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Analysis of Fas and Fas ligand expression and function in lung cancer cell lines

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Abstract

The aim of this study was to investigate the expression of Fas and Fas ligand (FasL) and to determine the significance of these molecules in lung cancer cell lines. Immunoblotting, RT-PCR and flow cytometric analyses were carried out to measure the expression of Fas and FasL and to examine their interactions and effects on cell growth and apoptosis. Fas and FasL were co-expressed in most of the cell lines but to varying degrees. Apoptosis induced by the agonistic anti-Fas antibody was significantly correlated with Fas expression ($P=0.0075$), whereas cisplatin-induced apoptosis was not. Upregulation of Fas and FasL expression by the administration of cisplatin was found in 7 of 11 (64%) and 9 of 11 (82%) cell lines, respectively. However, cisplatin-induced apoptosis was not suppressed by antagonistic anti-FasL antibody. Thus, our data indicated that Fas and FasL were co-expressed in lung cancer cell lines, and that Fas ligation induced by agonistic anti-Fas antibody is functional and induced apoptosis that was dependent on the levels of Fas expression. In contrast, Fas-FasL interactions appeared to be non-functional. Furthermore, our results suggest that cisplatin-induced apoptosis in lung cancer cells was independent of the Fas-FasL interaction.

Keywords: Lung cancer; Apoptosis; Fas; Fas ligand; Cisplatin

1. Introduction

Fas ligand (FasL) and its receptor (Fas, CD95) are a set of regulatory components in immune system [1]. Fas-FasL ligation results in apoptosis of cells expressing Fas [2], and this process has been shown to play a critical role in the maintenance of immunological homeostasis and peripheral tolerance by the destruction of activated T lymphocytes [3]. In addition, cells in immunologically privileged sites, such as Sertoli's cells of the testis and parenchymal cells of the anterior chamber of the eye, express FasL. Any activated T cells expressing Fas that enter such a site would encounter cells expressing FasL and receive a death signal, thereby preventing an immune response [4,5].

It has recently been demonstrated that FasL is expressed on the surface of cancer cells including lung cancer cells [6–9]. These studies suggested that the expression of FasL might play an important role in establishing immunologically privileged environments that allow tumours to escape the host's immune surveillance [10]. However, Fas is also constitutively expressed on the cell surface of many normal cells including lymphocytes, epithelial cells and several other tissues [11], whereas Fas expression in cancer cells is variable [12–14].

In a recent investigation, it was also observed that in leukaemia cells, there is both an upregulation of Fas and FasL accompanying doxorubicin-induced cell death and a direct correlation between Fas receptor density and drug sensitivity [15]. This suggests the autocrine and/or paracrine activation of Fas signalling, caused by the induction of FasL expression, as a potential mechanism in the mediation of drug-induced apoptosis. In a subsequent study, bleomycin- and methotrexate-induced apoptosis in hepatoma cells were also shown to involve the Fas-FasL system [16]. However, it has also been reported that chemotherapy-induced apoptosis is not dependent on Fas-FasL interactions [17,18].

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Although several groups [9,19] reported the expression of FasL in lung cancer cells, there have been no reports about the relationship between Fas and FasL and function of these molecules in cancer. Therefore, we examined the expression of both Fas and FasL, and the function of Fas-signalling in 11 lung cancer cell lines. In addition, we investigated whether Fas-FasL interaction is involved in chemotherapy-induced apoptosis, using cisplatin, a key chemotherapeutic drug for lung cancer [20].

