Reciprocal co-expression of Fas and Fas ligand in human cholangiocarcinoma

GEORGE PAN[1], EUN-YOUNG AHN[1], YABING CHEN[1], GONG FENG[1], VISHNU REDDY[1], NIRAG C. JHALA[1] and JAY M. McDONALD[1,2]

[1]Department of Pathology, University of Alabama at Birmingham, Birmingham, AL 35294; [2]Veterans Administration Medical Center, Birmingham, AL 35233, USA

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Abstract. We have previously characterized the role of Fas in tumorigenesis using two cholangiocarcinoma cell lines expressing high (Fas^H) and low (Fas^L) levels of Fas. Here we further characterize Fas ligand (FasL) expression and function in these two cell lines. The Fas^L cells expressed a high level of FasL, whereas the Fas^H cells expressed a low level of FasL showing reciprocal expression of Fas and FasL in tumor cells. FasL released from the Fas^L cells is capable of inducing apoptosis of lymphocytes, which is blocked by neutralizing Fas antibody. To study the underlying mechanism for the reciprocal expression of Fas and FasL, we examined the activities of both the Fas and FasL promoters. The activity of the Fas promoter is suppressed and the activity of the FasL promoter is stimulated in the Fas^L cells compared to the Fas^H cells. The inverse activities of Fas and FasL promoter in tumor cells are regulated by NF-κB, which inhibits Fas expression and increases FasL expression through binding to their respective promoters. The inverse expression of Fas and FasL in tumor cells is partially reversed by an NF-κB inhibitor. In conclusion, human cholangiocarcinoma cells reciprocally co-express functional Fas and FasL, which are the result of the activities of the Fas and FasL promoters being regulated by NF-κB. These findings provide a potential unifying molecular mechanism for modulating tumorigenesis via Fas/FasL expression.

Introduction

Cholangiocarcinoma represents a challenging primary malignant tumor with no effective medical therapy and a poor prognosis. The etiology of cholangiocarcinoma remains unknown. In our previous investigations, we have demonstrated that human cholangiocarcinoma cell lines heterogeneously express Fas antigen at the cell surface. We have isolated two subpopulations based on their Fas levels: one with low Fas expression (Fas^L) and the other with high Fas expression (Fas^H) (1,2). The Fas^L cells, but not Fas^H cells, are resistant to apoptosis induced by Fas activating antibody and by other apoptotic stimuli (3-5) and are capable of producing tumors when subcutaneously injected into nude mice (2,5). These results suggest that the down-regulation of Fas expression and resistance to Fas-activated apoptosis might play a crucial role in the tumorigenesis of cholangiocarcinoma.

Although decreased Fas expression in tumors is frequently associated with enhanced malignant growth or metastasis (6,7), a decrease in Fas expression alone is unlikely to be entirely responsible for the enhanced neoplastic growth and metastasis (8). Tumor cells must require additional molecular mechanisms to render them resistant to host immune attack (9). Malignant cells have several mechanisms to escape immune surveillance including expression of low levels of target tumor antigens (10). Expression of FasL on tumor cells is also one of the mechanisms by which malignant cells escape the host immune attack. It has been reported that many carcinomas aberrantly express FasL, whereas the parallel normal tissues do not (11-13). Expression of FasL by malignant tumors may counteract the host's anti-tumor immunity and favor tumor growth by killing infiltrating T lymphocytes through Fas-FasL interaction (14-16). When FasL-positive melanoma cells were injected into mice, tumors developed more rapidly in wild-type mice compared with Fas deficient *lpr* mutants, suggesting that functional FasL on tumors may be involved in the destruction of immune cells and in maintaining a state of tumor immune privilege (17).

