ANTICANCER RESEARCH *24*: 1569-1580 (2004)

The Breakdown of Apoptotic Mechanism in the Development and Progression of Colorectal Carcinoma

SANEHITO OGAWA, MITSUO NAGAO, HIROMICHI KANEHIRO, MICHIYOSHI HISANAGA, SAIHO KO, NAOYA IKEDA and YOSIYUKI NAKAJIMA

Nara Medical University, 840 Shi Jo-cho, Kashihara-city, Nara 634-8522, Japan

Abstract. *Background: Fas (APO-1/CD95) is a cell surface receptor that mediates apoptosis when it reacts with Fas ligand (FasL) or Fas antibody. Alterations of Fas and FasL expression have been demonstrated in various carcinomas. Materials and Methods: We examined the alteration of Fas and FasL expression in seventy-eight specimens of colorectal adenoma and carcinoma by immunohistochemistry and real-time reverse-transcriptase polymerase chain reaction (RT-PCR). Results: Our study revealed that the expression of Fas was reduced in colorectal adenoma and completely lost in some 60% of colorectal carcinomas. Fas expression was significantly down-regulated in liver metastasis compared with corresponding primary colorectal carcinoma. The expression of Fas significantly related to p53 status, tumor location and apoptosis in colorectal carcinoma. Up-regulation of FasL was not detected in colorectal adenoma, carcinoma cells and liver metastatic cancer cells. Conclusion: These results indicate that Fas may play an important role, not only in development but also progression, and that FasL is not always required for both development and progression in colorectal carcinomas.*

Apoptosis or programmed cell death selectively allows certain cells to undergo cell death following biological signals and plays an important role in development and homeostasis. In malignant cells, the physiological apoptotic pathways are often disturbed and, as a result, they acquire uncontrolled survival. The Fas receptor (Fas)/ligand (FasL) system is regarded as a key system in the regulation of apoptosis in the immune system (1). Fas-mediated apoptosis is involved in tolerance acquisition (2), T cell activation-induced cell death (3), T cell-mediated cytotoxicity (4) and immune response termination (5). Cross-linking of Fas with either FasL (6) or activating antibody (7) induces apoptosis in Fas-bearing cells. Fas is constitutively expressed in various human organs, *e.g.*, heart, liver, lung, colorectum and kidney (8). Normal colonic epithelial cells are relatively sensitive to Fas-mediated apoptosis (9).

Many solid tumor cell lines have been shown to constitutively express low levels of Fas (10). Ligation of Fas by specific antibody has been shown to induce apoptosis in a variety of tumor cell lines (11, 10) and to mediate tumor regression *in vivo* (11). In colorectal cancer, partial or complete loss of Fas expression has been detected (8, 12) and this loss is supposed to result in resistance of the tumor cells toward T cell cytotoxicity (13).

FasL expression was first considered to be restricted to the immune system including activated T cells (6), B cells (14) and natural killer cells (15). However, it has been found that cells in immunologically privileged sites, such as the anterior chamber of the eye (16) and Sertori's cells of the testis (17), expressed FasL. Any activated T cell expressing Fas that enters such a site would encounter cells expressing FasL and receive a death signal, thereby preventing an immune response. It has been reported that tumor cells originated from various tissues *e.g.*, colon (13), skin (18), liver (19), lung (20) and esophagus (21), could express FasL and induce apoptosis of Fas-expressing T cells. Accordingly, it was proposed that tumor cells could counterattack activated tumor-infiltrating lymphocytes expressing Fas and escape from the host immune rejection (13). Furthermore, Shiraki *et al.* have demonstrated that hepatic metastatic tumors of colon cancer expressed FasL and they suggested that FasL might also be important in colonization in the liver through induction of apoptosis in the surrounding Fas-expressing hepatocytes (22). However, the various antibodies used in those reports have been clearly shown to lack specificity (23) and recent studies have demonstrated that human melanoma cells did

Correspondence to: Sanehito Ogawa. Nara Medical University, 840 Shi Jo-cho, Kashihara-city, Nara 634-8522, Japan. Tel: (81) 744-29-8863, Fax: (81) 744-24-6866, e-mail: ogawasan@nmu-gw.naramed-u.ac.jp

Key Words: Fas, FasL, colorectal carcinoma, carcinogenesis, metastasis.

not express FasL (24). Accordingly, it is controversial whether the counterattack by FasL-expressing cancer cells accounts for the mechanism of escaping from the host immune rejection.

Most colorectal carcinomas develop from adenomatous polyps. These adenomas become gradually more dysplastic over a period of several years and ultimately develop into cancer. This adenoma-carcinoma sequence is a relatively well-defined example of multiple steps of carcinogenesis (25). Although it has been reported that the adenomatous polyps in colorectum expressed FasL (26), the involvement of the Fas/FasL system in this sequence is unclear. Furthermore, wild-type (wt) p53, the mutation frequently occurring in colorectal carcinomas, would be essential for Fas expression *in vitro* and *vivo* (27, 28). The aims of this study were to confirm the alterations of Fas/FasL in the development and progression of colorectal carcinoma and to re-confirm the role of the Fas/FasL system in the tumor counterattack hypothesis.

Materials and Methods

Patients and tissue collections. Fifty-three patients (age 26-80, mean 61.1 years; 38 men, 15 women) with colorectal carcinoma (including 28 colorectal adenocarcinomas with synchronous liver metastasis and 2 metachronous liver metastases) and 25 patients (age 50-78, mean 65.5 years; 22 men, 3 women) with colorectal polyps were included in this study. All patients with colorectal adenocarcinoma were treated with surgical resection at the first Department of Surgery, Nara Medical Universal Hospital (Nara, Japan) between 1995 and 2000, and colon polyps were taken by polypectomy through a colon fiberscope. None of the patients with colorectal adenocarcinoma had received chemo-, radio-, or immunotherapy prior to surgery. Colorectal polyps were classified as adenoma with mild atypia (n=6, 24%), moderate atypia (n=17, 68%), severe atypia (n=1,4%) and carcinoma in adenoma (n=1, 4%). Seventeen polyps (68%), 7 (28%) and 1 (4%) were located in the proximal and medial part of the colon (from ceacum to descending colon), sigmoid colon and rectum, respectively. Staging of colorectal carcinoma was classified according to the TNM classification of malignant tumors defined by the International Union Against Cancer. Normal colorectal epithelial tissue samples were collected from the site that was most distant from the tumors. All samples were obtained with informed consent.

Cell culture. SW480, a human colon cancer cell line, was obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were cultured in Dulbecco's modified Eagle's medium-Ham's F12 medium 1:1 (DMEM/F12) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100units/ml) and streptomycin sulfate (100 μg/ml), unless otherwise specified. All media supplies were from Life Technologies, Inc. All cultures were maintained at 37˚C with 5% carbon dioxide and 100% humidity.