2. Materials and methods

2.1. Cell culture and treatment

Human lung cancer cell lines, NCI-H157, NCI-H322, NCI-H460, NCI-H1299, NCI-N417 (kindly provided by A.F. Gazder, Hamon Cancer Center, University of Texas, Dallas, TX, USA), EBC1, PC9, A549, LK2 (obtained from Health Science Research Resources Bank, Tokyo, Japan) and QG56, QG90 (provided by National Kyushu Cancer Center, Fukuoka, Japan), were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS, GIBCO-BRL, Grand Island, NY, USA) and penicillin/streptomycin in a humidified incubator with a 5% CO_2 atmosphere at 37°C. The cell lines consisted of three adenocarcinomas (PC9, H322, A549), three squamous cell carcinomas (EBC-1, LK2, QG56), two large cell carcinomas (H460, H1299), one adenosquamous carcinoma (H157) and two small cell carcinomas (QG90, N417). For RT-PCR and flow cytometric analysis, cells were harvested by trypsinisation using 0.05% trypsin and 0.02% EDTA without Ca^{2+} and Mg^{2+}. The cells were fixed for at least 1 h in ice-cold 70% ethanol and resuspended in PBS for flow cytometric analysis of Fas and FasL. Cells (2×10^6) were cultured in 75 cm^2 flasks for 2 days before treatment with 25 μM cisplatin (Nippon Kayaku, Tokyo, Japan), 100 ng/ml agonistic anti-Fas antibody (CH-11, MBL, Nagoya, Japan) and 10 μg/ml antagonistic anti-FasL antibody (4H9, Pharmingen, San Diego, CA, USA). The amount of apoptosis occurring in the treated cancer cells was measured 24 h after a 1 h treatment with cisplatin or a 24 h treatment with CH-11. In some experiments, antagonistic anti-FasL blocking antibody (4H9) was added 1 h before the incubation with cisplatin.

2.2. MTT assay

The MTT [3-(4,5-dimethilthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma Chemical, St Louis, MO, USA) assay was carried out as previously described [21]. Briefly, cancer cells (1×10^4 cells) were incubated overnight in a 96-well plate and treated with anti-Fas MAb (CH-11, 100 ng/ml) or anti-FasL MAb (4H9, 10 μg/ml) in a humidified atmosphere of 5% CO_2 at 37°C. After 24 h, 0.1 mg/50 μl MTT was added, the plate incubated at 37°C for 4 h, centrifuged at 800*g* for 10 min and the media removed. MTT formazan crystals were then solubilised by adding 200 μl DMSO and the absorbance measured using an automated microplate reader at a wavelength of 540 nm (Easy Reader EAR 340; SLT-Labinstruments, Austria). The value of the sample with medium alone was regarded as 100% survival. Results were shown as the mean of six independent wells ± S.D.

2.3. RT-PCR for FasL

Total RNA was extracted from cancer cells by the Isogen RNA extraction kit (Nippon Gene, Tokyo, Japan). For the PCR analysis of RNA, cDNA was prepared by reverse transcription of 4 μg of each RNA sample in a 20 μl reaction volume containing 10 mM Tris HCl (pH 8.8), 50 mM KCl, 4 mM $MgCl_2$, 0.1% Triton X-100, 1 mM DTT, 0.25 mM dNTPs, 5 μM random hexamer primers, 0.1 U/μl of ribonuclease inhibitor (Promega, Madison, WI, USA) and 10 U/μl of Molony-murine leukaemia virus RT (MMLV-RT) (GIBCO-BRL, Gaithersburg, MD, USA). The reaction mixture was incubated at 42°C for 1 h and at 95°C for 5 min. The cDNAs were then diluted to 100 μl, and these cDNAs were used in all PCRs. The PCR amplifications were performed in a 50 μl reaction volume containing 5 μl of each cDNA, 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 or 2.5 mM $MgCl_2$, 0.1% Triton X-100, 0.2 mM dNTPs and 1.25 U of Taq polymerase (Takara, Ohtsu, Japan). The primers used were as follows: (a) β-actin: sense 5′-TCCTGTGGCATCCATGAAACT-3′; antisense 5′-CTTCGTGAACGCCACGTGCTA-3′, (b) FasL: sense 5′-ATCCCTCTGGAATGGGAAGA-3′, antisense 5′-CCATATCTGTCCAGTAGTGC-3′. The conditions for amplification were as follows: 93°C for 3 min for 1 cycle, 93°C for 1 min, 55°C for 1 min, 72°C for 2 min for 35 cycles and 72°C for 7 min for 1 cycle. PCR products for FasL were transferred to Hybond-N hybridisation transfer membranes (Amersham, Arlington Heights, IL, USA). The membranes were then hybridised with an oligonucleotide probe (5′-GAGGATCTGGTGCTAATGGA-3′) labelled with digoxigenin-ddUTP by using the DIG Oligonucleotide 3′-End Labelling Kit (Boehringer Mannheim Biochemicals, Indianapolis, IN, USA). The hybridisations were performed according to the manufacturer's recommendations. The digoxigenin-labelled probe which hybridised with the PCR products was detected with the DIG Nucleic Acid Detection Kit (Boehringer Mannheim).