The hypothesis that FasL is important for tumor growth and immune privilege has been challenged (18,19). Tumor cells transfected with the gene encoding FasL induced rapid tumor regression rather than fostering tumor development. However, the level of Fas expression by these transfected tumor cells was not indicated (14,20,21). We hypothesized that simultaneously decreased Fas expression and increased FasL expression in tumor cells are required for tumor growth or metastasis. Reciprocal expression of Fas and FasL in tumors has been observed in previous studies (22,23). However, the underlying mechanisms for their expression have not been

Correspondence to: Dr Jay M. McDonald, Department of Pathology, University of Alabama at Birmingham, 701 19 Street S., LHR 504, Birmingham, AL 35294, USA
E-mail: jmcdonald@path.uab.edu

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elucidated. In this study, we found a reciprocal relationship between Fas and FasL levels in the Fas^H and Fas^L human cholangiocarcinoma cell lines, which is associated with high levels of NF-κB transcription factor in the Fas^L tumor cells. Increased NF-κB transcription factor simultaneously inhibited Fas and stimulated FasL expression in the Fas^L tumor cells. Our results provided important insight into the underlying molecular mechanism regulating Fas and FasL expression and thus tumorigenesis.

Materials and methods

Cell culture. Human cholangiocarcinoma cells (SK-ChA-1) were generously provided by Dr A. Knuth (Ludwig Institute for Cancer Research, London, UK) and their growth conditions were described previously (1,2). Cells were subcloned by flow cytometric sorting into two subpopulations, those expressing low amounts of Fas (Fas^L) and those expressing high amounts of Fas (Fas^H), as previously reported (1,2).

Isolation of RNA and quantitative real-time RT-PCR. Total RNA was isolated with TRIzol reagent (Invitrogen, Palo Alto, CA) according to the manufacturer's protocol. First-strand cDNA was synthesized by annealing RNA (1 μg) with 250 ng of random primers by heating at 70˚C for 2 min. After denaturing, samples were incubated with reverse transcriptase, 10 mM dithiothreitol, RNasin (Promega, Madison, WI) and 0.5 mM dNTPs in a total volume of 20 μl for 1 h at 42˚C. The reaction mixture was diluted to a final volume of 100 μl and heat-inactivated at 95˚C for 5 min.

Real-time RT-PCR was performed on an ABI GeneAmp 5700 sequence detection system using SYBR-Green labeling primers (Applied Biosystems, Foster City, CA). In the total volume of 25 μl reaction mixture, 22.5 μl master mix, 2.5 μl cDNA (corresponding to 25 ng of total RNA), and 6 μM of forward and reverse primers were added. The reaction mixture was heated for 5 min at 95˚C and then 40 PCR cycles consisting of 15 sec at 95˚C and 60 sec at 60˚C were performed. The product accumulation was monitored by SYBR-Green fluorescence (24). The sequences of primers used for real-time RT-PCR were as follows: GAPDH 5'-GATTCCACCC ATGGGAATT-3' (forward), 5'-GGGTGGTGGAAGATGGT GAT-3' (reverse); Fas 5'-ACTTCGGAGGATTGCTCAACA-3' (forward), 5'-ACTTCGGAGGATTGCTCAACA-3' (reverse); and FasL 5'-AAAGTGGCCGATTTAACAGGC-3' (forward), 5'-GCAACAGACGTAAGAACCAGAGG-3' (reverse). The relative gene expression levels were determined from the standard curve and were calculated using software provided by the manufacturer (Applied Biosystems).

Northern blotting of Fas and FasL. Total mRNA (20 μg/lane) isolated from the cultured Fas^H and Fas^L cells was denatured and electrophoretically separated on 2.2% agarose gels containing 6% formaldehyde. The gels were then transferred to nitrocellulose membranes and fixed by UV light cross-linking. Membranes were prehybridized in QuikHyb hybridization buffer (Stratagene, La Jolla, CA) at 65˚C for 1 h and then hybridization was continued in QuikHyb hybridization buffer containing $8x10^5$ cpm/ml of [γ-^{32}P]-labeled Fas or FasL cDNA probes for a further 1 h. The Fas and FasL probes were generated with a T7 sequence using [γ-^{32}P]UTP and a T7 RNA polymerase. The labeled RNA transcripts were purified by G-25 Sephadex chromatography (Boehringer Mannheim, Indianapolis, IN). Membranes were washed 3 times for 20 min at 65˚C in 1X SSC and then exposed to film for 16 h.