Immunohistochemical staining. Detection of Fas, FasL and p53 was performed using a monoclonal antibody directed against Fas (UB2: MBL Co., Nagoya, Japan; 10 μg/mL), FasL (NOK2: Pharmingen Co., San Diego, CA, USA; 1:20 dilution) and p53 (DO-7: Dako Co., Glostrup, Denmark; 1:60 dilution), respectively. The resected specimens were immediately snap-frozen in liquid nitrogen and stored at -80˚C until analyzed. Cryosections (5-μm thick) were air-dried and fixed for 10 minutes in cold acetone, and then washed in phosphate-buffered saline. The endogenous peroxidase activity was blocked with methanol containing 0.3% hydrogen peroxidase for 20 minutes. After another washing, the sections were incubated for 60 minutes at room temperature with the primary monoclonal antibody. Immunostaining was performed by a labeled polymer method using a Histofine Simple Stein-PO(M) kit (Nichirei Co., Tokyo, Japan). The staining was visualized with diaminobenzidine-tetrachloride. The sections were finally counterstained with hematoxylin. A negative control slide was already included in each immunostaining, in which the first antibody was replaced by normal serum. The immunohistochemical investigation of Fas and FasL were performed twice on each specimen, and the equivalent results for positivity could be obtained in all cases, although the intensity of Fas and FasL expression was slightly altered between the two tests.

Evaluation. Staining was evaluated by two of us (M.N., K.H.). With regard to Fas staining, normal colonic mucosa, normal liver and T-lymphocytes strongly expressed Fas and those served as intrinsic positive controls. We designed the following evaluation system and divided the mode of Fas staining into 3 categories: (i) Fas expression was regarded as normal when the entire neoplastic population was strongly stained as well as normal mucosa and no unreactive sub-sets were observed; (ii) Fas expression was regarded as reduced whenever a sub-set of unstained tumor cells was detectable and/or the antigenic density (corresponding to the staining intensity) was reduced compared with intrinsic positive controls; (iii) Fas expression was regarded as lost when the tumor cell compartment was unreactive to anti-Fas antibody throughout (Table I). FasL and p53 expression was evaluated as positive if the stained cells were distributed in more than 10% of the cancer cells (28).

Apoptosis. The TUNEL assay to detect the fragmented DNA *in situ* was performed twice on cryosections according to the manufacturer's instructions (Apoptaq Plus Peroxidase kit; Intergen Company, Purchase, NY, USA). In each examination, 1,000 to 1,500 tumor cells were counted and the apoptotic index was calculated as the mean of the 2 counts.

Ki-67 labeling index. Because there was variation in the number of Ki-67-positive cells among the microscopic fields, the sections were scanned under low power to determine the areas that were most evenly and heavily labeled. The Ki-67 labeling index (LI) was determined by observing 1,000 nuclei in the selected areas, then the value was used for the analysis. Immunohistochemistry of Ki-67 LI was also performed twice, and the mean of the two counts of each examination was considered to represent the Ki-67 LI.

Real-time RT-PCR of Fas and FasL gene expression. To confirm the expression of Fas and FasL, real-time RT-PCR was performed on total RNA extracted from colorectal polyps, colorectal cancers, corresponding liver metastases and

Table I. *Fas expression in normal colorectal mucosa, colorectal adenoma, colorectal carcinoma, normal liver and liver metastasis as determined by immunohistochemistry.*

Mode of Fas mucosa	Normal expression	Adenoma carcinoma	Colorectal liver	Normal metastasis	Liver
Normal	63	0	0	30	0
Reduced	0	25	22	0	8
Lost	0	0	31	0	22

Table II. *The relationship of Fas expression between colorectal carcinoma and corresponding liver metastasis.*

		Liver metastasis	
		Reduced	Lost
Colorectal carcinoma	Fas Reduced	8	6
	Fas Lost	0	16
			$p=0.0005$*

*Fisher's exact test.

noncancerous tissues of colon and liver. The total RNA was isolated from about 20 mg of those tissues using a RNAeasy Mini kit and Rnase-Free Dnase Set (QIAGEN Inc., Hilden, Germany) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized by extension of the (dT)12-18 primer with 4 units of Omniscript Reverse Transcriptase (QIAGEN Inc., Hilden, Germany) in a mixture containing 1 μg of total RNA according to the manufacturer's instructions. The primers used for amplification of Fas, FasL and β-actin have been described recently (Fas: (29), FasL: (30), β-actin: (31)). The absence of non-specific amplification and equality of size between the actual PCR amplification products and the expected products, comprising 118, 82, 101 nucleotides (each Fas, FasL and β-actin), was confirmed by analyzing the PCR products by agarose gel electrophoresis. The SYBR Green PCR Core Reagent Kit (PE Applied Biosystems) was used for the relative quantitation of both Fas and FasL mRNA by real-time RT-PCR. Relative quantitation of both targets was performed using the standard-curve method according to the manufacturer's protocol. The quantitation of both targets was also normalized to the level of the endogenous control, β-actin, to account for variability in the initial concentration and quality of the total RNA and in the conversion efficiency of the reverse-transcription reaction. For quantitation normalized to β-actin, standard curves were prepared for both targets and β-actin using SW480, colon cancer cell line. For each experimental sample, the amounts of both targets and β-actin were determined from the standard curves, and both target amounts were divided by the amount of β-actin to obtain both normalized target values. All real-time RT-PCR reactions were performed in duplicate using the ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems). The amplification cycles were 50˚C for 2 minutes, 95˚C for 10 minutes, 40 cycles at 95˚C for 15 seconds and 60˚C for 1 minute. The duplicate values of the same sample were almost equivalent.

Statistical analysis. The relationships among the variables were analyzed with the χ^2 test (Fisher exact test), Student's *t*-test, Mann-Whitney *U*-test and Wilcoxon matched pairs signed-rank test where appropriate. The disease-free survival and overall survival curves were estimated by the Kaplan-Meier method and comparisons made by the log-rank test. The Cox proportional hazards model was used to determined the tumor characteristics that were most significantly correlated with survival.

Results

Fas and FasL expression. Immunohistochemical staining with anti-Fas antibody showed that normal colon epithlium consistently expressed Fas antigen, *i.e.*, both goblet and columnar epithelial cells strongly expressed Fas in the cytoplasm and on the basolateral surface. There was no difference in expression density from proximal to distal colon and from the basal parts of the crypts to the mucosal surface, respectively (Figure 1a).

All adenomas tested in this study showed heterogeneity and reduction of Fas expression. Almost all adenomas contained areas of reduced Fas expression and lacked Fas expression in many foci (Figure 1b).

Fas expression in colorectal carcinoma was remarkably down-regulated compared with adenoma (Figure 1c). Out of 53 carcinoma, 31(58.5%) were completely devoid of Fas expression. The remainder showed reduction of Fas (Table I).