2.4. Flow cytometric analysis

Cell surface expression of the Fas antigen was measured using fluorescein isothiocyanate (FITC)-con-

jugated anti-Fas MAb (UB-2, IgG_1, MBL) [22]. 2×10^6 cells were preincubated in 1 ml of PBS containing 1% BSA (bovine serum albumin) for 45 min at 4°C. FITC-conjugated mouse IgG_1 nonspecific control antibodies (Coulter Immunology, Hialeah, FL, USA) were used as a negative control. To analyse the expression of FasL, mouse anti-human FasL MAb (clone 33, Transduction Laboratories, Lexington, KY, USA) and FITC-labelled goat antimouse IgG antibody (Immunotech S.A., Marseille Cedex, France) were used [17]. Cells were incubated with anti-FasL MAb for 45 min at 4°C, and incubated with the second antibody for 30 min at 4°C. Samples measured with non-specific mouse IgG as the first antibody were used as a negative control for FasL. After incubation, cells were washed twice and immediately analysed using a flow cytometer (EPICS XL System II, Coulter). Ten thousand cells were analysed per sample, and the positive per cent was calculated by additional software, compared with the negative control.

Apoptosis was detected using flow cytometric assay to APO2.7 (7A6 antigen) which was the recently described mitochondrial membrane epitope that is expressed in cells undergoing apoptosis [23]. Cells were stained with PE-labelled APO2.7 monoclonal antibody (MAb) (Coulter) by a slightly modified method. In brief, 1×10^6 cells were incubated with 100 μg/ml digitonin in PBS containing 2.5% FBS for 20 min at 4°C for cell permeabilisation. The cells were washed three times in PBS and incubated with PE-APO2.7 mAb for 15 min in the dark room. The cells were stored on ice in the dark until analysis was carried out. After incubation, cells were washed twice and immediately analysed using a flow cytometer. Ten thousand cells were analysed per sample, and the positive per cent was calculated by additional software, compared with cells with medium alone.

2.5. Western blot analysis

Whole cell protein extracts were prepared, and 30 μg of protein per extract was loaded on to an 11.25% SDS-polyacrylamide gel, proteins separated were electrophoretically then transferred on to an Immobilon (polyvinylidene difluoride) membrane and non-specific binding was blocked by incubating this membrane in 5% nonfat powdered milk in TBST (20 mM Tris, pH 7.5, 150 mM NaC1, 0.1% Tween 20). The membrane was incubated with the mouse anti-FasL MAb (clone 33; Transduction Laboratories) diluted 1:1000 in 5% powdered milk in TBST. The membrane was then washed extensively with TBST and incubated with anti-mouse horseradish peroxidase diluted 1:1500 (Santa Cruz Biotech, Santa Cruz, CA, USA). Proteins were

