Soluble Fas in malignant pleural effusion and its expression in lung cancer cells

Kayo Mitani, Yasuhiko Nishioka, Kazue Yamabe, Hirohisa Ogawa, Toyokazu Miki, Hiroaki Yanagawa and Saburo Sone[1]

Department of Internal Medicine and Molecular Therapeutics, Course of Medical Oncology, University of Tokushima School of Medicine, 3 Kuramoto-cho, Tokushima 770-8503

(Received September 27, 2002/Revised January 8, 2003/Accepted January 10, 2003)

Soluble Fas (sFas) has the ability to block Fas-mediated apoptosis, suggesting that sFas at tumor sites might inhibit tumor cell-killing by immune effector cells. We examined the sFas level in pleural effusion associated with lung cancer. The level of sFas in malignant pleural effusion was significantly higher than those in transudate and tuberculous pleural effusion. There was no significant difference in the sFas concentration among various histological types of lung cancer. The cytotoxicity mediated by anti-Fas agonistic antibody against Jurkat cells was inhibited by the addition of malignant pleural effusion, being inversely correlated with the sFas concentration. When Fas expression was examined using flow cytometry, eight of ten (80%) lung cancer cell lines expressed cell surface Fas. On the other hand, sFas protein and mRNA were detected in six of ten (60%) lung cancer cell lines, but there was no correlation between Fas and sFas expression. Furthermore, although the expressions of Fas and sFas were clearly detected in tumor cells derived from malignant effusion, the sFas expression was down-regulated in an *in vitro* culture. These results suggest that sFas in malignant pleural effusion is at least in part produced by lung cancer cells, and might play a role in local immunosuppression by tumor cells. (Cancer Sci 2003; 94: 302–307)

Fas (APO-1, CD95) is a 48-kDa type I transmembrane protein which belongs to the TNF (tumor necrosis factor) receptor superfamily.[1)] The engagement of Fas and FasL, which is the ligand for Fas, or anti-Fas agonistic antibody is known to induce apoptosis of various cells.[2–5)] Abnormalities of the Fas/FasL system are associated with lympho-proliferative disorders and autoimmune diseases.[4, 5)] Furthermore, the Fas/FasL system was reported to be one of the target cell-killing mechanisms employed by natural killer (NK) cells and cytotoxic T lymphocytes (CTLs).[6, 7)] These effector cells could mediate tumor apoptosis through the engagement of FasL to Fas on target cells, though there were multiple mechanisms involved in tumor cell resistance to Fas-mediated death, including down-regulation of Fas and up-regulation of negative regulatory proteins, such as Bcl-2 family members, Fas-associated phosphatase 1 (FAP-1) and FADD-like-interleukin β-converting enzyme (FLICE)-inhibitory proteins (FLIP).[8, 9)]

Several isoforms of the soluble form of Fas (sFas) were reported to be generated by alternative mRNA splicing.[10–12)] The most predominant sFas isoform is derived from the deletion of exon 6 encoding the last 5 amino acids of the extracellular domain and 16 of 17 amino acids in the transmembrane (TM) domain.[10)] These sFas isoforms were reported to block apoptosis mediated by FasL or anti-Fas agonistic antibody,[10–12)] suggesting that the existence of sFas might protect tumor cells from host antitumor immunity.

In fact, several solid tumor cells, including osteosarcoma,[13)] melanoma[14)] and hepatoma[15)] and hematopoietic malignancies,[16, 17)] have been reported to express sFas. Recent studies have also reported that serum sFas levels of patients with various malignancies including solid tumors[18, 19)] and hematopoietic malignancies[20, 21)] were elevated. Furthermore, a correlation of the serum sFas level with the prognosis of patients was observed in several studies.[22–25)] However, little is known about the level of sFas in malignant pleural effusion and the sFas expression of lung cancer.

To evaluate the presence and immunosuppressive role of sFas in malignant pleural effusion, we examined the concentration of sFas in the pleural effusion associated with lung cancer compared with tuberculous pleurisy and transudate. Furthermore, we examined the expression of the surface Fas and sFas mRNA of lung cancer cell lines in addition to tumor cells from primary cultures of malignant pleural effusion by using flow cytometry and RT-PCR (reverse transcriptase-polymerase chain reaction), respectively.

Materials and Methods

Cell lines. The human lung cancer cell lines RERF-LC-OK (adenocarcinoma), RERF-LC-MS (adenocarcinoma), A549 (adenocarcinoma), PC-9 (adenocarcinoma), H69 (small cell carcinoma) and N291 (small cell carcinoma), and Jurkat (human T leukemia cell line) were obtained from the American Type Culture Collection (ATCC, Rockville MD).[26, 27)] The human small cell lung cancer cell lines, SBC3 and SBC5 were provided by Dr. K. Hirai (Okayama University, Okayama).[28)] The human lung squamous cell carcinoma RERF-LC-AI was provided by Dr. Akiyama (Radiation Effects Research Foundation, Hiroshima), and human lung adenocarcinoma PC-14 was from Dr. N. Saijo (National Cancer Institute, Tokyo).[27)] These cell lines were maintained in RPMI 1640 medium supplemented with 10% FBS (fetal bovine serum), penicillin and streptomycin (designated as CRPMI-1640) in a humidified incubator with a 5% CO_2 atmosphere at 37°C. YM98 and JT99 were established from malignant pleural effusion due to lung adenocarcinoma in our laboratory as described previously.[26)]

Patients. Sixty-five patients with pleural effusion were examined in Tokushima University Hospital after having obtained informed consent, as described previously.[29)] Exudative pleural effusion (with protein concentration above 30 g/liter, and with a total leukocyte count greater than 1×10^6/ml) with positive cytology, was designated as malignant pleural effusion in the present study. Forty-five patients had malignant pleural effusion associated with lung cancer (20 adenocarcinomas, 14 small cell carcinomas and 11 squamous cell carcinomas). Tuberculous pleural effusion (n=10) was diagnosed by bacteriological and histological examination or PCR analysis. Transudative pleural effusion (with a protein concentration below 30 g/liter and with a total leukocyte count of less than 1×10^6/ml) secondary to congestive heart failure was also examined.