On the other hand, normal hepatocytes intensely expressed Fas in the cytoplasm and weakly on the surface. Fas expression in liver metastasis was lost in 22(73.4%) out of 30 liver metastases tested (Figure 1d). The other cases showed reduction of Fas expression. The loss of Fas expression in liver metastasis was more frequently recognized than that in corresponding primary colorectal carcinoma (Table II). There was no case with more intensive expression of Fas in the liver metastasis compared with the corresponding primary colorectal carcinoma.

FasL expression was not detected entirely in normal colon mucosa, colorectal adenoma and cancer of all cases tested (Figure 2). Many mononuclear cells infiltrating around and into tumor expressed Fas and FasL (Figures 1 and 2).

Expression of Fas and FasL mRNA. Although real-time RT-PCR revealed a positive expression of Fas and FasL mRNA in 68 samples selected at random from all cases tested in this study, the relative amounts of Fas and FasL mRNA varied. Those of Fas mRNA in 3 noncancerous tissues of

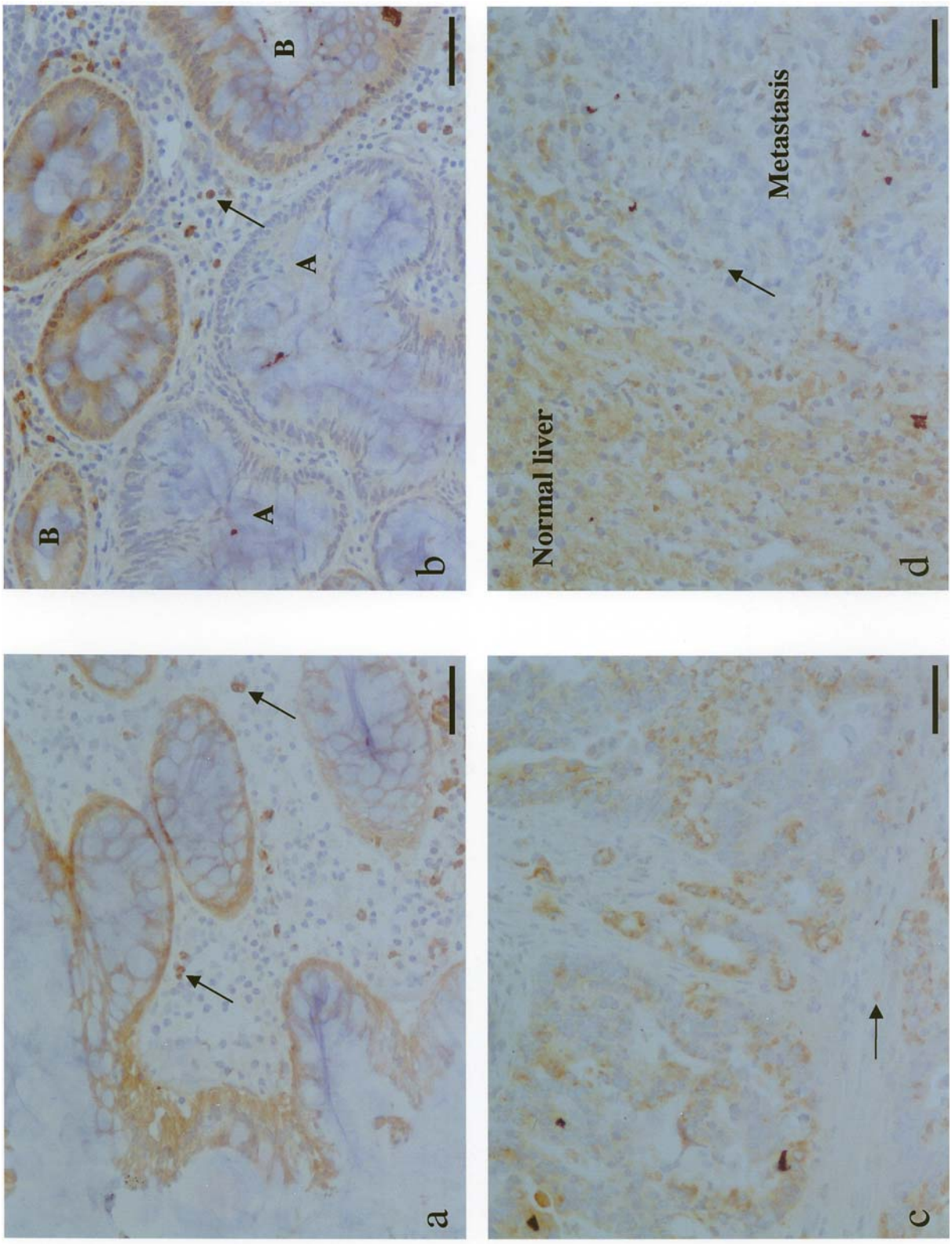

Figure 1. *Immunohistochemical staining for Fas in colon mucosa (a) colorectal adenoma (b) colorectal carcinoma (c) and liver metastasis (d).(a), Fas is detectable in equally high antigenic density in the cytoplasm and at the basolateral surface. (b), Fas expression is lost in some foci(A) and reduced in other foci(B). (c) Fas expression was regarded as reduced in this tumor, because of the presence of a subset of unstained tumor cells. (d) Normal hepatocyte strongly express Fas. Fas expression in liver metastasis was lost completely. Some infiltrating mononuclear cells were Fas-positive (arrows). Bar, 50 μm*