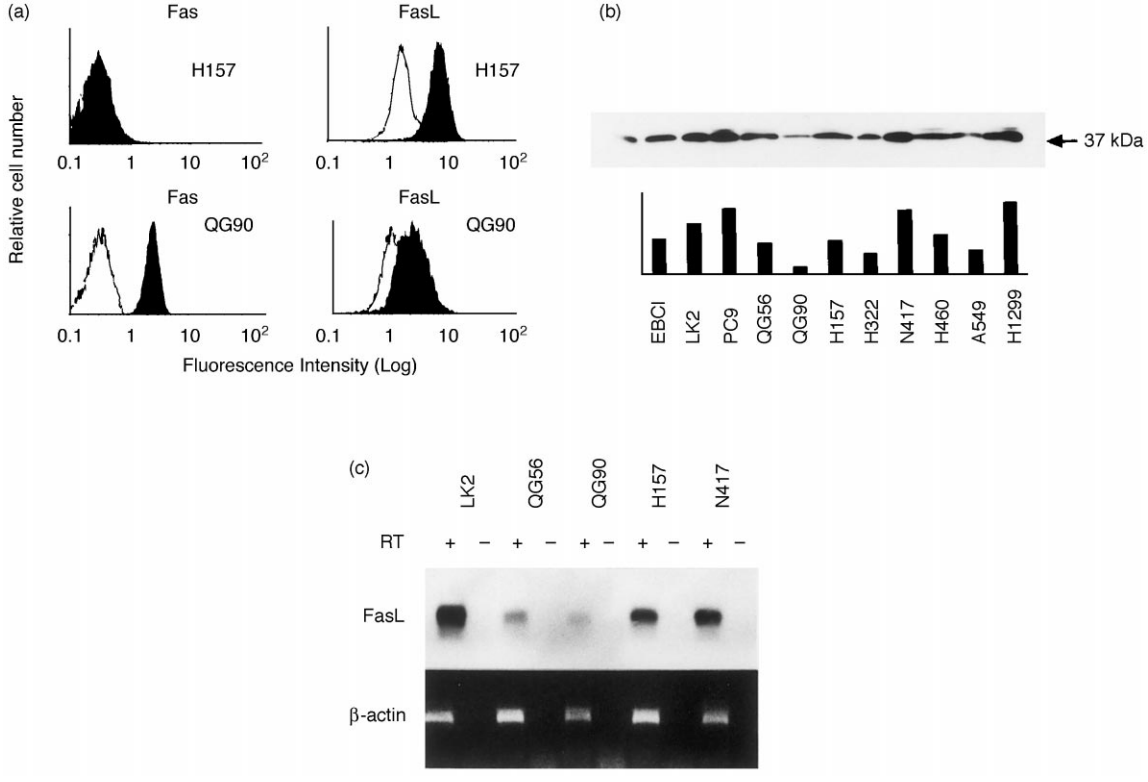

Fig. 1. (a) Surface expression of the Fas and FasL in lung cancer cell lines was analysed by flow cytometry. The open peaks represent the negative control and solid peaks are specific staining for Fas or FasL. (b) Western blotting analysis of FasL from 11 cancer cell lines. The left band shows the positive control of human endothelial cells. (c) Detection of FasL mRNA in 5 lung cancer cell lines by RT-PCR.

visualised by ECL (Amersham Pharmacia Biotech, Sweden) according to manufacturer's instruction. The density of FasL bands was assessed using the NIH Image 1.62.

2.6. Statistical analysis

The Spearman's rank correlation was used for correlation analyses. Quantitative experiments were analysed by use of the Student's *t*-test. All *P* values resulted from the use of the two-sided test. Flow cytometry data were analysed with the same statistical software package that was used for the expression analysis.

3. Results

3.1. Fas and FasL expression on lung cancer cell lines

Fas and FasL expression on the surface of tumour cells was analysed by quantitative flow cytometry experiments in 11 human lung carcinoma cell lines. Representative histograms of Fas and FasL are shown in Fig. 1(a). Fas and FasL expression in H157 were detected at 1.4% and 84.9%, respectively, compared with negative controls and at 87.5% and 25.7%, respectively, in QG90 cells. Fas and FasL were expressed in most of the cell lines but to different degrees (Table 1). Fas expression was detected at more than 20% in three of 11 (27%) cell lines, and FasL expression was detected at more than 20% in 11 of 11 (100%) cell lines. There was no correlation between Fas and FasL expression in lung cancer cell lines ($P = 0.25$). Western blotting also showed that all cell lines expressed different amounts of FasL protein (Fig. 1b). The results of the flow cytometry and Western blotting analyses were similar. In addition, FasL mRNA expression was examined by RT-PCR in 5 cell lines. Fig. 1(c) shows that FasL mRNA was detected in all of the five cell lines tested, although the levels of expression varied.

Table 1
Expression of Fas and FasL on 11 lung cancer cell lines, and upregulation of Fas and FasL on the cell surface of cancer cells treated with cisplatin. Values are the means from three determinations.