Reagents. FBS and RPMI 1640 were purchased from GIBCO (Grand Island, NY). The anti-Fas mAbs CH-11 and ZB4 were purchased from Medical Biochemical Laboratories Co., Ltd.

[1]To whom correspondence should be addressed.
E-mail: ssone@clin.med.tokushima-u.ac.jp

(Nagoya). The recombinant human Fas/Fc chimera was purchased from R&D Systems, Inc. (Minneapolis, MN). None of these materials contained endotoxins, as judged by *Limulus* amebocyte assay (Seikagaku Kogyo, Tokyo: minimum detection level, 0.1 ng/ml).

Sampling and storage of pleural effusions, and the establishment of tumor cell lines from malignant pleural effusions. Pleural effusion was collected via diagnostic thoracentesis.[29] Cells in the pleural effusions were counted using a hemocytometer. Differential counts were performed on a total of 200 cells stained with Wright and Giemsa. After centrifugation at 400*g* for 10 min, cell-free supernatant was separated and stored at −80°C until analysis. In some experiments, mononuclear cells in malignant pleural effusion were harvested by density centrifugation with Lymphocyte Separation Medium (LSM; Litton Bionectics, Kensington, MD), and cultured in CRPMI-1640 to establish tumor cell lines from the primary culture as previously described.[26]

Determinations of the sFas concentration using enzyme-linked immunosorbent assay (ELISA). The level of sFas in pleural effusion was measured with ELISA kits (sFas ELISA; Medical Biochemical Laboratories Co., Ltd.). ELISA was performed essentially as described previously, according to the manufacturer's instructions.[30] The lower limit of detections for sFas was 0.5 ng/ml.

To examine the production of sFas protein, lung cancer cells (3×10^6 cells/well) were cultured in 6-well plates. Their supernatant was harvested at various times and used for the examination of the sFas level with high-sensitivity ELISA (>20 pg/ml; R&D Systems, Inc., Minneapolis, MN) according to the manufacturer's instructions.

Flow cytometry. For flow cytometric analysis, lung cancer cells (5×10^5 cells) were resuspended in flow cytometry buffer (phosphate-buffered saline (PBS) with 0.5% rabbit serum and 10^{-3} m*M* NaN_3) and labeled for 30 min at 4°C with anti-Fas primary antibody CH-11 (20 μg/ml). The cells were washed twice with flow cytometry buffer, then labeled with FITC-conjugated anti-mouse secondary antibody (Biosource International, Camarillo, CA) diluted to 1:1000 in flow cytometry buffer. Flow cytometric analysis was carried out using a FACSCaliber flow cytometer with Cell Quest software (Becton Dickinson, Lincoln Park, NJ).[31] The cells were gated to exclude dead cells and debris, and 10 000 cells were examined for each determination.[30]

RT-PCR of Fas. Complementary DNA (cDNA) was synthesized from 1 μg of total RNA which was isolated using Isogen (Wako K.K., Kyoto). The primer set specific for Fas (sense; 5′-CACTTCGGAGGATTGCTCAACA-3′, antisense; 5′-TATGTTGGCTTCTTCAGCGCTA-3′) was used to amplify human Fas mRNA according to the previous method.[10] When used with these primers, two major bands of PCR products were detected. The stronger band was 1167 bp, which represented the full-length cDNA of the native Fas. The other was 1104 bp, which shows the alternatively spliced smaller fragment (sFas: FasΔTM-mRNA) lacking a 63 bp segment of the TM domain. The β-actin mRNA expression was also analyzed using the following primers: sense; 5′-AAGAGAGGCATCCTCACCCT-3′, antisense; 5′-TACATGGCTGGGGTGTTGAA-3′. A One-Step RNA PCR Kit (TaKaRa Biochemicals, Tokyo) was used for RT-PCR. cDNA was amplified in a total volume of 50 μl. The PCR reaction contained PCR buffer, 5 m*M* $MgCl_2$, 1 m*M* dNTP, 0.8 U/μl of RNase inhibitor, AMV-RTase XL 0.1 U/μl, 0.1 U/μl AMV-Optimized *Taq*, and 0.4 μ*M* of each primer. After an initial denaturation of 3 min at 94°C, amplification was performed for 30 cycles (sFas: 94°C for 3 min, 60°C for 2 min, and 72°C for 2 min 30 s, β-actin: 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s). Amplified products were electrophoresed in a 2% (for sFas) or 1.5% (for β-actin) agarose gel and visualized by ethidium bromide staining.

The cytotoxicity mediated by anti-Fas mAb. The Cell Counting Kit-8 (Dojin East, Tokyo) was used to determine anti-Fas mAb-mediated cytotoxicity. First, 100 μl of Jurkat cell suspension (5×10^3 cells/well) was added to 96-well flat-bottomed microtiter plates (Falcon 3072, Becton Dickinson), and each plate was incubated for 24 h at 37°C. After incubation, 2 μl of malignant pleural effusion were added to the 96-well plates, and 5 μl of anti-Fas agonistic mAb CH-11 was added to each well (final concentration: 2 ng/ml). After incubation for 48 h, 10 μl of Cell Counting Kit solution (consisting 5 m*M* WST-8, 0.2 m*M* 1-methoxy PMS and 150 m*M* of NaCl, Dojin East) was added to each culture well, and the cells were further incubated for 3.5 h at 37°C. The absorbance of each well was measured with a microtiter plate reader at 450/630 nm. The percent cytotoxicity was calculated using the following formula: Percentage cytotoxicity=[1−(absorbance of experimental wells/absorbance of control wells)]×100.

