AINO JOURNAL, Vol. 3, 2004, pp. 61 – 66

Review

The alteration of Fas receptor and ligand system in hepatocellular carcinoma

—— How do hepatoma cells escape from the host immune surveillance in vivo? ——

Hiroshige NAKANO *M. D. Aino University, Osaka, Japan*
Mitsuo NAGAO *M. D. Department of Surgery, Nara Medical University, Nara, Japan*

Key words: apoptosis, intrahepatic metastasis, p53, soluble Fas, soluble FasL

Introduction

The escape from the immune system reacting to the tumor cells may play an important role in tumor outgrowth and metastasis. The tumor cells escape from immunological rejection by a diversity of mechanisms. Fas receptor (Fas)/ligand (FasL) system is regarded as one of such mechanisms. Fas/FasL system plays an important role in T lymphocyte cytotoxicity as well as B- and T-lymphocyte development and maturation[1-12]. Cross-linking of Fas with either FasL or with activating antibody induces apoptosis of Fas-bearing cells[13-16]. Fas is constitutively expressed in various human organs, e.g., heart, liver, lung and kidney[17].

Many solid-tumor cell lines have been shown to constitutively express low levels of Fas[17, 18]. Ligation of Fas by specific antibody has been shown to induce apoptosis in a variety of hematopoietic and non-hematopoietic tumor cell lines, and to mediate tumor regression in vivo[18-20]. In hepatocellular carcinoma (HCC), partial or complete loss of Fas expression has been detected[21]. Midis et al.[22] have reported that soluble Fas (sFas), generated by alternative mRNA splicing and antagonizing FasL killing in a dose dependent manner[23-26], is increased in patients with solid tumors in a manner reflective of the disease stage and tumor burden. They proposed the hypothesis that sFas can be synthesized and released both systemically and locally within the tumor microenvironment. In patients with HCC, elevation of the serum sFas levels has been reported[27].

It has been found that cells in immunologically privileged sites, such as Sertori's cells of the testis and parenchymal cells of the anterior chamber of the eye, express FasL[28, 29]. Any activated T cell bearing Fas that enters such a site would encounter cells expressing FasL and receive a death signal, thereby preventing an immune response. Recent studies have demonstrated that tumor cells express FasL as possible means of actively destroying T cell and creating immune privileged site[30-34]. In HCC, expression of the functional FasL has been also detected, and production of soluble FasL (sFasL) acting in a paracrine fashion has been suggested[32]. Indeed, the presence of sFasL has been described in serum samples from patients with hematological malignancies[35]. However, the relationship between Fas/FasL system including sFas/sFasL and the clinicopathological features including hepatic metastasis and outcome has been scarcely studied and less attention has been paid to the correlation of Fas/FasL expression and the role of Fas/FasL interaction in vivo. In addition, a study using transduction of p53 has demonstrated that the wild-type p53 activity could induce Fas expression[36]. Therefore, we investigated the tissue expression of Fas, FasL and p53 and the alteration of sFas and sFasL in patients with HCC to reveal what advantage is achieved by alteration of Fas/FasL system involving modulation of the soluble forms and whether Fas expression is dependent on the wild-type p53 activity in vivo.

Materials and Methods

Forty-four patients suffering from HCC received radical resection at the first department of surgery, Nara Medical University Hospital from 1994 to 1998 and these cases were enrolled in this study. In all cases, HCC was confirmed histologically, and the noncancerous liver tissues were diagnosed as chronic hepatitis or liver cirrhosis. The patients after hepatic resection were strictly followed up. The median follow-up period for all patients was 25.7 months with a range of 3–49 months.

PCR analysis of Fas and FasL mRNA

RT-PCR was performed on total RNA extracted from the noncancerous liver tissues and HCCs in 20 cases selected at random from 44 cases. The primers used for amplification of Fas and FasL have been described recently[40, 41]. Amplification of human β-actin served as a control for sample loading and integrity. The PCR products were analyzed on a 2% agarose gel and visualized by ethidium bromide staining.