Cell line	Fas (%)	FasL (%)	Cisplatin-induced Fas-upregulation (%)	Cisplatin-induced FasL-upregulation (%)
EBC1	12.5	23.1	1.7	0
LK2	0.9	49.8	0.1	37.3
PC9	28.4	86.4	44.2	13.7
QG56	47.3	66.1	57.3	18.1
QG90	87.5	25.7	19.9	18.5
H157	1.4	84.9	1.9	20.4
H322	4.4	80.4	1.8	0.4
N417	6.1	87.1	19.0	53.5
H460	15.3	96.4	42.4	58.0
A549	15.8	62.4	59.0	60.5
H1299	13.1	86.6	15.5	62.7

3.2. The influence of the Fas-FasL interaction on the proliferation of cancer cells

Fig. 2 shows the proliferation of the QG56 cell line, in which both Fas and FasL were highly expressed (Table 1).

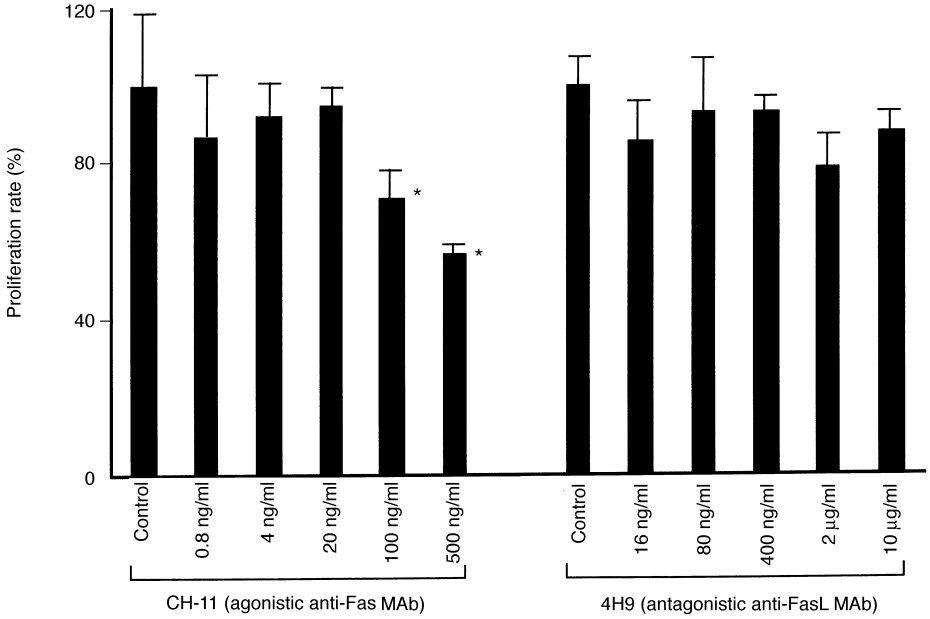

Fig. 2. Effect of antagonistic anti-FasL MAb (4H9) or agonistic anti-Fas MAb (CH-11) on the cellular proliferation rate of QG56 cells treated with CH-11 or 4H9 at various concentrations for 24 h. The cell viability was evaluated by MTT assay. The value of the sample with medium alone was regarded as 100% survival. Results are shown as the mean of six independent wells ±S.D. $*P < 0.05$ compared with the control.

These cells were cultured with 4H9 (antagonistic anti-FasL antibody) or CH-11 (agonistic anti-Fas antibody) and the effects of these antibodies on cellular proliferation was measured. Whilst CH-11-induced cell death was dependent on the concentration of antibody, 4H9 did not affect the proliferation of QG56 cell line. 10 other cell lines were also tested. In all cell lines, CH-11-induced cell death was dependent on the concentration of antibody to varying degrees whereas 4H9 did not affect the proliferation of the cell lines (data not shown). The isotype matched control antibodies (CH-11; mouse IgM, 4H9; mouse IgG) did not affect the proliferation of all cancer cell lines.

3.3. Relationship between Fas expression and CH-11 or cisplatin-induced apoptosis in lung cancer cells

Apoptosis induced by CH-11 or cisplatin was measured by APO2.7 flow cytometry. Representative results of apoptosis induced by CH-11 are shown in Fig. 3. The percentage of apoptosis was calculated at 3.2% in H322 and 91.6% in QG56, respectively. The number of apoptotic cells measured by flow cytometry induced by CH-11 or cisplatin was correlated with data obtained from a trypan-blue dye exclusion test (data not shown). In 11 cell lines, CH-11-induced apoptosis was significantly correlated with the Fas expression ($R = 0.883$, $P = 0.0075$, Fig. 4a). However, cisplatin-induced apoptosis was not correlated with Fas expression ($R = 0.044$, $P = 0.774$, Fig. 4b).