Immunoprecipitation and western blotting. The cell supernatants were collected from confluent cultures of lung cancer cell lines in 10-cm dishes. The concentrated supernatants were immunoprecipitated using anti-Fas mAb (ZB4) and Protein G Sepharose 4 Fast Flow at 4°C for 2 h. Protein G beads were collected by centrifugation and washed five times with RIPA buffer (10 m*M* Tris HCl pH 7.4, 1% NP40, 0.1% sodium deoxycholate, 0.1% SDS (sodium dodecyl sulfate), 0.15 m*M* NaCl, 1 m*M* EDTA, 10 μg/ml aprotinin), and suspended in 2× Laemmli sample buffer. These samples were boiled for 3 min and subjected to 10% SDS-PAGE (polyacrylamide gel electrophoresis) under a nonreducing condition, and the proteins were blotted onto Hybond-C membrane (Amersham, Arlington Heights, IL). The membrane was blocked with 5% nonfat dry milk powder in PBS at room temperature for 2 h and incubated with anti-Fas antibody (ZB4). The membrane was washed and incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG mAb (Santa Cruz Biotech, Santa Cruz, CA), and protein bands were developed using the enhanced chemiluminescence (ECL) method (Amersham).

Statistical analysis. Comparisons of sFas between malignant effusions and transudative effusions were assessed by use of the Mann-Whitney *U* test. The correlations between the levels of sFas and the numbers of macrophages and lymphocytes in the malignant effusion were evaluated with Pearson's correlation test. Statistical analysis was performed with Statview software.

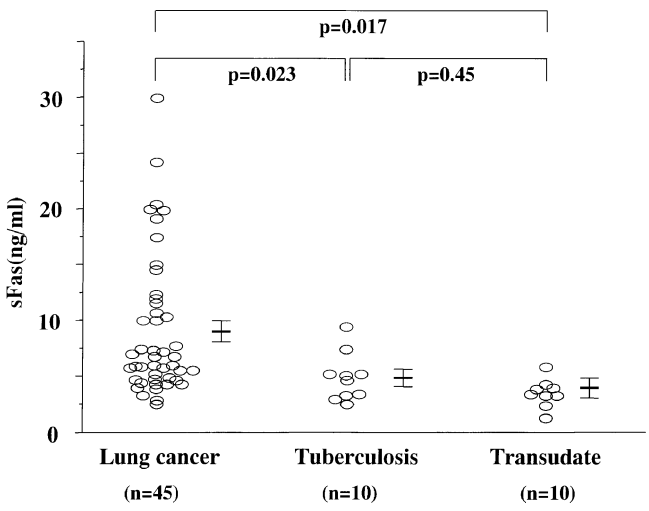

Fig. 1. The sFas level in pleural effusion due to various diseases. The concentration of sFas in pleural effusion was measured by ELISA. Each open circle (○) indicates the data from an individual donor. The scale in this figure shows mean±SE.

Differences were considered statistically significant if P values were less than 0.05.

Results

Elevated sFas level in malignant pleural effusion due to lung cancer. We first examined the levels of sFas in the pleural effusions. Transudative and tuberculous pleural effusions contained detectable levels of sFas, as shown in Fig. 1. However, the level of sFas in malignant pleural effusion was significantly higher than that of transudative effusion (9.19±0.95 vs. 4.20±0.49 ng/ml, P=0.017). Furthermore, the concentration of sFas in malignant pleural effusion was also higher than that of tuberculous pleural effusion (9.19±0.95 vs. 5.11±0.67 ng/ml, P=0.023).

Next, the sFas level in malignant pleural effusion was examined in three histological types of lung cancer (Fig. 2). There was no significant difference among the histological types. Several authors have reported that peripheral blood lymphocytes could produce sFas protein.[10, 11] Thus, we examined the correlation of the sFas level and the numbers of lymphocytes and macrophages in malignant pleural effusions. As shown in Fig. 3, we could not detect any correlation between the level of sFas and the numbers of lymphocytes and macrophages.

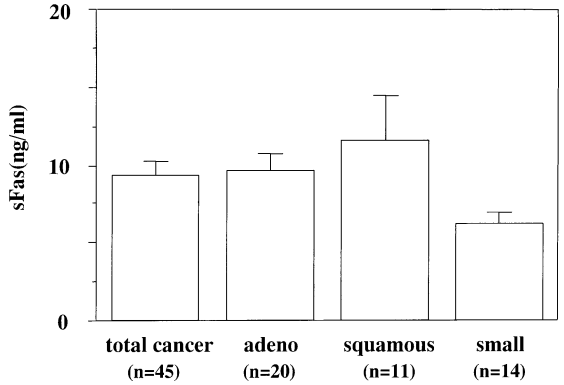

Fig. 2. The sFas level in malignant pleural effusion due to lung cancer with various histologies. The concentration of sFas in pleural effusion was measured by ELISA. Values are shown as means±SE. There was no significant difference among the histological types.

