+$ T cells (γ=0.31, P<0.05), CD8$^+$ T cells (γ=0.49, P<0.01), and neutrophils (γ=0.45, P<0.01). FasL expression was also positively correlated with the number of CD8$^+$ T cells distributed within cancer cell nests (γ=0.4, P<0.01).

Immunoreactive Fas was observed in cancer cells in 27 of 58 cases, and in stromal inflammatory cells, particularly small round cells (lymphocytes) (Fig. 1d). However, no statistically significant correlation was found between the expression of FasL in stromal cells and Fas in cancer cells (γ=0.21).

***In situ* hybridization for FasL** mRNA for FasL was localized in cancer cells in 7 of 10 cases examined, which is consistent with the previous report,[22] but in contrast to the absence of immunoreactive FasL protein as shown above. By adopting a more efficient fixation method,[18] we newly detected signals for FasL in the area of stromal cells near the invasive margin in 10 of 10 cases (Fig. 3c, asterisks). As shown in Fig. 3, c and e, this area of FasL$^+$ stromal cells corresponded to that of macrophages in serial sections immunostained for CD68.

M30$^+$ apoptotic cancer cells Monoclonal antibody M30 CytoDEATH, recognizing caspase-degraded cytokeratin 18, sporadically labeled cancer cells in cancer nests as shown previously (Fig. 3g, arrowheads).[23] We also detected small, round, dissociated M30$^+$ bodies along the invasive margin, separately from cancer cell nests (Fig. 3f). Considering the specificity of M30 to epithelial cells, we assumed that these were apoptotic cancer cells. As shown in Fig. 3g, the areas of dissociated M30$^+$ cancer cells were overlapped with those of macrophages. The semiquantitative scoring confirmed a positive correlation between dissociated M30$^+$ cancer cells and CD68$^+$ macrophages (γ=0.5, P<0.01) in 58 cases analyzed with frozen sections. These macrophages expressed B7-2 (CD86) as shown previously,[3] but were negative for mature dendritic cell marker CD83. Double staining revealed that dissociated M30$^+$ cancer cells were distributed in the area with B7-2$^+$ cells along the invasive margin, and further confirmed that B7-2$^+$ large stromal cells, which were identified as macrophages in our previous study,[3] contained M30$^+$ bodies in their cytoplasm (Fig. 3h). Similar results were obtained by double staining for HLA-DR/M30 (data not shown) since these macrophages also expressed HLA-DR.[2]

Clinicopathologic analysis on dissociated M30$^+$ cancer cells in paraffin-embedded sections To analyze the relationship between dissociated M30$^+$ cancer cells and occurrence of hematogenous metastasis, we performed a clinicopathological study as described in "Materials and Methods." First, we confirmed a positive correlation between the semiquantitative scoring of dissociated M30$^+$ cancer cells and the number of macrophages along the invasive margin in 123 cases of colorectal cancer (γ=0.44, P<0.001; Fig. 4). As shown in Fig. 5, dissociated M30$^+$ cancer cells were less abundant in cases with metachronous hematogenous metastasis than in control cases (P<0.0005).

***In situ* nick end-labeling** Apoptotic cells were also identified among cancer cells and in the stroma. Double labeling of these cells and CD3-immunostaining revealed that positive apoptotic signals were also detected among CD3$^+$ T cells (data not shown).

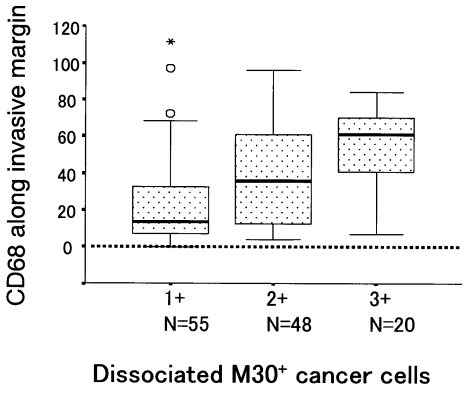

Fig. 4. Box-whisker plot showing a positive correlation between the semiquantitative scoring of dissociated M30$^+$ apoptotic cancer cells and the number of macrophages along the invasive margin of colorectal cancer (correlation coefficient, 0.44). A central horizontal bar in the box represents a median value. *X*-axis, subdivision of all metastasis and control cases into 3 by the scoring of dissociated M30$^+$ cancer cells along the invasive margin. Statistical test, Spearman's test. r=0.44.

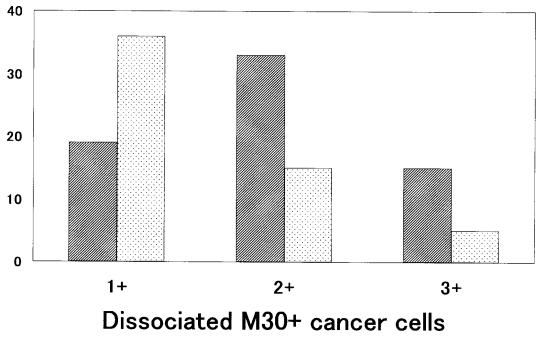

Fig. 5. Relationship between dissociated $M30^+$ apoptotic cancer cells and metachronous hematogenous metastasis. *X*-axis, subdivision of cases into 3 by the semiquantitative scoring of $M30^+$ cancer cells along the invasive margin. ▨, control group. □, group of metachronous hematogenous metastasis. *Y*-axis, number of cases. Statistical test, Kruskal-Wallis test. Metastasis<Control ($P<0.0005$).