Western blotting of Fas and FasL. Western blotting was performed using lysates (20 μg protein) from the Fas^H and Fas^L cells as described previously (3-5). Primary antibodies were rabbit anti-human Fas (diluted 1: 200, clone C-20) or mouse anti-human FasL (diluted 1:250, clone N-20; Santa Cruz Biotechnology, Santa Cruz, CA). After washing, membranes were incubated with a goat anti-rabbit (or goat anti-mouse) IgG-antibody conjugated to horseradish peroxidase at a 1:5,000 dilution as the second antibody. Anti-GAPDH antibody was used for loading control.

Chromium release assay. The Fas^H and Fas^L cells were grown to 80% confluence. These effector cells (E) were then resuspended in RPMI-1640 complete medium at $1x10^7$/ml. Fas-bearing sensitive A20 ($2x10^6$) target cells (T) were suspended in 0.5 ml RPMI-1640 complete medium and labeled with 25 μl ^{51}Cr (30 μCi) at 37˚C for 1 h. After washing twice with 50 ml complete medium, labeled A20 cells were resuspended at a concentration of $1x10^6$/ml and then seeded into a 96-well plate ($1x10^5$ cells/0.1 ml/well). The ^{51}Cr-labeled A20 cells (T) were incubated with 0.1 ml Fas^H or Fas^L cells (E) at E/T ratios of 3:1, 6:1, 12:1 and 24:1 overnight. The plate was centrifuged and 100 μl supernatant was removed to determine radioactivity by a liquid scintillation counter. The maximum and minimum radioactivity were determined by counting ^{51}Cr in the supernatant of ^{51}Cr-A20 cells alone in the presence (maximum) and absence (minimum) of 0.1% SDS. The specific toxicity was calculated as follows: Specific toxicity = [(sample CPM) - (minimum CMP)]/[(maximum CPM) - (minimum CPM)] x 100.

Isolation of FasL from cell culture supernatants. FasL from tumor cell supernatants was isolated as described previously (9,25,26). Briefly, cell culture supernatants collected from the Fas^H and Fas^L tumor cells were centrifuged twice at 200 x g for 20 min at 4˚C followed by ultracentrifuging at 47,000 x g at 4˚C for 3 h (Beckman, T50). The microvesicle pellet was washed with PBS and then treated with or without 1% Triton X100 at 4˚C for 30 min. The microvesicle supernatant was collected and stored at -80˚C after centrifuging at 23,000 x g at 4˚C for 1 h.

Assessment of released FasL bioactivity. The function of FasL released from tumor cells was determined as described previously (26). Jurkat cells were cultured with the indicated volumes of the prepared microvesicle supernatants overnight. The medium and Fas activating antibody (50 ng/ml) were used as a negative and a positive control. After incubation, apoptosis was determined by an Annexin V assay as per the manufacturer's instructions.

Electrophoretic mobility shift analysis (EMSA). Nuclear extracts were prepared as described previously (27). The double-stranded oligonucleotides containing the consensus