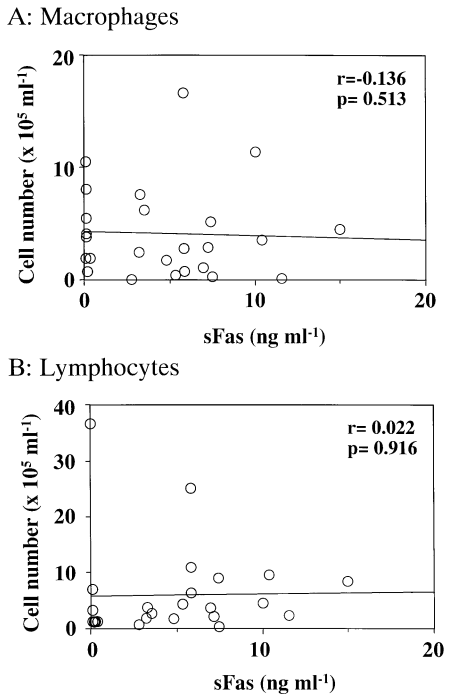

Fig. 3. The correlation between the level of sFas and the numbers of lymphocytes or macrophages in malignant pleural effusion. Values were analyzed using Pearson's correlation test with Statview software.

Effect of malignant pleural effusion on the cytotoxicity mediated by anti-Fas mAb. The Fas/FasL system plays an important role in the tumor cell-killing by NK cells and CTLs.[10, 11] Next, we examined whether Fas-mediated cytotoxicity was affected by the addition of malignant pleural effusion. To test this, we used anti-Fas antibody, which has an agonistic effect on apoptosis induction through the engagement of Fas molecules. We first confirmed whether the sFas inhibited the anti-Fas-mediated cytotoxicity against Jurkat cells using a recombinant Fas/Fc chimera. As shown in Fig. 4, the addition of Fas/Fc chimera resulted in the inhibition of anti-Fas-mediated cytotoxicity. Next, an aliquot of malignant pleural effusion from several donors was added to the culture of Jurkat cells in the presence of anti-Fas mAb. As shown in Table 1, the addition of malignant pleural effusion inhibited the cytotoxicity induced by anti-Fas mAb, and the Fas-mediated cytotoxicity was inversely correlated with the concentration of sFas (Fig. 5).

Expression of Fas on the cell surface of lung cancer cell lines. To test the cell surface expression of Fas antigen on lung cancer cell lines, flow cytometric analysis using anti-Fas mAb was performed. Furthermore, two tumor cells derived from the primary

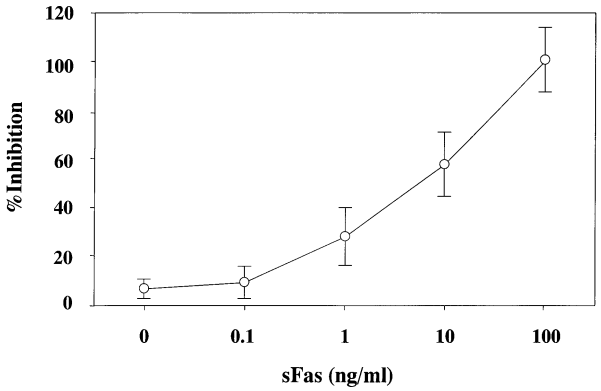

Fig. 4. The addition of Fas/Fc chimera inhibits anti-Fas-mediated cytotoxicity. Various concentrations of recombinant human Fas/Fc chimera were added to the culture to examine anti-Fas mAb (CH-11)-mediated cytotoxicity against Jurkat cells. The cytotoxicity was determined using a Cell Counting Kit as described in "Materials and Methods." Values are shown as means ±SD of triplicate cultures. Data are representative of two separate experiments.

Table 1. The addition of malignant pleural effusion inhibits the cytotoxicity of anti-Fas mAb against Jurkat cells[1)]

Pleural effusion	sFas (ng/ml)	% Cytotoxicity against Jurkat cells
(—)	—	97.8±1.8[2)]
Patient 4	30.0	22.3±8.8[3)]
Patient 31	2.9	65.7±0.4[4)]
Patient 24	2.5	56.2±3.2[4)]

1) Anti-Fas mAb (CH-11) was used to induce the apoptosis of Jurkat cells. An aliquot of pleural effusion was added to the culture of CH-11 and Jurkat cells, and the cytotoxicity was determined as described in "Materials and Methods."
2) Mean±SD for triplicate culture.
3) P<0.005 compared with the cytotoxicity without the addition of pleural effusion.
4) P<0.001 compared with the cytotoxicity without the addition of pleural effusion.

culture of malignant pleural effusion were also examined. As shown in Fig. 6, Fas expression was detected on the cell surface of eight of ten (80%) lung cancer cell lines, irrespective of the variety of their expression levels. Tumor cells (YM98 and JT99) derived from malignant pleural effusion also expressed the surface Fas antigen.

Detection of sFas expression in lung cancer cell lines. We know of no study reporting the production of sFas protein from lung cancer cells. To examine this, we harvested the culture supernatant of lung cancer cell lines and measured the level of sFas by ELISA. sFas protein was clearly detected in the supernatant of five of ten (50%) lung cancer cell lines (Fig. 7 and Table 2). However, the expression level of sFas did not correlate with Fas expression (Table 2).

Furthermore, to confirm the expression of Fas and sFas, RT-PCR analysis was also performed. sFas mRNA represents a transcript which lacks the transmembrane domain of exon 6 and is 63 bp shorter than full-length Fas mRNA. As shown in Fig. 8, A and B, all lung cancer cell lines expressed detectable levels of native Fas mRNA, though the level of expression varied. sFas mRNA was detected in six of ten (60%) lung cancer cell lines. Three cell lines, SBC3, A549 and RERF-LC-AI, did not express sFas mRNA although Fas expression was confirmed by both flow cytometry and RT-PCR.