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, USA ; 1 : 20 dilution), and p53 (DO-7 : Dako Co., Glostrup, Denmark ; 1 : 60 dilution), respectively. Immunostaining was performed by the streptavidin-biotin (SAB) method using a Histofine SAB-PO(M) kit (Nichirei Co., Tokyo, Japan). The specimen was evaluated as Fas-positive when it stained as intensively as the normal liver tissues. FasL and p53 expression was evaluated as positive if the stained cells were distributed in more than 10% of the cancer cells.

Apoptosis

TUNEL assay to detect the fragmented DNA in situ was performed twice on cryosections according to the manufacturer's instructions (Apoptag Plus Peroxidase kit ; Oncor, Gaithersburg, USA).

Measurement of sFas and sFasL

Serum concentration of sFas and sFasL was measured in 37 patients with HCC, 16 patients bearing liver cirrhosis (LC) without HCC, and 31 healthy volunteers. The serum concentrations of sFas and sFasL were measured by sandwich ELISA as described previously[38, 42].

Results

Fas and FasL expression

Fas expression of the noncancerous liver tissues was upregulated compared with the normal liver tissues. FasL expression on hepatocytes of the noncancerous liver tissues was detected in six (16.2%) cases although that was not detected in the normal liver. Many mononuclear cells infiltrating into or around HCC expressed Fas and FasL.

Fas expression was positive in 15 (34.1%) of 44 HCCs tested. FasL expression was positive in 19 (43.2%) of 44 HCCs. FasL expression was mainly observed on the periphery or the perivascular area of the tumor. An inverse relationship was noted between Fas and FasL expression in HCCs (Table 1).

RT-PCR revealed that HCCs expressed Fas in 7 (35%) cases, and FasL in 11 (55%) cases of 20 cases tested (Fig. 1). The analysis of Fas and FasL mRNA expression by RT-PCR was

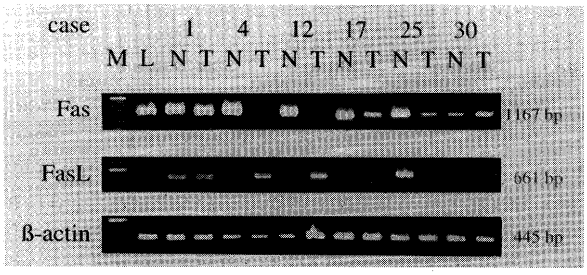

Fig. 1 Representative cases of Fas and FasL expression in human hepatocellular carcinomas detected by RT-PCR. Lane M, molecular marker; lane L, normal liver bearing a metastatic liver tumor; lanes N and T, noncancerous liver tissues and HCC, respectively. Expression of ß-actin served as a posivive control

Table 1 The relationship between Fas and FasL expression in HCC

	Fas positive (n=15 ; 34.1%)	Fas negative (n=29 ; 65.9%)
FasL positive (n=19 ; 43.2%)	2	17
FasL negative (n=25 ; 56.8%)	13	13

p=0.005*

*Fisher exact test

equivalent to the positivity of Fas and FasL expression by immunohistochemistry.

Clinicopathological findings and Fas/FasL expression

Table 2 shows the relationship between Fas status and the clinicopathological features. None of Fas positive cases showed intrahepatic metastatic foci, but eight cases of Fas negative cases showed intrahepatic metastatic foci ($p=0.034$). The disease-free survival rate of Fas positive cases was significantly higher than that of Fas negative cases (Fig. 2).