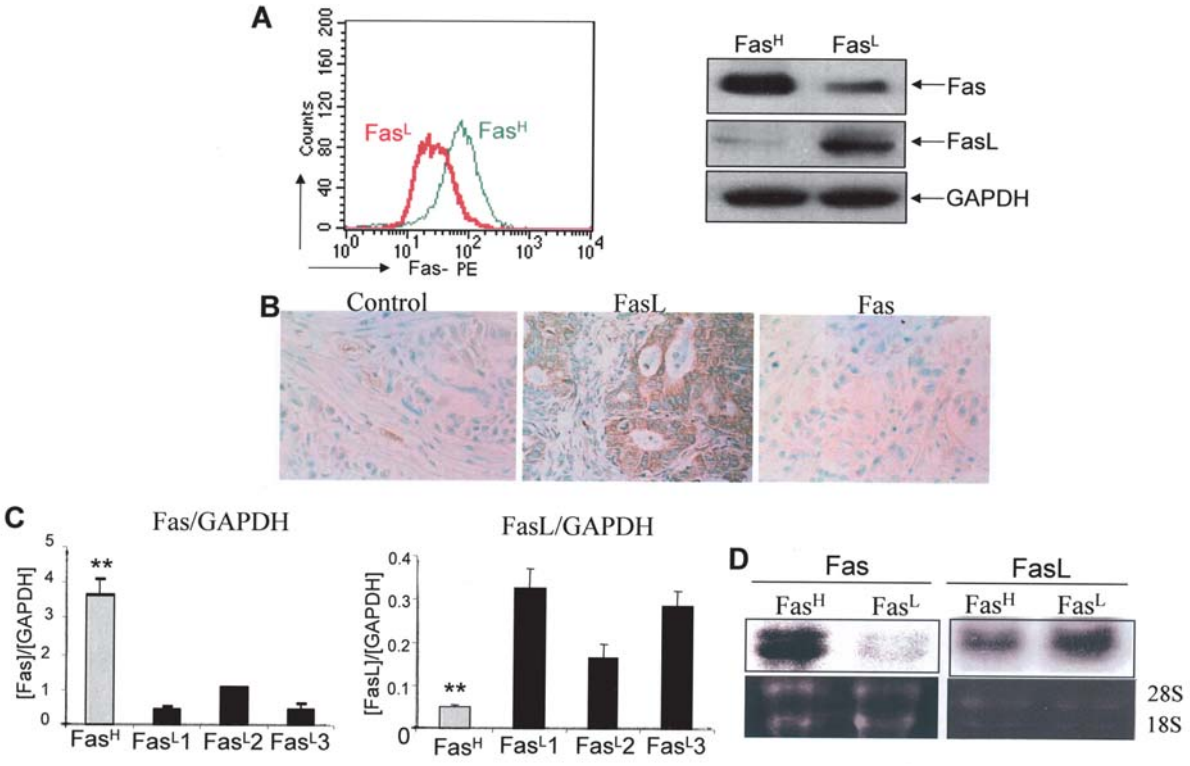

Figure 1. Fas^H and Fas^L human cholangiocarcinoma cells inversely co-express Fas and FasL proteins. (A) Cultured human cholangiocarcinoma cells (SK-ChA-1) were stained for Fas using a PE-conjugated mouse anti-Fas antibody. Cells were sorted and cloned into the Fas^H clones with a high expression level of Fas and the Fas^L cells with a low expression level of Fas. The mean Fas expression in the Fas^L clone (red) is shown compared to the Fas^H clone (green). Cultured Fas^H and Fas^L human cholangiocarcinoma cells were lysed, the levels of Fas and FasL were assayed in the lysates (20 μg protein) by Western blotting. GAPDH was performed as a control. (B) Tissue sections of paraffin-embedded cholangiocarcinoma engraftments were stained for Fas and FasL. Tumors were grown in nude mice using cultured Fas^L cells for injection (2,5). Immunostaining for Fas and FasL was visualized by DAB and hematoxylin. Left, negative control without Fas or FasL antibodies shows well-differentiated cholangiocarcinoma and a low background. Middle, the distribution of FasL protein is distinctly observed in tumor cells as a brown staining. Right, cells probed with Fas antibody show a low level of Fas protein is expressed in tumor engraftment as a slight brown color. (C) Total RNAs were isolated from a clone of Fas^H and three clones of Fas^L cells using a TRIzol reagent according to the manufacturer's protocol. One microgram of total RNA was used for first-strand cDNA synthesis. The cDNA (25 ng) was then used as templates for quantitative real-time PCR amplification of human Fas, FasL and GAPDH. The results are expressed as the mean ± SE of the ratio of Fas transcripts (left) or FasL transcripts (right) to GAPDH transcripts in three independent experiments. $^{**}p<0.01$ compared to the individual Fas^L cell clones. (D) Total mRNA of 20 μg from representative clones of Fas^H and Fas^L cells was analyzed by Northern blotting with cDNA probes for human Fas and FasL as described in Materials and methods. Fas mRNAs of 1.4 kb and FasL mRNAs of 1.6 kb (upper panel) and 28S and 18S (lower panel) are shown.

NF-κB binding site in the Fas and FasL promoters were labeled by a kinase reaction with T4 polynucleotide kinase and [γ-^{32}P]-ATP. EMSA was performed using 2 ng of labeled double-strand oligonucleotides containing an NF-κB consensus binding motif (10,000 cpm, Promega, Madison, WI), and 5 μg of nuclear extract according to Promega's gel shift assay core system.