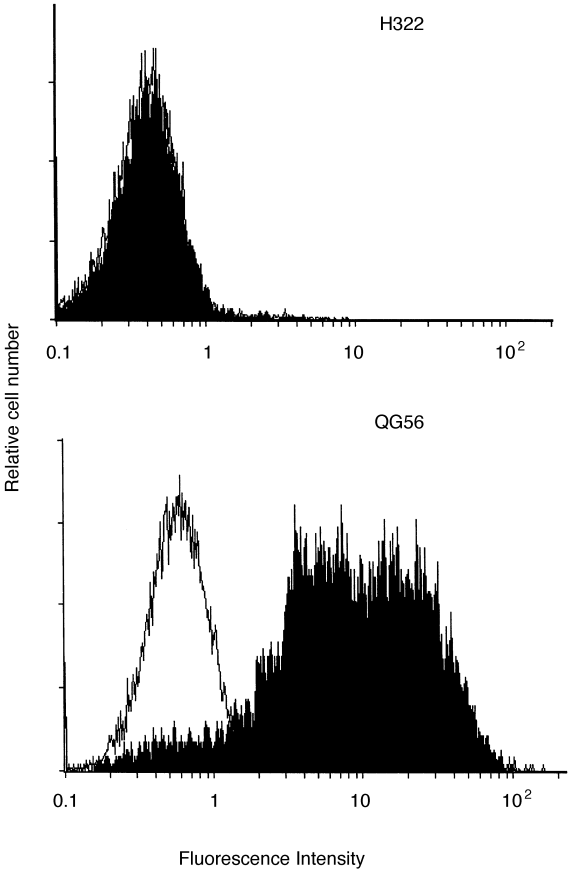

Fig. 3. Apoptosis induced by CH-11 (agonistic anti-Fas antibody) in lung cancer cells as measured by flow cytometry. Cancer cells were incubated for 24 h with CH-11 at concentrations of 100 ng/ml. Apoptosis was then analysed by flow cytometry for APO2.7. The histograms obtained from the CH-11 treatment are shaded. Open peaks denote a histogram of cells without CH-11 treatment, which shows the basal level of APO2.7 in control cancer cells.

3.4. 4H9 (Fas-FasL blocking antibody) does not effect cisplatin-induced apoptosis

We examined the effect of cisplatin on the expression of Fas and FasL on cancer cells. As shown in Table 1, varying levels of upregulation of Fas and FasL in 11 lung cancer cell lines following cisplatin treatment was observed. Fas and FasL were upregulated more than 20% in 4/11 (36%) and 6/11 (55%) cell lines, respectively. Rate of upregulation of Fas or FasL was not correlated with the degree of expression of Fas or FasL in the resting cells. The upregulation of Fas was not correlated with that of FasL in the same cell line.