Table 3 shows the relationship between FasL status and the clinicopathological features. FasL positive cases were more frequent

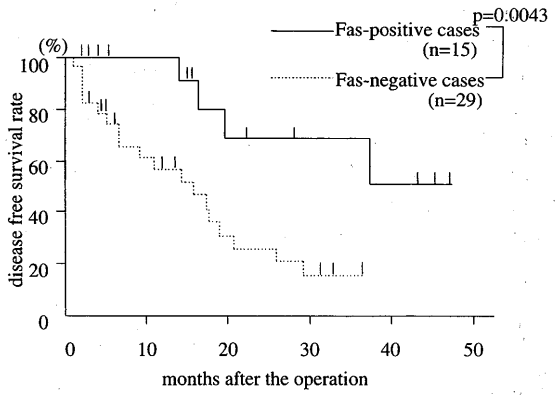

Fig. 2 Disease-free survival curves for Fas-positive and -negative patients, respectively. The disease-free survival rate of Fas-positive patients was significantly higher than that of Fas-negative patients ($p=0.0043$)

Table 2 Clinicopathological features of HCC with regard to Fas expression

Factors	Fas positive (n=151 ; 34.1%)	Fas negative (n=29 ; 65.9%)	p value
Gender (male/female)	12/3	21/8	0.722[3]
Age (mean±SD)	63.7±10.0yr.	64.5±9.0yr.	0.777[4]
Hepatitis viral infection (B/C/NBNC)[1]	3/8/5	8/19/3	0.192[3]
AFP (median)	13.6ng/ml	53.2ng/ml	0.105[5]
(range)	(1.5–13600)	(1.3–41600)	
Tumor size (mean±SD)[2]	3.9±2.8cm	5.3±3.4cm	0.197[4]
stage I/II/III/IV	2/9/2/2	2/15/8/4	0.452[5]
Cases with multiple tumors	26.7% (4/15)	13.8% (4/29)	0.414[3]
Cases with histological capsular formation	73.3% (11/15)	65.5% (19/29)	0.735[3]
Cases with histological capsular infiltration	72.7% (8/11)	89.5% (17/19)	0.638[3]
Histology (well/moderately/poorly)[2]	2/10/3	1/13/15	0.093[3]
Cases with histological portal invasion	66.7% (10/15)	69.0% (20/29)	0.999[3]
Cases with histological metastatic foci	0%	26.9% (8/29)	0.037[3]

[1] B, positive for hepatitis B surface antigen; C, positive antibody against hepatitis C virus and NBNC, negative B antigen nor C antibodies (including two cases with both positive of B and C)
[2] In case of multiple tumors, tumor size and histology are expressed by examining the largest tumor
[3] Chi square test or Fisher exact test
[4] Student's t test
[5] Mann-Whitney U test

Table 3 Clinicopathological features of HCC with regard to FasL expression

Factors	FasL positive (n=19 ; 43.2%)	FasL negative (n=25 ; 56.8%)	p value
Gender (male/female)	15/4	18/7	0.732[3]
Age (mean±SD)	65.7±10.8yr.	63.1±7.9yr.	0.369[4]
Hepatitis viral infection (B/C/NBNC)[1]	1/15/3	10/12/5	0.030[3]
AFP (median)	40.4ng/ml	44.3ng/ml	0.487[5]
(range)	(3–21600)	(1.3–41600)	
Tumor size (mean±SD)[2]	5.7±3.3cm	4.1±3.0cm	0.111[4]
Stage I/II/III/IV	1/10/4/4	3/14/6/2	0.124[5]
Cases with multiple tumors	21.1% (4/19)	16.0% (4/25)	0.710[3]
Cases with histological capsular formation	68.4% (13/19)	68.0% (17/25)	0.999[3]
Cases with histological capsular infiltration	76.9% (10/13)	88.2% (15/17)	0.676[3]
Histology (well/moderately/poorly)[2]	0/9/10	3/14/8	0.169[3]
Cases with histological portal invation	68.4% (13/19)	68.0% (17/25)	0.999[3]
Cases with histological metastatic foci	21.1% (4/19)	16.0% (4/25)	0.710[3]

[1] B, positive for hepatitis B surface antigen; C, positive antibody against hepatitis C virus and NBNC, negative B antigen nor C antibodies (including two cases with both positive of B and C)
[2] In case of multiple tumors, tumor size and histology are expressed by examining the largest tumor
[3] Chi square test or Fisher exact test
[4] Student's test
[5] Mann-Whitney U test

in patients with hepatitis C infection and less in those with hepatitis B infection, compared with FasL negative cases (p=0.030). The disease-free survival rate of FasL positive cases tended to be lower than that of FasL negative cases (Fig. 3). Fas status, multiple tumors, portal vein invasion and capsular formation were identified as significant factors indicative of a low disease-free survival on multivariate analysis (Table 4).