Plasmids, transient transfection and luciferase assay. PCR-amplified fragments of the Fas promoter (1.8 kb) and FasL promoter (1.7 kb) were subcloned into *KPN*I and *Hind*III restriction sites of the luciferase reporter vector pGL3-basic (Promega). The activities of the Fas and FasL promoters in the Fas^H and Fas^L cells were determined by a dual-luciferase reporter assay system (Invitrogen, Carlsbad, CA). Luciferase reporter pGL3-basic vectors containing the Fas promoter or the FasL promoter were transfected into the Fas^H and Fas^L cells using Lipofectamine and Plus™ reagent per the manufacturer's instructions. A luciferase reporter plasmid containing Renilla-Luciferase driven by an SV40 promoter (pRF-SV40) was used as an internal control for transfection efficiency. Two days after transfection, the luciferase activity in 50 μl of cell lysate was assayed and normalized to Renilla-Luciferase activity for transfection efficiencies.

Immunohistochemical staining of Fas and FasL. Immunohistochemical staining of Fas and FasL was performed in tumor specimens produced by subcutaneous injection of the Fas^L cells into nude mice as previously described (1,2). Briefly, tumor specimens were fixed in 10% formalin, embedded in paraffin, and cut into 6-μm sections (15). After dewaxing and rehydration, the slides were incubated with 0.5% hydrogen peroxide in water for 30 min to block endogenous peroxidase activity and 5% BSA blocking buffer for 30 min to reduce non-specific binding. Expression of Fas and FasL was determined using anti-human monoclonal antibodies (BD Biosciences, Palo Alto, CA). The secondary horseradish peroxidase (HRP)-conjugated goat anti-mouse serum (Dako) was used at 1/200 for 30 min. The color reaction was developed using 3,3'-diaminobenzidine tetrahydrochloride (DAB). Negative control slides were prepared without the primary antibody but retaining all of the other steps.

Results

Reciprocal expression of Fas and FasL proteins and transcripts in the Fas^H and Fas^L cells. We generated two cell clones, Fas^H and Fas^L cells, based on their relative expression level of Fas (2). We first examined the expression of Fas in the Fas^L and the Fas^H cells by flow cytometry. A representative result is shown in Fig. 1A. Fas expression in the Fas^L cells (red line) was lower than in the Fas^H cells (green line) confirming the different expression of Fas in the Fas^H and Fas^L cells. Using these Fas^H and Fas^L cells the expression of FasL was determined by Western blotting. The level of FasL protein in the Fas^H cells was decreased compared to the Fas^L cells (Fig. 1A).

We previously demonstrated that the Fas^L, but not the Fas^H, cells produced tumors following subcutaneous injection into nude mice (2). To confirm the reciprocal expression of Fas and FasL in engraftments, we determined the expression of Fas and FasL in formalin-fixed paraffin embedded engraftments, produced by the injection of Fas^L cells into the flanks of nude mice (1,2). Tumor cells were stained intensely by FasL antibody as indicated by the intense brown staining (Fig. 1B, FasL panel). As expected, Fas expression in the tumor engraftments was not detectable (Fig. 1B, Fas panel). The negative control was incubated with the secondary antibody only (Fig. 1B, control panel).

To determine whether there is a reciprocal relationship between the Fas and FasL transcripts, the relative concentrations of Fas and FasL mRNAs in the Fas^H and Fas^L cells were determined by quantitative real-time RT-PCR and Northern blotting assay. The ratio of Fas and FasL to GAPDH gene transcripts in the Fas^H and three Fas^L cell lines are shown in Fig. 1C. The Fas transcripts in the Fas^H cells were approximately four times higher than in the Fas^L cells, whereas the FasL transcripts in the Fas^H cells were decreased compared to the Fas^L cells. This reciprocal concentration of Fas and FasL mRNA transcripts was also confirmed by Northern blotting (Fig. 1D).

The function of surface FasL in the Fas^H and Fas^L cells. To assess whether the higher levels of FasL in the Fas^L cells are capable of killing Fas-bearing cells, a cytotoxicity assay using ^{51}Cr-labeled target cells was performed. Fig. 2 shows that the percentage of ^{51}Cr released from A20 cells induced by the Fas^L cells was markedly higher than that induced by the Fas^H cells at all ratios of tumor (E) to A20 (T) cells ($p<0.001$). The maximum ^{51}Cr release by the Fas^L cells was 50-60%, whereas the maximum ^{51}Cr release by the Fas^H cells was 10-20% suggesting that the increased FasL on the Fas^L cells is functional in killing Fas-bearing T lymphocytes.