We further examined the expression of Fas and sFas mRNA of total cells and tumor cells in primary cultures of malignant pleural effusion. The results are shown in Fig. 8C. In all samples tested, Fas transcript was clearly detected. sFas mRNA expression was observed in 2 of 3 malignant pleural effusions. Furthermore, primary culture tumor cells (adenocarcinomas; YM98 and JT99) expressed sFas mRNA in short-term *in vitro*

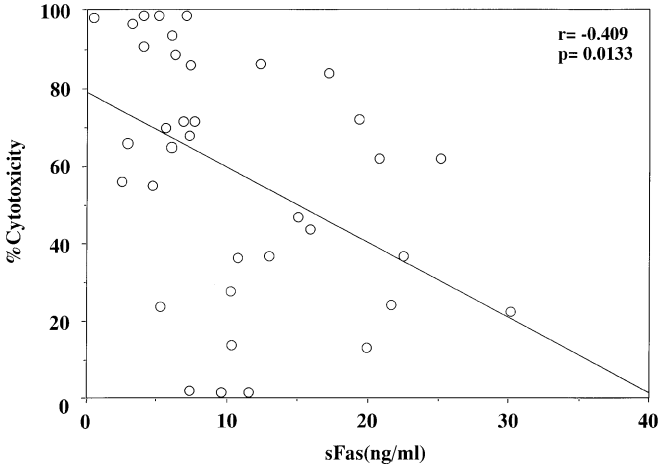

Fig. 5. The correlation between the level of sFas in malignant pleural effusion and its inhibitory effect on the cytotoxicity mediated by anti-Fas mAb against Jurkat cells. An aliquot of malignant pleural effusion from 26 lung cancer patients was added to the culture to examine anti-Fas mAb (CH-11)-mediated cytotoxicity against Jurkat cells. The cytotoxicity was determined using a Cell Counting Kit as described in "Materials and Methods." Values were analyzed using Pearson's correlation test with Statview software.

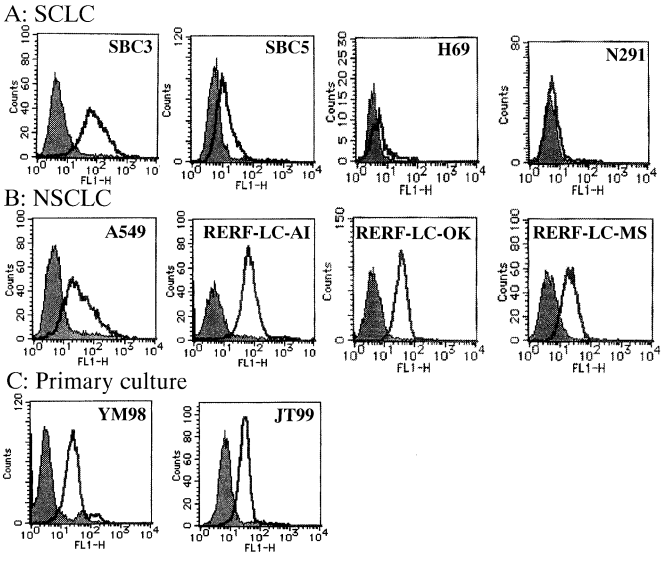

Fig. 6. Flow cytometric analysis of surface Fas expression on lung cancer cells. Lung cancer cell lines or tumor cells established from malignant pleural effusion due to lung cancer were stained with anti-Fas mAb (bold line) or control mAb (hatched area), and the expression of Fas was analyzed by flow cytometry as described in "Materials and Methods."

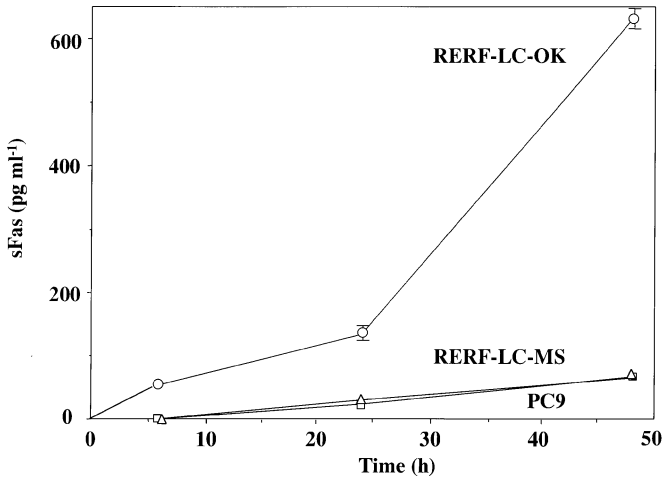

Fig. 7. The production of sFas protein from lung cancer cell lines. RERF-LC-OK (○), RERF-LC-MS (△) and PC9 (□) (3×10^6 cells per well) were cultured in 6-well plates. The supernatant was harvested at various times, and the sFas level was examined using ELISA (sensitivity: >20 pg/ml). Values are shown as means±SD of triplicate cultures. Data are representative of three separate experiments.