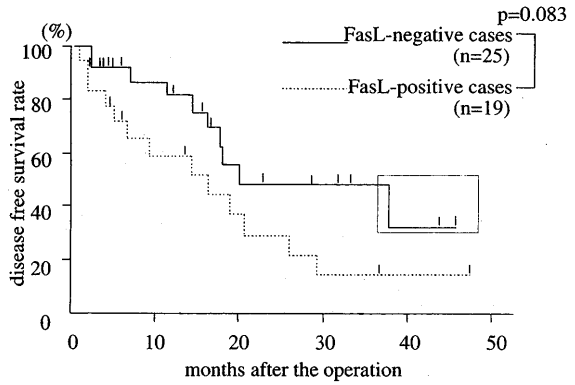

Fig. 3 Disease-free survival curves for FasL-positive and -negative patients, respectively. The disease-free survival rate of FasL positive cases tended to be lower than that of FasL negative cases (p=0.083)

Apoptosis and p53 status

Fas positive cases showed significantly higher apoptotic index than Fas negative cases (Table 5). p53 positive cases were observed in 12 (27.3 %) of 44 HCCs. All of p53 positive cases were Fas negative, and conversely all of Fas positive cases were p53 negative. On the other hand, no correlation between p53 and FasL expression was recognized.

Serum sFas and sFasL levels

Fig. 4 shows the distribution of individual concentrations of serum sFas and sFasL levels. The median of serum sFas levels was 228.2, 302.4, and 712.4 pg/ml in the controls, LC patients and HCC patients, respectively. On the other hand, the median of sFasL were 32.2, 32.4, and 19.0 pg/ml in the controls, LC patients and HCC patients, respectively. Significant difference of serum sFas and sFasL levels was detected among these groups. Both sFas and sFasL levels correlated with neither Fas/FasL status nor apoptotic index in HCC tis-

Table 4 Results of multivariate analysis for predictive factors associated with intrahepatic recurrence

variables		Hazard ratio	95% confidence intervals	p value[1]
Fas	positive vs. negative	0.042	0.005 - 0.312	0.002
FasL	positive vs. negative	1.686	0.563 - 5.053	0.351
Histological grading	well, moderately vs. poorly	0.277	0.061 - 1.256	0.096
Histological portal invasion	positive vs. negative	4.428	1.193 - 16.437	0.026
Histological capsular formation	positive vs. negative	0.194	0.058 - 0.652	0.008
Size (cm)	continuous variables	1.039	0.854 - 1.264	0.702
AFP (ng/ml)	continuous variables	1.000	1.000 - 1.000	0.149
Multiplicity	solitary vs. multiple	9.174	1.468 - 57.307	0.018

[1] Cox proportional hazards regression model

Table 5 Relationshp between Fas/FasL system and apoptotic index and p53 status

	Fas		p value	FasL		p value
	positive (n=15)	negative (n=29)		positive (n=19)	negative (n=25)	
apoptotic index median (range)	3.0 (0.8-4.0)	1.1 (0.4-2.2)	p=0.004[1]	1.1 (0.4-3.1)	1.6 (0.4-4.0)	0.092[1]
p53 positivity	0%	41.4% (12/29)	p=0.003[2]	36.8% (7/19)	20% (5/25)	0.308[2]

[1] Mann-Whitney U test
[2] Fisher exact test

Table 6 Relationship between Fas/FasL tissue expression and serum sFas/sFasL in HCC patients

	Fas		p value[1]	FasL		p value[1]
	positive (n=9)	negative (n=17)		positive (n=13)	negative (n=13)	
sFas (pg/ml) median (range)	573.7 (83-1115)	537.8 (300-1051)	0.797	537.8 (318-1051)	573.7 (83-1115)	0.923
sFasL (pg/ml) median (range)	17.9 (0-24.9)	18.2 (0-206)	0.700	17.7 (0-206)	17.9 (0-43)	0.808