The function of FasL released from the Fas^H and Fas^L cells. A recent report has indicated that epithelial ovarian cancer cells secrete functional FasL in the form of microvesicles capable of inducing apoptosis of Fas-bearing immune cells (26). To determine whether Fas^L and Fas^H cells secrete functional FasL in microvesicles, FasL-containing microvesicles were prepared from culture supernatants collected from the Fas^H and Fas^L cells (26). FasL was released from these microvesicles using a lysis buffer containing 1% Triton X-100 for 30 min (Fas^H/Sup and Fas^L/Sup). PBS (Fas^H/PBS and Fas^L/PBS,) was used as negative controls. Fig. 3A shows a representative experimental result. The Triton-treated supernatants from the Fas^L (Fas^L/Sup), but not from the Fas^H (Fas^H/Sup), cells profoundly activated apoptosis of Jurkat cells (right hand panels). However, the PBS-treated supernatants from both the Fas^H (Fas^H/PBS) and Fas^L (Fas^L/PBS) cells were unable to induce apoptosis of Jurkat cells. Fig. 3B shows a summary of three independent experiments. The percentage of apoptotic Jurkat cells was markedly increased when Fas antibody was added. The supernatants from the Fas^L, but not from the Fas^H, tumor cells induced apoptosis of T lymphocytes. The amount of apoptosis induced by supernatants from the Fas^L cells was dependent on the volume of supernatant used (Fig. 3C).

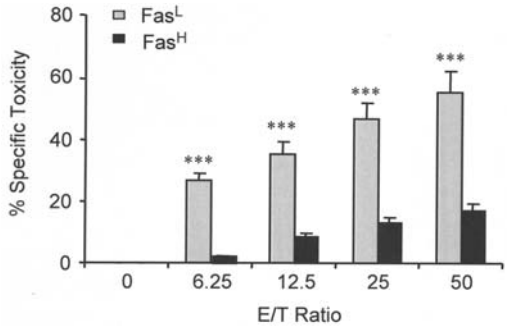

Figure 2. Function of surface FasL in the Fas^H and Fas^L cholangiocarcinoma cells. The Fas^H and Fas^L human cholangiocarcinoma cells (E) were cocultured with ^{51}Cr-labeled A20 cells (T) at the indicated ratio of E/T for 12 h. The released radioactivity in the supernatants was measured. The Y-axis represents the percentage of specific toxicity calculated by the formula given in the Materials and methods. The X-axis represents the ratio of (E/T). The results are shown as the mean ± SE from three independent experiments. $^{***}p<0.001$ compared to the relative Fas^L cells in Student t-test.

To further confirm whether apoptosis of Jurkat cells is induced by FasL released from the Fas^L cells, a blocking assay was performed using a neutralizing Fas antibody. Supernatants from the Fas^L cells (Fas^L/Sup) and Fas antibody (Fas Ab)-treated cells profoundly induced apoptosis of Jurkat cells. Treatment with a neutralizing Fas antibody (nFas Ab) markedly inhibited this apoptosis ($p<0.01$, Fig. 3D). Apoptosis induced by a Fas antibody was reduced from 84% to 23% by a neutralizing Fas antibody. Similarly, a neutralizing Fas antibody also reduced apoptosis by the supernatants from the Fas^L cells from 73% to 37%.

Promoter activity of the Fas and FasL in the Fas^H and Fas^L cells. To begin elucidating the mechanism(s) for the inverse expression of Fas and FasL in the Fas^L and Fas^H cells, the activities of both the Fas and the FasL promoters were determined. We constructed plasmids containing a luciferase reporter gene regulated by the Fas promoter (Faspromoter-Luc) or FasL promoter (FasLpromoter-Luc). These plasmids were transfected into the Fas^L and Fas^H cells and luciferase activities were determined at 48 h post-transfection.