Table 2. The expressions of Fas and sFas in lung cancer cell lines

Cell line		Fas (MFI)[1)]	sFas (pg/ml)[2)]
SCLC	SBC3	16.18	<20
	SBC5	2.49	<20
	H69	1.74	<20
	N291	1.27	<20
NSCLC	A549	5.90	67.7±1.5[3)]
	RERF-LC-AI	11.80	98.3±15.9
	RERF-LC-OK	7.34	631.7±16.4
	RERF-LC-MS	4.27	64.7±1.5
	PC-9	2.94	60.7±1.2
	PC-14	2.47	<20

1) The flow cytometric analysis was performed as described in "Materials and Methods." The relative mean fluorescence intensity (MFI) was expressed as the ratio of MFI of cells stained with anti-Fas mAb (CH-11) to MFI of cells stained with control IgM.
2) The supernatants of lung cancer cell lines were harvested after 24 h culture and the level of sFas was measured by ELISA as described in "Materials and Methods." The sensitivity of ELISA was >20 pg/ml.
3) Mean±SD for triplicate culture.

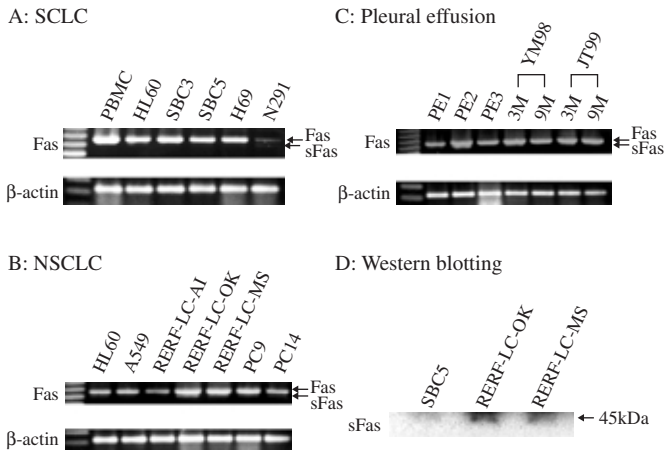

Fig. 8. The expressions of Fas and sFas mRNA and protein in lung cancer cell lines. Total RNA was extracted from various lung cancer cell lines (A:SCLC and B:NSCLC), MNCs from malignant pleural effusions and primary lung cancer cell lines established from malignant effusions after *in vitro* culture for 2 to 6 months (C). RT-PCR for Fas was performed using specific primers as described in "Materials and Methods." Ten microliters of PCR product was electrophoresed in agarose gel and stained with ethidium bromide. Two major bands, which correspond to Fas and sFas, were detected. In Fig. C, PE 1 to 3 indicate the individual patients. In Fig. D, the Fas protein of the culture supernatant of lung cancer cells was immunoprecipitated using anti-Fas antibody (ZB4). These samples were run on a 10% SDS-PAGE and transferred onto Hybond-C membrane. The blot was developed with anti-Fas mAb and HRP-conjugated anti-mouse IgG mAb using the ECL method.

culture. However, sFas expressions of their tumor cells were decreased after long-term culture for up to 6 months (Fig. 8C). Finally, to further confirm the production of sFas from lung cancer cell lines, we performed western blotting of sFas protein using immunoprecipitated samples of the supernatants of lung cancer cell lines. As shown in Fig. 8D, a protein band of 43 to 45 kDa was detected in some of the lung cancer cell lines.

Discussion

In the present study, we demonstrated that the level of sFas is elevated in malignant pleural effusion due to lung cancer when compared with transudate and tuberculous effusion, and that sFas is expressed in tumor cells derived from malignant pleural effusion in addition to lung cancer cell lines. Furthermore, the addition of malignant pleural effusion blocked the tumor cell-killing mediated by anti-Fas agonistic mAb *in vitro*.

The Fas-FasL system plays an important role in the killing mechanisms of NK cells and CTLs.[5] Thus, blocking of the signaling pathway through Fas-FasL might be one possible mechanism of the tumor cell's escape from host immune surveillance. An important inhibitor for this pathway is likely to be sFas, which is produced by an alternative splicing and lacks the transmembrane domain of the native Fas cDNA fragment. sFas has been shown to antagonize both anti-Fas mAb- and FasL-mediated tumor cell lysis through competition for the binding of surface Fas to FasL.[10, 11] There are several studies reporting an elevated sFas level in the serum of patients with solid tumors as well as hematopoietic malignancies.[14–16] However, there is no known study reporting the level of sFas in pleural effusion. We have demonstrated the elevation of sFas in malignant pleurisy for the first time. Furthermore, the level of Fas in malignant effusion correlated with its blocking ability for Fas-mediated cytotoxicity. Although other factors in the malignant pleural effusion, which could block the Fas-FasL pathway, might be involved in this suppressive effect, these results together with those of previous studies reporting the correlation of the serum sFas level and a poor prognosis of patients suggest a role of sFas as an immunosuppressive factor.