[1] Mann-Whitney U test

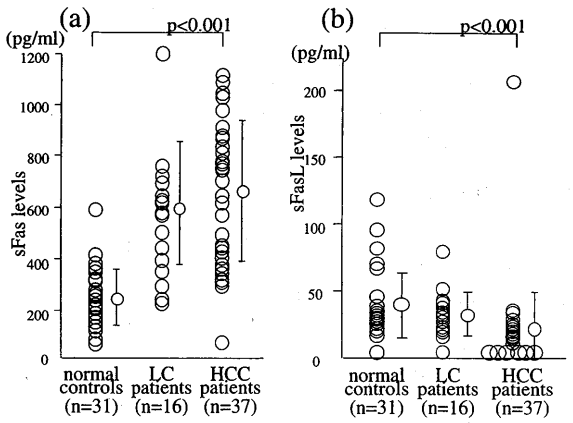

Fig. 4 Serum levels of soluble Fas (sFas) (a) and FasL (sFasL) (b) in patients carrying liver cirrhosis (LC) with or without HCC. Sera from patients carrying LC with or without HCC and from healthy volunteers were assayed by ELISA for sFas and sFasL. Significant differences of serum sFas and sFasL levels were detected among the controls, LC patients and HCC patients (Kruskal Wallis test). The values are expressed as mean± SD

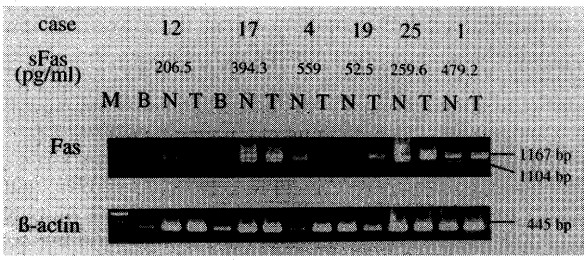

Fig. 5 Fas and Fas splice variant expression in HCC detected by RT-PCR. Lane M, molecular marker; lane B, N, and T, peripheral blood mononuclear cells before the operaton, noncancerous liver tissues, and HCC, respectively. The minor band (1104bp) corresponds to the Fas mRNA splice variant, which encodes a soluble form of Fas. In cases 1 and 4, where sFas level was high, Fas mRNA splice variant was more clearly detected in the noncancerous liver tissues than in HCC

sues (Table 6).

To investigate the origin of sFas in HCC patients, RT-PCR was performed. A distinct smaller DNA fragment, adding the anticipated full-length Fas cDNA fragment (1167 base pair), on RT-PCR was detected in HCCs and the noncancerous liver tissues but not in the circulating mononuclear cells (Fig. 5).

Discussion

Hepatoma cells may eliminate Fas expression on themselves and let the hepatocytes and infiltrating mononuclear cells generate sFas to escape from the immune system and to produce metastasis. FasL might contribute to malignant transformation in some circumstances because hepatocytes in the pericancerous pseudolobules expressed FasL.