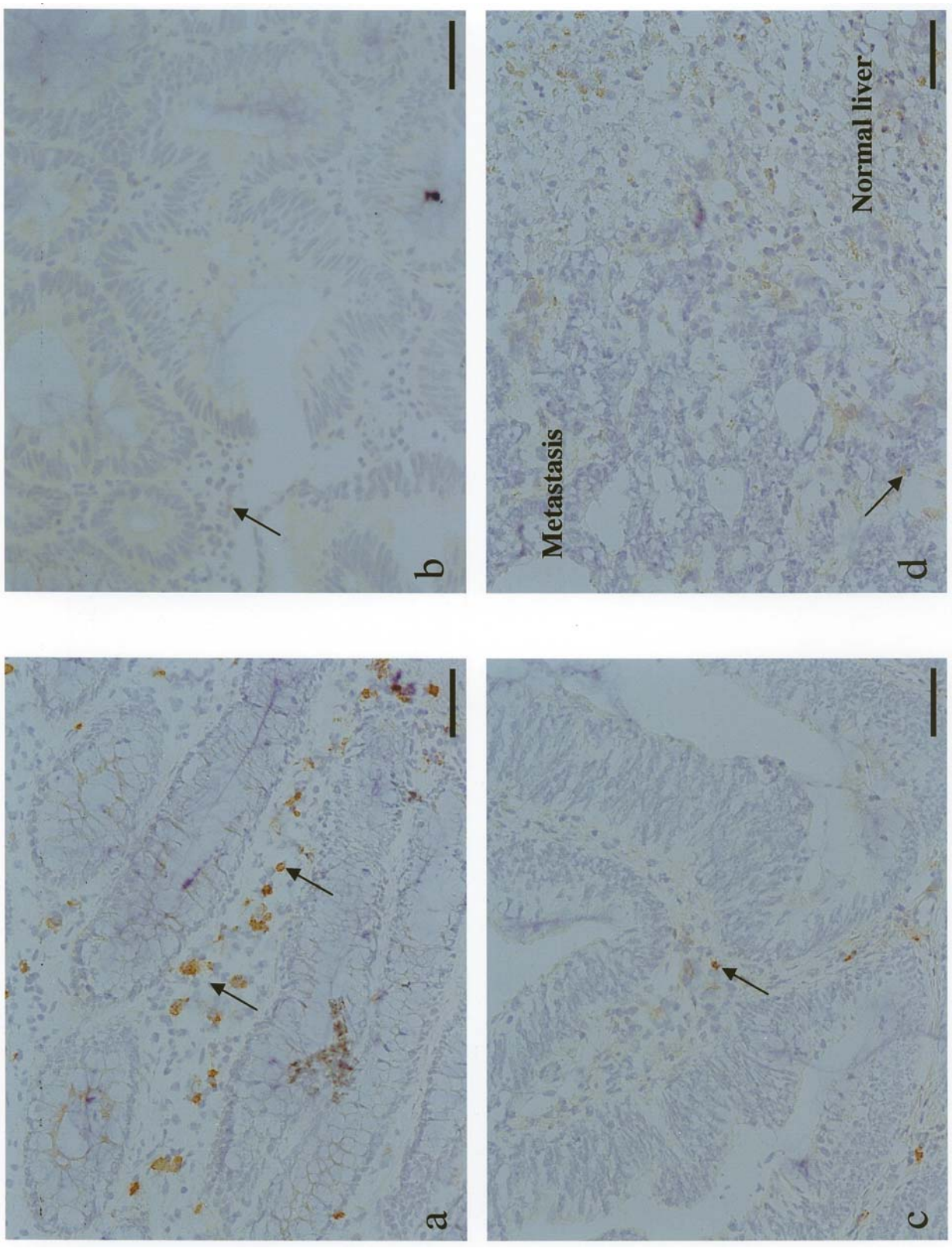

Figure 2. *Immunohistochemical staining for FasL in colon mucosa (a), colorectal adenoma (b), colorectal carcinoma (c) and liver metastasis (d). FasL expression was not detectable in all cases. Some infiltrating mononuclear cells were FasL-positive (arrows). Bar, 50 μm*

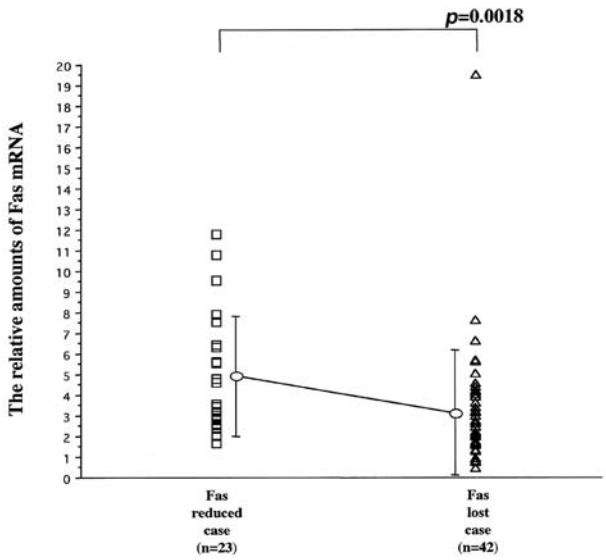

Figure 3. *Relationship between the relative amounts of mRNA and protein expression in Fas. Relative amounts of Fas mRNA significantly differed between the groups divided by the immunohistochemistry (Mann-Whitney U-test). The values are expressed as mean±SD.*

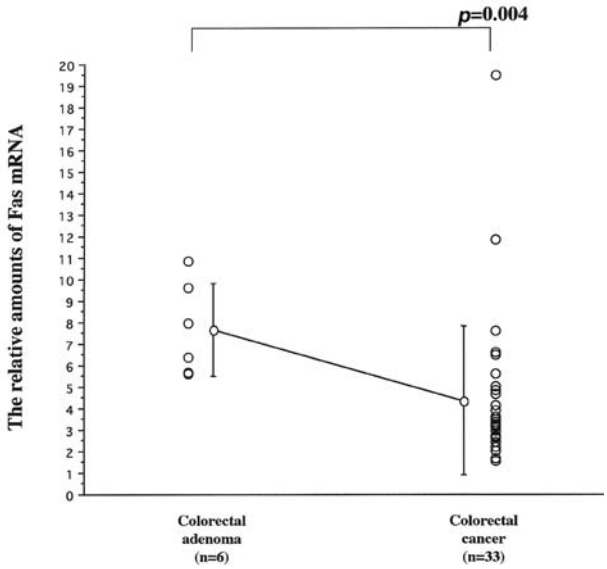

Figure 4. *The relative amounts of Fas mRNA in colorectal adenoma and carcinoma. The relative amounts of Fas mRNA in carcinoma are significantly lower than those in adenoma (Mann-Whitney U-test). The values are expressed as mean±SD.*

colon and liver ranged from 3.67 to 4.76 and from 4.59 to 6.22, respectively. The median amount (the range) of Fas mRNA in 23 regarded as reduced by immunohistochemistry and 42 cases regarded as lost was 3.50 (1.66 to 11.7) and 2.44 (0.36 to 19.3), respectively. The results of real-time RT-PCR were in accord with those of immunohistochemistry (Figure 3). The relative amounts of Fas mRNA in colorectal carcinoma (median 3.20, range 1.44-19.3) were significantly more reduced than those in colorectal adenoma (median 7.89, range 5.51-10.7) ($p=0.004$) (Figure 4), which was similar to the result of immunohistochemistry. The relative amounts of Fas mRNA in liver metastasis (median 1.93, range 0.36-5.56) were significantly more reduced than those in corresponding primary colorectal carcinoma (median 3.24, range 1.45-19.38) ($p=0.009$) (Figure 5).

On the other hand, the relative amounts of FasL mRNA in 3 noncancerous tissues of colon and liver ranged from 0.45 to 0.53 and from 0.65 to 2.65, respectively. Those of FasL mRNA in colorectal carcinoma (median 0.38, range 0.07-1.83) did not increase compared with those in colorectal adenoma (median 1.04, range 0.44-4.11) and normal colorectal epithelium (data not shown). No correlation between liver metastasis (median 0.29, range 0.02-8.84) and corresponding primary colorectal carcinoma (median 0.33, range 0.07-1.83) was recognized in the relative amounts of FasL mRNA ($p=0.87$) (Figure 6). In two liver metastases with high relative amounts of FasL mRNA, immunohistochemistry study revealed strong infiltration of FasL-bearing lymphocytes into the tumors.