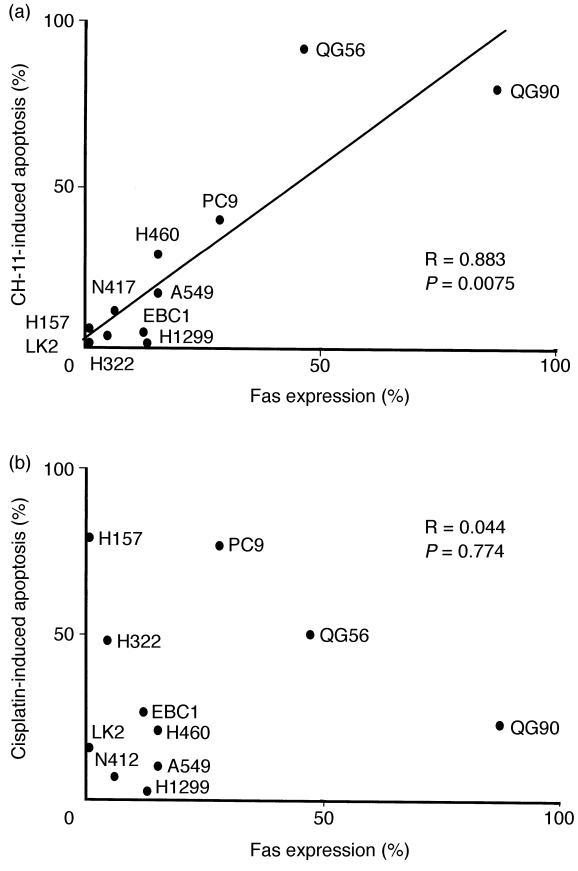

Fig. 4. Correlation between percentage of Fas positivity and percentage of CH-11-induced apoptosis (a) and cisplatin-induced apoptosis (b). Apoptosis was detected 24 h after a 1 h treatment with 25 μM cisplatin or a 24 h treatment with 100 ng/ml CH-11.

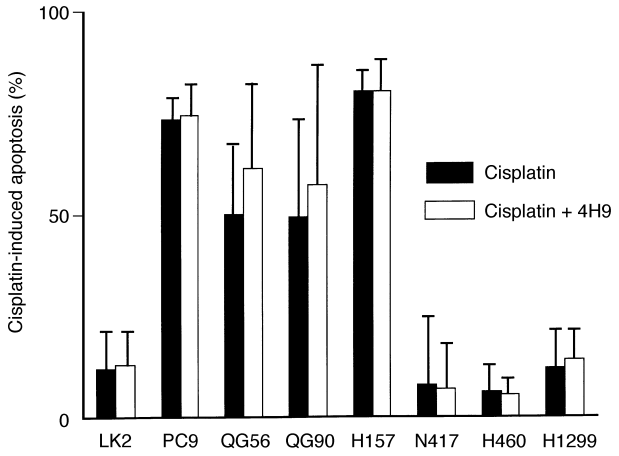

Fig. 5. Effects of antagonistic anti-FasL antibody (4H9) on cisplatin-induced apoptosis in lung cancer cell lines. 4H9 (10 μg/ml) was added 1 h before cisplatin treatment (1 h). Apoptosis was detected by APO2.7 flow cytometry after 24 h culturing. Values are the means from three determinations; bars, SEM.

Finally, we examined the effect of the blockade of the Fas-FasL system on cisplatin-induced apoptosis in eight cell lines. Addition of 10 μg/ml 4H9 (antagonistic anti-FasL antibody) had no effect on cisplatin-induced apoptosis (Fig. 5).

4. Discussion

Recently, constitutive expression of FasL has been found in various cancer cells including melanoma [6], hepatocellular carcinoma [7] and colon cancer [8]. Niehans and colleagues reported human lung cancer cells also express FasL [9]. They demonstrated that all human lung cancer cell lines tested (16/16) expressed FasL as determined by immunoblotting analysis and the majority of resected tumours (23/28) showed positive staining for FasL by immunohistochemistry. Furthermore, they confirmed these results using RT-PCR for FasL mRNA and by functional analysis using the Jurkat cell line. We also confirmed that the majority of lung cancer cell lines tested expressed FasL using flow cytometry, and RT-PCR.

In tumours tested to date, a wide spectrum of Fas expression and sensitivity to Fas-mediated apoptosis is found. This ranges from: minimal or no expression of Fas in melanoma [6,11]; downregulation of Fas in hepatocellular carcinoma [7,24]; expression of Fas, but resistance to Fas-mediated apoptosis in colon carcinoma [8,12] and pancreatic adenocarcinoma [25]; to frequent sensitivity to Fas-mediated death in glioma cell lines [26]. In lung carcinomas, Hellquist and colleagues reported that Fas was expressed in all squamous carcinomas [13], whilst Nambu and colleagues reported that cell surface expression of Fas was lacking in pulmonary adenocarcinomas [14]. In our data, the expression of Fas appeared to be varied in the 11 lung cancer cell lines, which was independent of the cell type. Similar to colon carcinoma, Fas-expression tended to be weak in some lung cancer cells [8].