References

1) Nagata S, Golstein, P. The Fas death factor. Science (Washington DC) 1995 ; 267 : 1449–1456.
2) Krammer PH, Behrmann I, Daniel P, Dhein D, Dehatin K-M. Regulation of apoptosis in the immune system. Curr. Opin. Immunol. 1994 ; 6 : 279–289.
3) Lowin B, Hahne M, Mattmann C, Tschopp J. Cytolytic T-cell cytotoxicity is mediated through perforin and Fas lytic pathway. Nature 1994 ; 370 : 650–652.
4) Kägi D, Vignaux F, Ledermann B, Bürki K, Depraetere V, Nagata S, Hengartner H, et al. Fas and perforin pathways as major mechanisms of T-cell mediated cytotoxicity. Science 1994 ; 265 : 528–530.
5) Rouvier E, Luciani M-F, Golstein P. Fas involvement in Ca2+-independent T cell-mediated cytotoxicity. J. Exp. Med. 1993 ; 177 : 195–200.
6) Hanabuchi S, Koyanagi M, Kawasaki A, Shinohara N, Matsuzawa A, Nishimura Y, Kobayashi Y, et al. Fas and its ligand in a general mechanism of T-cell mediated cytotoxicity. Proc. Natl. Acad. Sci. USA 1994; 91 : 4930–4934.
7) Ju ST, Cui H, Pauka DJ, Ettinger R, Marshak-Rothstein A. Participation of target Fas protein in apoptosis pathway induced by CD4+ Th1 and CD8+ cytotoxic T cells. Proc. Natl. Acad. Sci. USA 1994 ; 91 : 4185–4189.
8) Stalder T, Hahn S, Erb P. Fas antigen is the major target molecule for CD4+ T-cell mediated cytotoxicity. Immunology 1994 ;152 :1127–1133.
9) Dhein J, Walczak H, Bäumler C, Debatin K-M, Krammer PH. Autocrine T-cell suicide mediated by APO-1/(Fas/CD95). Nature 1995 ; 373 : 438–441.
10) Brunner T, Mogil RJ, LaFace D, Yoo NJ, Mahboubi A, Echeverri F, Martin SJ., et al. Cell-autonomous Fas (CD95)/Fas-ligand interaction mediates activation-induced apoptosis in T-cell hybridomas. Nature 1995; 373 : 441–444.
11) Ju S-T, Panka DJ, Cui H, Ettinger R, El-Khatib M, Sherr DH, Stanger BZ, et al. Fas (CD95)/FasL interactions required for programmed cell death after T-cell activation. Nature 1995 ; 373 : 444–448.
12) Alderson MR, Tough TW, Davis-Smith T, Braddy S, Falk B, Schooley KA., Goodwin RG, et al. Fas ligand mediates activation-induced cell death in human T lymphocytes. J. Exp. Med. 1995 ; 181 : 71–77.
13) Yonehara S, Ishii A, Yonehara M. A cell-killing monoclonal antibody (anti-Fas) to a cell surface antigen co-downregulated with the receptor of tumor necrosis factor. J. Exp. Med. 1989 ; 169 : 1747–1756.
14) Itoh N, Yonehara S, Ishii A, Yonehara M, Mizushima S-I, Sameshima M, Hase A, et al. The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. Cell 1991 ; 66 : 233–243.
15) Oehm A, Behrman I, Falk W, Pawlita M, Maier G, Klas C, Li-Weber M, et al. Purification and molecular cloning of the APO-1 cell surface antigen, a member of the tumor necrosis factor/nerve growth factor receptor family : sequence identify with the Fas