Clinicopathological findings and protein expression. Table III shows the relationship between Fas status and clinicopathological features in colorectal carcinoma. The expression of Fas significantly correlated with tumor location ($p=0.004$). Actually in only 3 out of 13 right colon cancers (Ceacum ~ Descending colon), Fas expression was lost. On the other hand, in 14 out of 20 left colon cancers (both Sigmoid colon and Rectum), Fas expression was lost. The other variables did not correlate with the status of Fas expression. Furthermore, there was no relationship between disease-free survival rate and the status of Fas expression (data not shown). There was no significant relationship between Fas status and clinicopathological features of both colorectal adenoma and liver metastasis (data not shown).

Apoptosis, Ki67 and p53 status. The cases with reduced expression of Fas showed a significantly higher apoptotic index than those with loss of Fas in both colorectal carcinoma ($p=0.0019$) and hepatic metastasis ($p=0.026$) (Figure 7). No correlation between Fas expression and Ki67 labeling index was recognized in both colorectal carcinoma (Table III) and hepatic metastasis (data not shown). p53-positive cases were observed in 27 out of 53 colorectal carcinomas. Twenty cases out of 27 cases with p53-positive expression lost Fas expression completely and, conversely, 20 out of 31 cases with loss of Fas were p53-positive. There was significant correlation between Fas and p53 status in colorectal carcinoma (Table IV).

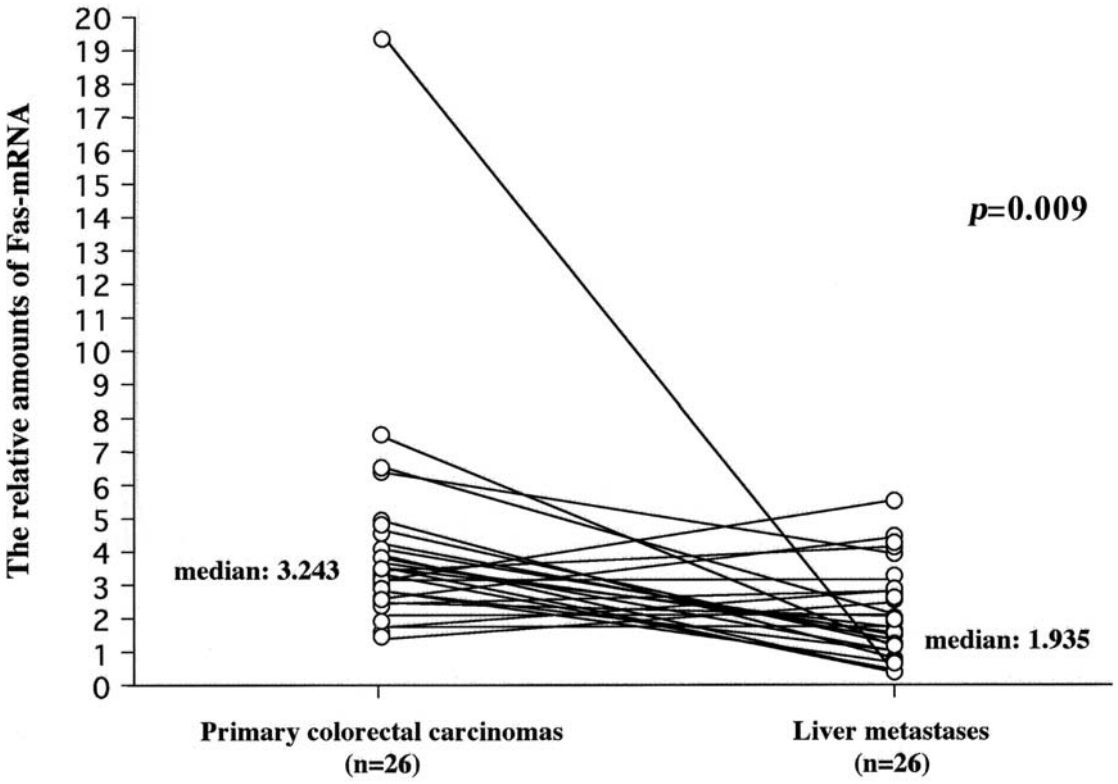

Figure 5. *Relationship between primary colorectal carcinomas and corresponding liver metastases in the relative amounts of Fas mRNA. The relative amounts of Fas mRNA in liver metastasis are significantly lower than those in corresponding primary colorectal carcinoma (Wilcoxon matched pairs signed-rank test).*

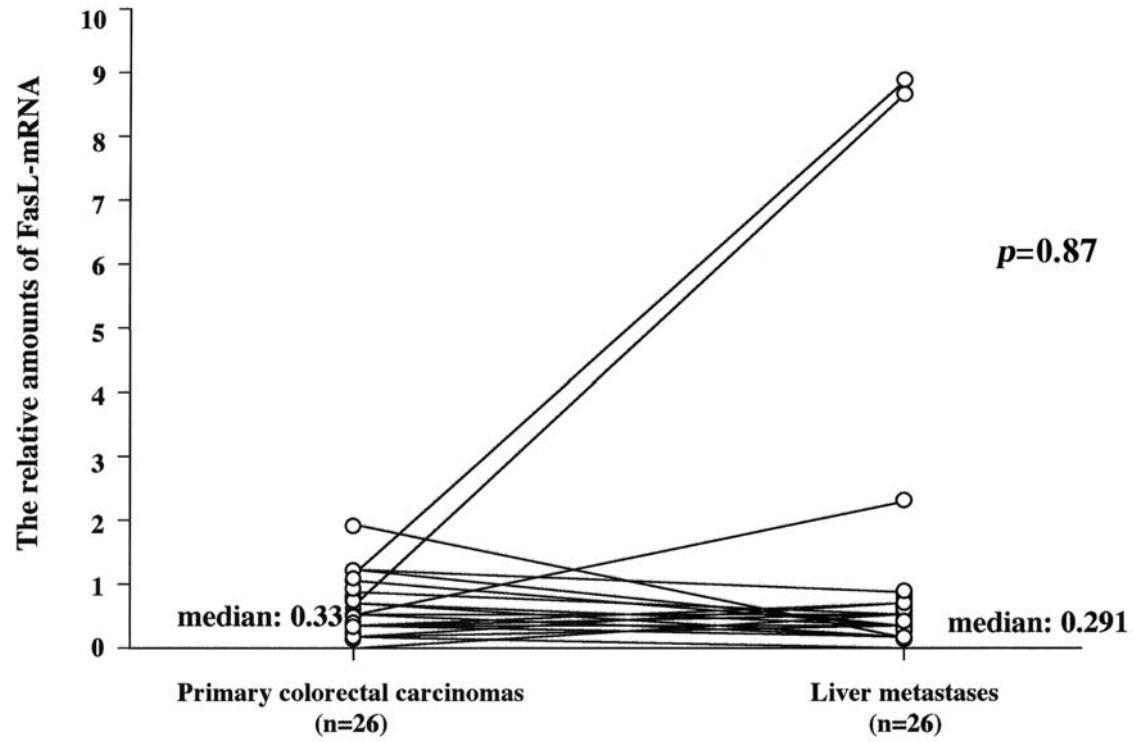

Figure 6. *Relationship between primary colorectal carcinomas and corresponding liver metastases in the relative amounts of FasL mRNA. No correlation between liver metastasis and corresponding primary colorectal carcinoma was recognized (Wilcoxon matched pairs signed-rank test).*