DISCUSSION

The present study revealed that $CD68^+$ macrophages (tumor-associated macrophages) distributed along the invasive margin of colorectal cancer expressed FasL on their surfaces, and $M30^+$ apoptotic cancer cells were colocalized in the same area. Some of the macrophages were apparently phagocytosing $M30^+$ apoptotic bodies that were probably derived from cancer cells, and expressed the costimulatory molecule B7-2 on their surfaces simultaneously. There was a positive correlation between the degree of appearance of $M30^+$ apoptotic bodies and the numbers of $CD68^+$ macrophages along the invasive margins, and the degree of apoptotic bodies derived from cancer cells was inversely correlated with the hematogenous metastases.

Most of the previous histochemical studies on FasL were performed to substantiate the so-called counterattack theory, and they mainly focused on the localization of FasL protein in cancer cells, resulting in the demonstration of cytoplasmic localization of FasL in cancer cells.[24–27] However, the localization of FasL protein along the plasma membrane should be more important for the function of this receptor-ligand molecule. We tried to explore this discrepancy between the presumed function (attack on lymphocytes) and cytoplasmic localization of FasL by using pre-fixed frozen sections and mouse monoclonal antibody specific for human FasL, clone NOK-2.[20] With these methods, we successfully detected clear membrane staining in macrophages, rather than in cancer cells, and this was confirmed at the immunoelectron microscopic level. We further detected FasL mRNA expression in macrophages. Thus, we concluded that macrophages in the invasive margins of human colorectal cancer synthesize and express FasL on their cell surfaces. The expression of FasL in macrophages has been well documented.[28–30] The absence of staining for FasL protein in cancer cells despite the presence of its mRNA may be due to insufficient translation or rapid degradation of the protein in cancer cells. Similar absence of FasL protein on the cell surface despite the presence of FasL mRNA was reported in colon cancer cell lines.[14]

There may be at least two different explanations for the above correlations among the degree of cancer cell apoptosis, macrophage infiltration, and clinical behavior of cancer. The simplest interpretation is that the $FasL^+$ macrophages are effector cells inducing apoptotic death of Fas^+ cancer cells, and thereby suppressing the spread of cancer at the front line of host defense, the invasive margins. This explanation is consistent with the previous concept that activated macrophages display a killing activity on tumor cells.[31] The second explanation is that macrophages that phagocytose cancer cell-derived apoptotic bodies could present tumor antigens to T cells, thereby activating T cell-mediated effector mechanisms. This would include both local activation and proliferation of T lymphocytes through stimulation by these macrophages expressing B7-2, HLA-DR, CD11c and ICAM-1,[2,3] and through migration of tumor antigen-laden macrophages into draining lymph nodes followed by priming of naive T cells. The latter may involve presentation of macrophage-processed antigens by dendritic cells or differentiation of the migrated macrophages into mature dendritic cells.[32] If effector T cells are re-activated and proliferate locally, as we have suggested,[3] they may suppress the invasive growth of cancer cells at the front line, and thereby reduce the rate of metastases. On the other hand, if naive T cells are activated in draining lymph nodes by the stimulation of dendritic cells, they might have systemic effects in surveying hematogenous cancer cell spread and their settlement into target organs.[5]

Our present results suggest that the invasive margin (tumor-host interface) is important not only for the mechanism supporting tumor cell invasion,[33] but also for the host defense against tumor spread. It is necessary to take these two distinct aspects into consideration in interpreting the observations obtained from human cancer tissues.

The net function of tumor-associated macrophages has been generally regarded to be ambiguous, since they may be either inhibitory to or supportive of tumor growth.[34] Therefore, we need to analyze the functions of these macrophages in relation to their location, i.e., along the tumor-host interface in this case, and their detailed phenotypes, since tumor-associated macrophages could be heterogeneous in their function. $FasL^+$ macrophages may not only work as cytotoxic effectors against cancer cells but may also induce apoptosis in neighboring Fas^+ lymphocytes. Thus, apoptosis in tumor-infiltrating lymphocytes in colo-

rectal cancer as reported by Okada *et al.*[27] and observed in our study may be exerted by macrophages. This could be explained as a negative regulatory mechanism by macrophages against T cells distributed in the same areas.

We have also observed a positive correlation between FasL expression and neutrophil infiltration along the invasive margin. This correlation may be directly related to the proinflammatory effect of FasL, since overexpression of FasL could induce neutrophil infiltration to the locale as shown by experimental studies using FasL transfection transgene models.[16, 35] On the other hand, the observed accumulation of neutrophils may also reflect the possible production of chemokines and proinflammatory cytokines from activated macrophages or T-lymphocytes. In either case, neutrophils accumulating along the invasive margin may also be inhibitory to tumor spread.[36]

To conclude, the present study has shown that macrophages are a predominant source of FasL in human colorectal cancer. The observations shown here do not support the counter-attack theory, although we do not have enough evidence to negate this theory. Based on the clinicopathologic analyses, we propose that FasL$^+$ macrophages have the potential to suppress tumor spread. Further studies to elucidate the functions of these tumor-associated macrophages would be important to improve anti-tumor immunotherapy.

ACKNOWLEDGMENTS

The authors are grateful to Mr. Katsuhiko Ono, Mr. Takunori Sato, Mr. Kenji Honkura for their assistance in the present study. This work was partly supported by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology, Japan (#11670164) and a Grant from the Cancer Research Institute, NY.

(Received October 12, 2001/Revised December 6, 2001/ Accepted December 11, 2001)

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