antigen. J. Biol. Chem. 1992 ; 267 : 10709–10715.
16) Suda T, Takahashi T, Golstein P, Nagata S. Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family. Cell 1993 ; 75 : 1169–1178.
17) Leithäuser F, Dhein J, Mechtersheimer G, Koretz K, Bruderlein S, Henne C, Schmidt A, et al. 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. 1993 ; 69 : 415–429.
18) Owen-Schaub LB, Radinsky R, Kruzel E, Berry K, Yonehara S. Anti-Fas on non-hematopoietic tumors : levels of Fas/APO-1 and bcl-2 are not predictive of biological responses. Cancer Res. 1994 ; 54 : 1580–1586.
19) Trauth BC, Klas C, Peters AMJ, Matzku S, Moller P, Falk W, Debatin K-M, et al. Monoclonal-antibody-mediated tumor regression by induction of apoptosis. Science 1989 ; 245 : 301–305.
20) Coney LR, Daniel PT, Sanborn D, Dhein J, Debatin K-M, Krammer PH, Zurawski VR. Apoptotic cell death induced by a mouse-human anti-APO-1 chimeric antibody leads to tumor regression. Int. J. Cancer 1994 ; 58 : 562–567.
21) Higaki T, Yano H, Kojiro M. Fas antigen expression and its relationship with apoptosis in human hepatocellular carcinoma and noncancerous tissues. Am. J. Pathlogy 1996 ; 149 : 429–436.
22) Midis GP, Shen Y, Owen-Schaub LB. Elevated soluble Fas (sFas) levels in nonhematological human malignancy. Cancer Res. 1996 ; 56 : 3870–3874.
23) Cheng J, Zhou T, Liu C, Shapiro JP, Brauer MJ, Kiefer MC, Barr PJ, et al. Protection from Fas-mediated apoptosis by a soluble form of the Fas molecule. Science (Washington DC) 1994 ; 263 : 1759–1762.
24) Owen-Schaub LB, Angelo LS, Radinsky R, Ware CF, Gesner TG, Bartos DP. Soluble Fas/APO-1 in tumor cells : a potential regulator of apoptosis? Cancer Lett. 1995 ; 94 : 1–8.
25) Cascino I, Fiucci G, Papohh G, Ruberti G. Three functional soluble forms of human apoptosis-inducing Fas molecule are produced by alternative splicing. J. Immunol. 1995 ; 154 : 2706–2713.
26) Natoli G, Ianni A, Costanzo A, De Petrillo G, Ilari I, Chirillo P, Balsano C, et al. Resisyance to Fas-mediated apoptosis in human hepatoma cells. Oncogene 1995 ; 10 : 1157–1164.
27) Jodo S, Kobayashi S, Nakajima Y, Matsunaga T, Nakayama N, Ogura N, Kayagaki N, et al. Elevated serum levels of soluble Fas/APO-1 (CD95) in patients with hepatocellular carcinoma. Clin. Exp. Immunol. 1998 ; 112 : 166–171.
28) Griffith TS, Brunner T, Hetcher SM, Green DR, Ferguson TA. Fas ligand-induced apoptosis as a mechanism of immune privilege. Science 1995 ; 270 : 1189–1192.
29) Donald B, Daniel G, Helena S, Jodena M, Alex F, Richard CD. A role for CD95 ligand in preventing graft rejection. Nature 1995 ; 377 : 630–632.
30) Hahne M, Rimoldi D, Schroter M, Romero P, Schreier M, French LE, Schneider P, et al. Melanoma cell expression of Fas (APO-1/CD95) ligand : implications for tumor immune escape. Science (Washington DC) 1996 ; 274 : 1363–1366.
31) O'Connell J, O'Sullivan GC, Collins JK, Shanahan F. The Fas counterattack : Fas-mediated T cell killing by colon cancer cells expressing Fas ligand. J. Exp. Med. 1996 ; 184 : 1075–1082.
32) Strand S, Hofman WJ, Hug H, Muller M, Otto G, Strand D, Mariani SM, et al. Lymphocyte apoptosis induced by CD95 (APO-1/Fas) ligand-expressing tumor cells-a mechanism of immune evasion? Nat. Med. 1996 ; 2 : 1361–1366.
33) Niehans GA, Brunner T, Frizelle SP, Liston JC, Salerno CT, Knapp DJ, Green DR, et al. Human lung carcinomas express Fas ligand. Cancer Res. 1997 ; 57 : 1007–1012.
34) Gratas C, Tohma Y, Barnas C, Taniere P, Hainaut P, Ohgaki H. Up-regulation of Fas (APO-1/CD95) ligand and down-regulation of Fas expression in human esophageal cancer. Cancer Res. 1998 ; 58 : 2057–2062.
35) Tanaka M, Suda T, Haze K, Nakamura N, Sato K, Kimura F, Motoyoshi K, et al. Fas ligand in human serum. Nat. Med.1996 ; 2 : 317–322.
36) Owen-Schaub LB, Zhang W, Cusack JC, Angelo LS, Santee SM, Fujiwara T, Roth JA, et al. Wild-type human p53 and a temperature-sensitive mutant induce Fas/APO-1 expression. Mol. Cell. Biol. 1995; 15: 3032–3040.