Table III. *Clinocopathological features of colorectal carcinoma with regard to Fas expression in colorectal carcinoma.*

Factor	Fas reduced (n=22;41.5%)	Fas lost (n=31;58.5%)	*p*
Gender (male/female)	14 / 8	24 / 7	0.357†
Age (mean ± SD)	57.8 ± 12.5	63.3 ± 10.1	0.082§
CEA / CA19-9 (median)	23.5 ng/ml / 43.35 ng/ml	15.0 ng/ml / 18.8 ng/ml	0.860§§ / 0.692§§
(range)	(1.6 ~ 1033.8) / (1.0 ~ 4116.5)	(1.0 ~ 1146.0) / (1.0 ~ 572.0)	
Tumor size (mean ± SD)	5.49 ± 1.79	5.04 ± 1.86	0.376§
Location (the right colon / the left colon)*	10 / 12	3 / 28	0.004†
Differentiation degree (well/mod/poor)	6 / 16 / 0	11 / 16 / 4	0.131†
Depth of invasion (≤T2 / ≥T3)	1 / 21	5 / 26	0.381†
Lymph nodes metastasis (n- / n+)	9 / 13	14 / 15	0.381†
Distant metastasis (M0 / M1)	8 / 14	17 / 14	0.265†
Ki67 labeling index (mean ± SD)	33.43 ± 9.64	33.19 ± 11.17	0.936§

* the right colon, ceacum ~ descending colon; the left colon, sigmoid colon and rectum
† χ^2 test or Fisher's exact test.
§ Student's test.
§§ Mann-Whitney *U*-test.

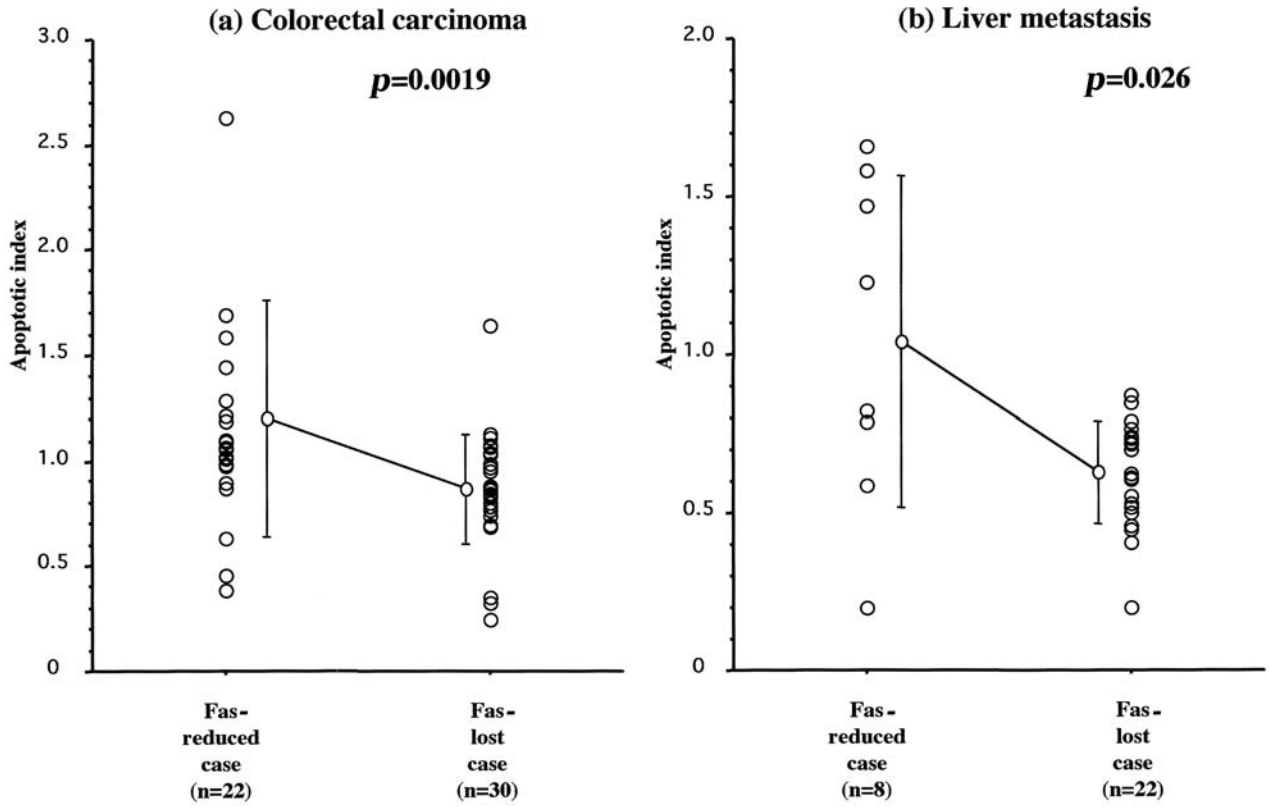

Figure 7. *Relationship between apoptosis and status of Fas expression. Apoptotic index in Fas-reduced cases was significantly higher than that in Fas-lost cases in both colorectal carcinoma (a) and liver metastasis (b) (Mann-Whitney U-test). The values are expressed as mean±SD.*

Table IV. *The relationship between Fas status and p53 status in colorectal carcinoma.*

		Fas	
		reduced (n=22)	lost (n=31)
p53	positive (n=27)	7	20
	negative (n=26)	15	11
			$p=0.026$*

*Fisher's exact test.

Discussion

This study demonstrated that Fas expression was already reduced in colorectal adenoma and that Fas expression was more reduced in colorectal carcinoma compared with adenoma. Furthermore, our study revealed that loss of Fas expression was recognized more frequently in left colon cancer than right and that loss of Fas expression significantly correlated with mutant p53 in colorectal carcinoma. Generally, most colorectal carcinomas develop from adenomatous polyps and the accumulation of multiple gene alterations is associated with multiple steps of carcinogenesis in colorectal carcinoma. The adenoma-carcinoma sequence is supposed to relate to carcinogenesis of the left colon cancer and mutation of p53 is thought to occur at the late event of the adenoma-carcinoma sequence. The previous reports demonstrated that wt-p53 would be essential for Fas expression *in vitro* and *vivo* (27, 28). Taken together, Fas might play an important role in the adenoma-carcinoma sequence under the control of p53. However, this study demonstrated that Fas expression was completely lost in 11 out of 26 p53-negative cases in colorectal carcinoma. This implies that other factors (*e.g.*, IFN-γ and TNF-α) apart from wt p53 may also be involved in Fas expression (12), although wt p53 may be necessary for Fas expression.