To analyze the source of sFas in malignant effusion, we examined the expressions of surface Fas and sFas of lung cancer cell lines and tumor cells from primary culture of malignant pleural effusions. Hellquist *et al.*[32] reported Fas expression in all of eight lung squamous cell carcinomas using immunohistochemistry. Nambu *et al.*[33] also reported the expression of Fas protein in 20 of 42 lung adenocarcinoma tissues (47.6%), and sFas mRNA in four of seven tumor samples. However, the production of sFas from lung cancer cells at the protein level has not been demonstrated. In the present study, we detected the surface Fas expression in 10 of 12 tumor cells as tested by flow cytometry, and sFas mRNA expression in 7 of 12 lung cancer cells including primary cultures. Consistent with previous findings,[33, 34] sFas expression of A549 cells was not clearly detected, whereas three adenocarcinoma cell lines, RERF-LC-OK, RERF-LC-MS and PC-9 expressed substantial levels of sFas mRNA. It is of importance that sFas protein was detected in their culture supernatant by ELISA, even in that of A549. Furthermore, high expression of sFas mRNA was also found in tumor cells (YM98 and JT99) from malignant pleural effusions. Both the Fas and sFas expressions of NSCLC cells tended to be higher than those of SCLC cell lines. These results suggest that lung cancer cells could have the ability to produce sFas *in vitro* and probably *in vivo* (in the pleural cavity). Furthermore, it is likely that sFas expression of tumor cells is enhanced in pleural effusion and reduced by *in vitro* culture, since two primary-culture cell lines showed reduced expression of sFas after *in vitro* culture, as shown in Fig. 8C. The exact mechanisms involved in the regulation of sFas expression remains unclear. It has been reported that the expression of Fas on T cells or mammary carcinoma cells was up-regulated by treatment with PHA or cytokines, including interferon (IFN)-γ or TNF-a, respectively.[35, 36] Osorio *et al.*[17] also reported that the treatment of B-CLL (B-cell chronic lymphocytic leukemia) with SAC (*Staphylococuss aureus* Cowan)+interleukin (IL)-2 or TPA (12-O-tetradecanoylphorbol-13-acetate)+IL-2 enhanced the expression of both Fas and sFas. Although we could not detect cytokines such as IL-2, IFN-γ and TNF-a in malignant pleural effusion,[37] other factors or interactions with host cells might enhance sFas expression of lung cancer cells *in vivo*.

In the present study, there was no correlation of the sFas level and the number of host cells in pleural effusion. However, we could not rule out the possibility that elevated sFas in malignant effusions was responsible for the production of lymphocytes and macrophages, since there are several studies showing that reported activated PBMCs (peripheral blood mononuclear cells) can produce sFas protein. Furthermore, the considerable level of sFas in transudate suggests that normal cells including lymphocytes and macrophages might be mainly responsible for the production of sFas *in vivo*. Further studies are required to explore the mechanisms of the elevation of sFas *in vivo*.

In summary, sFas, which is likely to be produced by tumor cells, might play a role in the local immunosuppression in malignant pleural effusion associated with lung cancer progression.

This work was supported by grants from the Ministry of Health, Labour and Welfare and the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by a grant from the Public Trust Haraguchi Memorial Cancer Research Fund. The authors thank the medical staff of the Department of Internal Medicine and Molecular Therapeutics, Course of Medical Oncology, University of Tokushima School of Medicine for their kind help.