We found that apoptosis of cancer cells induced by CH-11 (agonistic anti-Fas antibody) was significantly correlated with Fas expression. However, in general, Fas expression in cancer cells is downregulated and also in some cases non-functional. Many mechanisms for a developing resistance to apoptosis have been suggested in malignant cells including: lack of cell-surface Fas protein expression; synthesis of anti-apoptotic proteins such as the Bcl-family of proteins; alterations in intracellular Fas-signalling pathways; production of a soluble form of Fas. In our study, 8/11 cell lines, in which Fas was expressed at a low level, might utilise one of these mechanisms. Resistance to apoptosis (through low Fas expression, for example) may be a mechanism for lung cancer cells to escape attack by activated cytotoxic T cells, which are frequently present in these tumours.

In our study, Fas and FasL co-expression was found in most cell lines, and Fas-signalling in these cells with agonistic anti-Fas antibody was functional. In the literature, FasL expressed on the surface of most of cancer cells is functional [6–9]. It is not clear why culture of a cell line that expresses both Fas and FasL does not trigger cell death, although since the lung cancer cells in our study expressed relatively low levels of Fas, they may escape from binding to FasL and, therefore, the subsequent cell death. There have been only a few studies reporting co-expression of Fas and FasL [10,27,28]. Recently Ungefroren and colleagues reported that Fas and FasL were co-expressed in human pancreatic adenocarcinomas, but were resistant to Fas-mediated apoptosis due to FAP-1, a Fas-associated phosphatase that can block the apoptotic function of Fas [29]. However, in our study, Fas-mediated signalling was functional in lung cancer cells. There is a possibility that some cancer cells may lack an accessory molecule that is critical for the induction of cell lysis [28,30] or that agonistic antibody and FasL mediate different sensitivities to death in the signalling pathways of Fas [31,32].

Owen-Schaub and colleagues reported that Fas-FasL interactions may not uniformly result in apoptosis but can accelerate growth in some human tumours [25]. However, although our results suggested that co-expression of Fas and FasL did not induce autocrine cell death, blockade of the Fas-FasL system also did not affect the proliferation of the cancer cells.

Recent studies indicate that widely used chemotherapeutic agents induce apoptosis in susceptible cells [33,34]. It is suggested that the Fas-FasL system and chemotherapeutic agents activate the same downstream apoptotic pathways. However, the exact relationship between the pathways of chemotherapy-induced apoptosis and Fas/FasL-triggered apoptosis is unclear [18].

We demonstrated that upregulation of Fas or FasL expression was found in several cell lines following treatment with cisplatin, but blockade of the Fas-FasL system did not affect cisplatin-induced apoptosis. Several reports have described that chemotherapeutic agents upregulate the expression of Fas and/or FasL, but that their effects on apoptosis of the target cells were variable [17,35,36]. This disparity might reflect differences concerning either the cell lines and/or the drugs used. Upregulation of Fas and FasL expression after the application of chemotherapeutic agents might play a part in the rescue/survival mechanisms of some cancer cells. Muller and colleagues described induction of FasL in *p53* wild-type, *p53* mutant, and *p53* deficient cell lines in response to anticancer drugs. In contrast, upregulation of the Fas receptor was observed only in cells with wild-type *p53*, not in cells with mutant or no *p53* [16]. Likewise, in the cell lines used in this study, cells with mutant *p53* (EBC1, LK2, H157, H322, N417) or lacking *p53* (H1299) tended to express low levels of Fas protein and exhibited a weak upregulation of Fas following treatment with cisplatin, compared with the cells with wild-type *p53* (H460, A549). However, in contrast, there was no correlation between FasL expression and *p53* status. Thus, Fas expression or upregulation following cisplatin treatment probably requires the function of wild-type *p53*.

To conclude, this is the first report in which co-expression of Fas and FasL has been demonstrated and the relationship between Fas and FasL expression has been analysed in several cancer cell lines. Expression of Fas was low in almost all lung cancer cell lines. There was no correlation between Fas and FasL expression. Although Fas-ligation using agonistic anti-Fas antibody induced apoptosis in cells that express Fas, blockade of the Fas-FasL system had no effect on the proliferation of cancer cells. Cisplatin induced the upregulation of Fas and FasL expression on cancer cells. However, blocking of the Fas-FasL system did not affect cisplatin-induced apoptosis of cancer cells. Regarding the co-expression of Fas and FasL, unknown intracellular factors which modulate Fas-signalling may exist in cancer cell lines.

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