The present study demonstrated that Fas expression was much more down-regulated in liver metastasis than corresponding primary colorectal carcinoma. The metastatic mechanism is a complex series of processes including angiogenesis, intravasation of tumor cells, transport by the circulation, adhesive interaction with the endothelial cells and growth as colonies inside the vessel (32). Beside these processes of metastasis, an evasion of the host immune surveillance may also be important for the metastasis of cancer cells, because cancer cells are directly exposed to the host immune surveillance when they enter the circulating blood to be transported by the circulation. Therefore, metastatic cancer cells entering the blood would undergo apoptosis by lymphocytes expressing FasL when metastatic cancer cells express Fas. This suggests that Fas may play a role in a metastatic mechanism to evade the host immune surveillance. However, this study showed some metastatic cancer cells expressed Fas, although many cells lost Fas expression. These cancer cells may acquire diverse mechanisms to escape Fas-mediated apoptosis without down-regulation of Fas expression (33). Indeed, Cohen *et al.* have reported that death-associated protein kinase (DAP-K) played an important role in apoptosis induced by Fas (34). Inbal *et al.* have reported that hypermethylation of DNA encoding DAP-K reduced the activities of this protein and provided cancer cells with metastatic capacity (35). Therefore, the hypermethylation of DAP-K might result in resistance to Fas-mediated apoptosis in case with Fas expression. Studies are in progress to investigate the relationship of the expression of DAP-K, Fas and apoptosis.

Our present study did not detect FasL expression in colorectal adenoma, carcinoma and liver metastasis except for tumor-infiltrating lymphocytes (TILs). Furthermore, FasL-mRNA in colon adenoma, cancer and liver metastasis did not up-regulate compared with normal colorectal epithelium. These results do not agree with the previous reports that showed FasL expression in colorectal adenomas, carcinomas and liver metastases (26, 22). The reason for the difference may be the specificity of the antibody for FasL. Indeed, we confirmed that a polyclonal antibody against FasL (a rabbit polyclonal anti-human FasL, Santa Cruz Biotechnology, Santa Cruz, CA, USA) used in those previous reports reacted to intrahepatic bile duct as well as cancer cells and weakly reacted to muscle layer in colorectum and normal hepatocytes (data not shown). Those reaction are non-specific because it is generally believed that normal cells expressing FasL may be limited to activated lymphocytes and cells at immunologically privileged sites, such as the testis, eye and brain. The other study showed that human colon cancer cells did not induce apoptosis of Fas-expressing target cells and that FasL-protein was not detected on the surface of colon cancer cells (36). Furthermore, we revealed that there was no up-regulation of FasL-mRNA in cancerous tissues of colorectum or liver compared with its corresponding normal tissues. These results suggest that the counter-attack by FasL-expressing cancer cells may not be related to colorectal carcinogenesis and a metastatic mechanism. This study revealed both positive expression of Fas and FasL on tumor-infiltrating lymphocytes (TILs) in almost all cases tested. Therefore, activated TILs may try to kill the tumor cells using FasL, but the tumor cells reducing Fas expression may dexterously escape from FasL-expressing TILs and survive. It is possible that TILs

up-regulate Fas by reactivation and undergo apoptosis in the event of failure to kill tumor cells, because the outcome of recurrent interaction with antigenic tumor cells can be expected to lead to the deletion of TIL by activation-induced cell death (37). In fact, we detected apoptosis of TILs in this study (data no shown). Furthermore, recent studies have shown that FasL is expressed by T lymphocytes upon activation after tumor cell recognition, causing them to kill themselves and each other (24, 38). Therefore, it may not be necessary that colorectal cancer cells express FasL in order to escape from the host immune surveillance.

In summary, we revealed that down-regulation of Fas expression was already recognized in colorectal adenoma and that Fas expression was progressively reduced during cancer progression. On the other hand, we showed that FasL expression was not up-regulated in a series of adenoma-carcinoma sequence and liver metastases. We conclude that the alteration of Fas but not FasL expression is related to the development and the progression of colorectal carcinoma.

References

1 Nagata S and Golstein P: The Fas death factor. Science *267*: 1449-1456, 1995.

2 Mountz JD, Zhou T, Bluethmann H, Wu J and Edwards CK: Apoptosis defects analyzed in TcR transgenic and Fas transgenic *lpc* mice. Int Rev Immunol *11*: 321-342, 1994.

3 Alderson MR, Tough TW, Davis-Smith T, Braddy S, Falk B, Schooley KA, Goodwin RG, Smith CA, Ramsdell F and Lynch DH: Fas ligand mediates activation-induced cell death in human T lymphocytes. J Exp Med *181*: 71-77, 1995.

4 Ju ST, Cui H, Panka DJ, Ettinger R and Marshak-Rothstein A: Participation of target Fas protein in apoptosis pathway induced by CD4+ Th1 and CD8+ cytotoxic T cells. Proc Natl Acad Sci *91*: 4185-4189, 1994.

5 Daniel PT and Krammer PH: Activation induces sensitivity toward APO-1 (CD95) –mediated apoptosis in human B cells. J Immunol *152*: 5624-5632, 1994.

6 Suda T, Takahashi T, Golstein P and Nagata S: Molecular cloning and expression of the Fas ligand, a novel member of tumor necrosis factor family. Cell *75*: 1169-1178, 1993.

7 Itoh N, Yonehara S, Ishii A, Yonehara M, Mizushima S, Sameshima M, Hase A, Seto Y and Nagata S: The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. Cell *66*: 233-243, 1991.

8 Leithauser F, Dhein J, Mechtersheimer G, Koretz K, Bruderlein S, Henne C, Schmidt A, Debatin K-M, Krammer PH and Moller P: Constitutive and induced expression of APO-1, a new member of the nerve-growth-factor/tumor-necrosis-factor-receptor superfamily, in normal and neoplastic cells. Lab Invest *69*: 415-429, 1993.

9 S Strater J, Wellisch I, Riedl S, Walczak H, Koretz K, Tandara A, Krammer PH and Moller P: CD95 (APO-1/Fas)-mediated apoptosis in colon epithelial cells: a possible role in ulcerative colitis. Gastroenterology *113*: 160-167, 1997.

10 Owen-Schaub LB, Radinsky R, Kruzel E, Berry K and Yonehara S: Anti-Fas on non-hematopoietic tumors: levels of Fas/APO-1 and bcl-2 are not predictive of biological response. Cancer Res *54*: 1580-1586, 1994

11 Trauth BC, Klas C, Peters AM, Matzku S, Moller P, Falk W, Debatin KM and Krammer PH: Monoclonal-antibody-mediated tumor regression by induction of apoptosis. Science *245*: 301-305, 1989.