1. Nagata S. Fas and Fas ligand: a death factor and its receptor. *Adv Immunol* 1994; **57**: 129–44.
2. Itoh N, Yonehara S, Ishii A, Yonehara M, Mizushima S, Sameshima M, Hase A, Seto Y, Nagata S. The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. *Cell* 1991; **66**: 233–43.
3. Oehm A, Behrmann I, Falk W, Pawlita M, Maier G, Klas C, Li-Weber M, Richards S, Dhein J, Trauth BC, Ponstingl H, Krammer PH. Purification and molecular cloning of the APO-1 cell surface antigen, a member of the tumor necrosis factor/nerve growth factor receptor superfamily: sequence identity with the Fas antigen. *J Biol Chem* 1992; **267**: 10709–15.
4. Nagata S, Golstein P. The Fas death factor. *Science* 1995; **267**: 1449–56.
5. Nagata S. Apoptosis by death factor. *Cell* 1997; **88**: 355–65.
6. Kagi D, Vignaux F, Ledermann B, Burki K, Depraetere V, Nagata S, Hengartner H, Golstein P. Fas and perforin pathways as major mechanisms of T cell-mediated cytotoxicity. *Science* 1994; **265**: 528–30.
7. Arase H, Arase N, Saito T. Fas-mediated cytotoxicity by freshly isolated natural killer cells. *J Exp Med* 1995; **181**: 1235–8.
8. Hu S, Vincenz C, Ni J, Gentz R, Dixit VM. I-FLICE, a novel inhibitor of tumor necrosis factor receptor-1- and CD95-induced apoptosis. *J Biol Chem* 1997; **272**: 17255–7.
9. Whiteside TL, Rabinowich H. The role of Fas/Fasl in immunosuppression induced by human tumors. *Cancer Immunol Immunother* 1998; **46**: 175–84.
10. Cheng J, Zhou T, Liu C, Shapiro JP, Brauer MJ, Kiefer MC, Barr PJ, Mountz JD. Protection from Fas-madiated apoptosis by a soluble form of the Fas molecule. *Science* 1994; **263**: 1759–62.
11. Cascino I, Fiucci G, Papoff G, Ruberti G. Three functional soluble forms of the human apoptosis-inducing Fas molecule are produced by alternative splicing. *J Immunol* 1995; **154**: 2706–13.
12. Papoff G, Cascino I, Eramo A, Starace G, Lynch DH, Ruberti G. An N-terminal domain shared by Fas/APO-1 (CD95) soluble variants prevents cell death *in vitro*. *J Immunol* 1996; **156**: 4622–30.
13. 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.
14. Midis GP, Shen Y, Owen-Schaub LB. Elevated soluble Fas (sFas) levels in nonhematopoietic human malignancy. *Cancer Res* 1996; **56**: 3870–4.
15. Krams SM, Fox CK, Beatty PR, Cao S, Villanueva JC, Esquivel CO, Martinez OM. Human hepatocytes produce an isoform of Fas that inhibits apoptosis. *Transplantation* 1998; **65**: 713–21.
16. Knipping E, Debatin KM, Stricker K, Heilig B, Eder A, Krammer PH. Identification of soluble APO-1 in supernatants of human B- and T-cell lines and increased serum levels in B- and T-cell leukemias. *Blood* 1995; **85**: 1562–9.
17. Osorio LM, Aguilar-Santelises M, De Santiago A, Hachiya T, Mellstedt H, Jondal M. Increased serum levels of soluble Fas in progressive B-CLL. *Eur J Haematol* 2001; **66**: 342–6.
18. Mizutani Y, Yoshida O, Bonavida B. Prognostic significance of soluble Fas in the serum of patients with bladder cancer. *J Urol* 1998; **160**: 571–6.
19. Jodo S, Kobayashi S, Nakajima Y, Matsunaga T, Nakayama N, Ogura N, Kayagaki N, Okumura K, Koike T. Elevated serum levels of soluble Fas/APO-1 (CD95) in patients with hepatocellular carcinoma. *Clin Exp Immunol* 1998; **112**: 166–71.
20. Yufu Y, Choi I, Hirase N, Tokoro A, Noguchi Y, Goto T, Uike N, Kozuru M. Soluble Fas in the serum of patients with non-Hodgkin's lymphoma: higher concentrations in angioimmunoblastic T-cell lymphoma. *Am J Hematol* 1998; **58**: 334–6.
21. Kamihira S, Yamada Y, Tomonaga M, Sugahara K, Tsuruda K. Discrepant expression of membrane and soluble isoforms of Fas (CD95/APO-1) in adult T-cell leukaemia: soluble Fas isoform is an independent risk factor for prognosis. *Br J Haematol* 1999; **107**: 851–60.
22. Ueno T, Toi M, Tominaga, T. Circulating soluble Fas concentration in breast cancer patients. *Clin Cancer Res* 1999; **5**: 3529–33.
23. Kimura M, Tomita Y, Imai T, Saito T, Katagiri A, Tanikawa T, Takeda M, Takahashi, K. Significance of serum-soluble CD95 (Fas/APO-1) on prognosis in renal cell cancer patients. *Br J Cancer* 1999; **80**: 1648–51.
24. Konno R, Takano T, Sato S, Yajima A. Serum soluble Fas level as a prognostic factor in patients with gynecological malignancies. *Clin Cancer Res* 2000; **6**: 3576–80.
25. Ugurel S, Rappl G, Tilgen W, Reinhold U. Increased soluble CD95 (sFas/CD95) serum level correlates with poor prognosis in melanoma patients. *Clin Cancer Res* 2001; **7**: 1282–6.
26. Mizuno K, Sone S, Orino E, Mukaida N, Matsushima K, Ogura T. Spontaneous production of interleukin-8 by human lung cancer cells and its augmentation by tumor necrosis factor alpha and interleukin-1 at protein and mRNA levels. *Oncology* 1994; **51**: 467–71.
27. Miki T, Yano S, Hanibuchi M, Sone S. Bone metastasis model with multiorgan dissemination of human small-cell lung cancer (SBC-5) cells in natural killer cell-depleted SCID mice. *Oncol Res* 2000; **12**: 209–17.
28. Yonei T, Ohnoshi T, Hiraki S, Ueoka H, Kiura K, Moritaka T, Shibayama T, Tabata M, Segawa Y, Takigawa N. Antitumor activity of platinum analogs against human lung cancer cell lines and tumor specimens. *Acta Med Okayama* 1993; **47**: 233–41.
29. Yanagawa H, Takeuchi E, Suzuki Y, Ohmoto Y, Bando H, Sone S. Vascular endothelial growth factor in malignant pleural effusion associated with lung cancer. *Cancer Immunol Immunother* 1999; **48**: 396–400.
30. Nishioka Y, Yano S, Fujiki F, Mukaida N, Matsushima K, Tsuruo T, Sone S. Combined therapy of multidrug-resistant human lung cancer with anti-P-glycoprotein antibody and monocyte chemoattractant protein-1 gene transduction: the possibility of immunological overcoming of multidrug resistance. *Int J Cancer* 1997; **71**: 170–7.
31. Nishimura N, Nishioka Y, Shinohara T, Sone S. Enhanced efficiency by centrifugal manipulation of adenovirus-mediated interleukin 12 gene transduction into human monocyte-derived dendritic cells. *Hum Gene Ther* 2001; **12**: 333–46.
32. Hellquist HB, Olejnicka B, Jadner M, Andersson T, Sederholm C. Fas receptor is expressed in human lung squamous cell carcinomas, whereas bcl-2 and apoptosis are not pronounced: a preliminary report. *Br J Cancer* 1997; **76**: 175–9.
33. Nambu Y, Hughes SJ, Rehemtulla A, Hamstra D, Orringer MB, Beer DG. Lack of cell surface Fas/APO-1 expression in pulmonary adenocarcinomas. *J Clin Invest* 1998; **101**: 1102–10.
34. Kawasaki M, Kuwano K, Nakanishi Y, Hagimoto N, Takayama K, Pei XH, Maeyama T, Yoshimi M, Hara N. Analysis of Fas and Fas ligand expression and function in lung cancer cell lines. *Eur J Cancer* 2000; **36**: 656–63.
35. Owen-Schaub LB, Yonehara S, Crump WL, Grimm EA. DNA fragmentation and cell death is selectively triggered in activated human lymphocytes by Fas antigen engagement. *Cell Immunol* 1992; **140**: 197–205.
36. Leithauser F, Dhein J, Mechtersheimer G, Koretz K, Bruderlein S, Henne C, Schmidt A, Debatin KM, Krammer PH, 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* 1993; **69**: 415–29.
37. Yanagawa H, Haku T, Takeuchi E, Suzuki Y, Nokihara H, Sone S. Intrapleural therapy with MDP-lys(l-18), a synthetic derivative of muramyl dipeptide, against malignant pleurisy associated with lung cancer. *Lung Cancer* 2000; **27**: 67–73.