12 Moller P, Koretz K, Leithauser F, Bruderlein S, Henne C, Quentmeiner A and Krammer PH: Expression of APO-1(CD95), a member of NGF/TNF receptor superfamily, in normal and neoplastic colon epithelium. Int J Cancer *57*: 371-377, 1994.

13 O'Connell J, O'Sullivan GC, Collins JK and Shanahan F: The Fas counterattack: Fas-mediated T cell killing by colon cancer cells expressing Fas ligand. J Exp Med *184*: 1075-1082, 1996.

14 Hahne M, Renno T, Schroeter M, Irmler M, French L, Bornard T, MacDonald HR and Tschopp J: Activated B cells express functional Fas ligand. Eur J Immunol *26*: 721-724, 1996.

15 Loughran TPJ: Clonal diseases of large granular lymphocytes. Blood *82*: 1-14, 1993.

16 Griffith TS, Brunner T, Hetcher SM, Green DR and Ferguson TA: Fas ligand-induced apoptosis as a mechanism of immune privilege. Science *270*: 1189-1192, 1995.

17 Donald B, Daniel G, Helena S, Jodena M, Alex F and Richard CD: A role for CD95 ligand in preventing graft rejection. Nature *377*: 630-632, 1995.

18 Hahne M, Rimoldi D, Schroter M, Romero P, Schreier M, French LE, Schneider P, Bornand T, Fontana A, Lienard D, Cerottini J and Tschopp J: Melanoma cell expression of Fas (APO-1/CD95) ligand: implications for tumor immune escape. Science *274*: 1363-1366, 1996.

19 Strand S, Hofman WJ, Hug H, Muller M, Otto G, Strand D, Mariani SM, Stremmel W, Krammer PH and Galle PR: Lymphocyte apoptosis induced by CD95 (APO-1/Fas) ligand-expressing tumor cells-a mechanism of immune evasion? Nat Med *2*: 1361-1366, 1996,

20 Niehans GA, Brunner T, Frizelle SP, Liston JC, Salerno CT, Knapp DJ, Green DR and Kratzke RA: Human lung carcinomas express Fas ligand. Cancer Res *57*: 1007-1012, 1997.

21 Gratas C, Tohma Y, Barnas C, Taniere P, Hainaut P and Ohgaki H: Up-regulation of Fas (APO-1/CD95) ligand and down-regulation of Fas expression in human esophageal cancer. Cancer Res *58*: 2057-2062, 1998.

22 Shiraki K, Tsuji N, Shioda T, Isselbacher KJ and Takahashi H: Expression of Fas ligand in liver metastases of human colonic adenocarcinomas. Proc Natl Acad Sci USA *94*: 6420-6425, 1997.

23 Fiedler P, Schaetzlein CE and Eibel H: Constitutive expression of FasL in thyrocytes. Science *279*: 2015a, 1998. .

24 Chappell DB, Zaks TZ, Rosenberg SA and Restifo NP: Human melanoma cells do not express Fas (APO-1/CD95) ligand. Cancer Res *59*: 59-62, 1999.

25 Vogelstein B, Fearon ER, Hamilton SR, Kern SE, Preisinger AC, Leppert M, Nakamura Y, White R, Smith AMM and Bos JL: Genetic alterations during colorectal-tumor development. N Engl J Med *319*: 525-532, 1988.

26 Bennett MW, O'Connell J, Houston A, Kelly J, O'Sullivan GC, Collins JK and Shanahan F: Fas ligand upregulation is an early event in colonic carcinogenesis. J Clin Pathol *54*: 598-604, 2001.

27 Owen-Schaub LB, Zhang W, Cusack JC, Angelo LS, Santee SM, Fujiwara T, Roth JA, Deisseroth AB *et al*: Wild-type human p53 and a temperature-sensitive mutant induce Fas/APO-1 expression. Mol Cell Biol *15*: 3032-3040, 1995.
28 Nagao M, Nakajima Y, Hisanaga M, Kayagaki N, Kanehiro H, Aomatu Y, Ko S, Yagita H, Yamada T, Okumura K and Nakano H: The alteration of Fas receptor and ligand system in hepatocellular carcinoma: How do hepatoma cells escape from the host immune surveillance *in vivo*? Hepatology *30*: 413-421, 1999.
29 Das H, Koizumi T, Sugimoto T, Chakraborty S, Ichimura T, Hasegawa K and Nishimura R: Quantitation of Fas and Fas ligand gene expression human ovarian, cervical and endometrial carcinoma using real-time quantitative RT-PCR. Br J Cancer *82*: 1682-1688, 2000.
30 Reimer T, Herrnring C, Koczan D, Richter D, Bernd G, Kabelitz D, Friese K and Thiesen HJ: FasL: Fas ratio-A prognostic factor in breast carcinomas. Cancer Res *60*: 822-828, 2000.
31 Nagao M, Nakajima Y, Kanehiro H, Hisanaga M, Aomatu Y, Ko S, Tatekawa Y, Ikeda N, Kanokogi H, Urizono Y, Kobayasi T, Shibaji T, Kanamura T, Ogawa S and Nakano H: The impact of interferon gamma receptor expression on the mechanism of escape from host immune surveillance in hepatocellular carcinoma. Hepatology *3*: 491-500, 2000.
32 Al-Mehdi AB, Tozawa K, Fisher AB, Shientag L, Lee A and Muschel RJ: Intravascular origin of metastasis from the proliferation of endothelium-attached tumor cells: a new model for metastasis. Nat Med *6*: 100-102, 2000.
33 von Reyher U, Strater J, Kittstein W, Gschwentdt M, Krammer PH and Moller P: Colon carcinoma cells use different mechanisms to escape CD95-mediated apoptosis. Cancer Res *58*: 526-534, 1998.
34 Cohen O, Inbal B, Kissil JL, Raveh T, Berissi H, Spivak-Kroizaman T, Feinstein E and Kimchi A: DAP-kinase participates in TNF-α- and Fas-induced apoptosis and its function requires the death domain. J Cell Biol *146*: 141-148, 1999.
35 Inbal B, Cohen O, Polak-Charcon S, Kopolovic J, Vadai E, Eisenbach L and Kimchi A: DAP kinase links the control of apoptosis to metastasis. Nature *390*: 180-184, 1997.
36 Favre-Felix N, Fromentin A, Hammann A, Solary E, Martin F and Bonnotte B: Cutting edge: The tumor counterattack hypothesis revisited: Colon cancer cells do not induce T cell apoptosis *via* the Fas (CD95, APO-1) pathway. J Immunol *164*: 5023-5027, 2000.
37 Lenardo MJ: The molecular regulation of lymphocyte apoptosis. Semin Immunol *9*: 1-5, 1997.
38 Zaks TZ, Chappel DB, Rosenberg SA and Restifo NP: Fas-mediated suicide of tumor-reactive T cells following activation by specific tumor: selective rescue by caspase inhibition. J Immunol *162*: 3273-3279, 1999.

Received April 7, 2003
Accepted November 25, 2003