As shown in Fig. 4, both Fas and FasL promoters contain NF-κB binding motifs. Luciferase activity driven by the Fas promoter in the Fas^H cells was significantly greater than in the Fas^L cells ($p<0.001$) indicating a strong Fas promoter

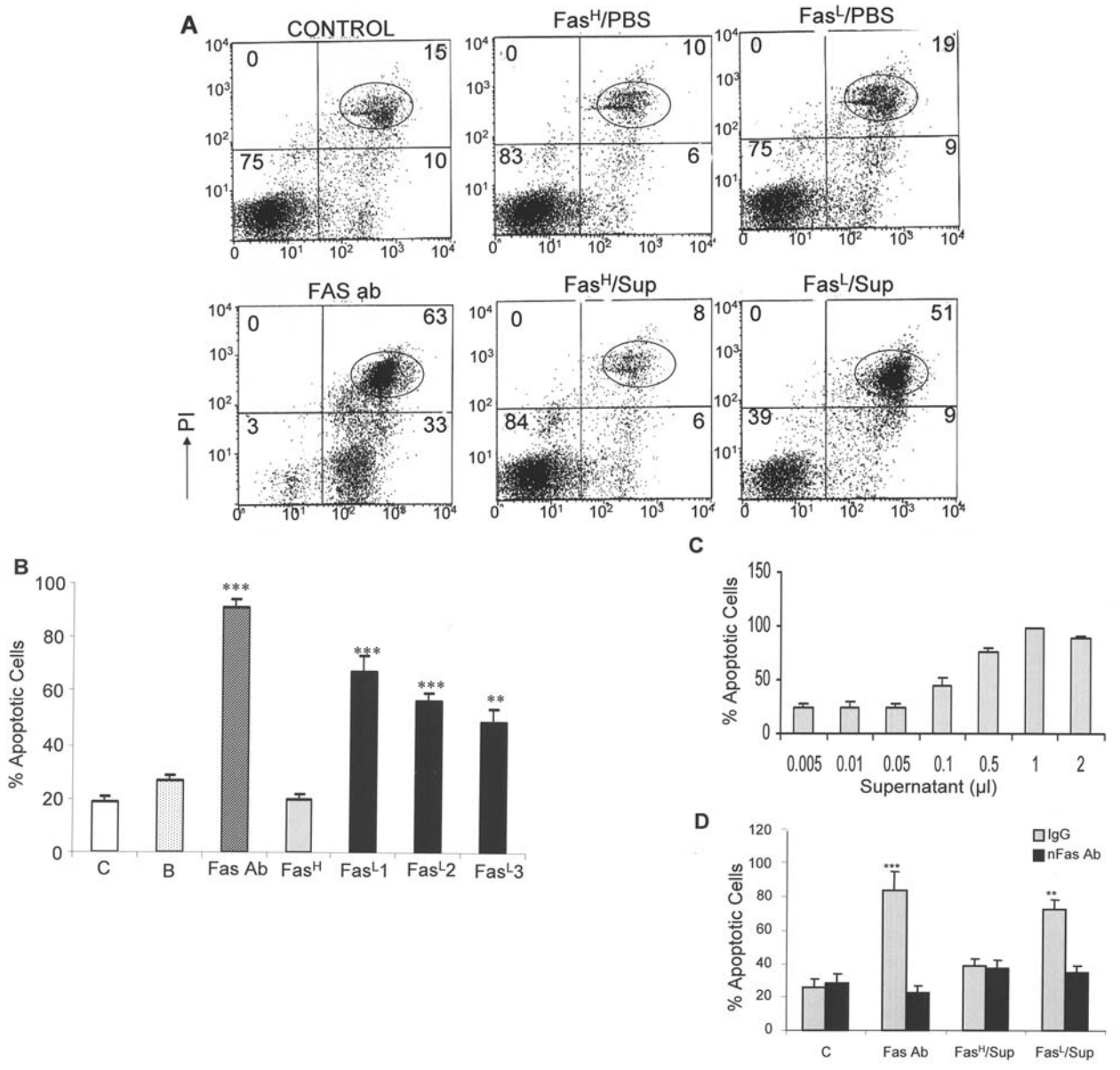

Figure 3. FasL secreted from the Fas^{L} tumor cells is functional. (A) Microvesicles were prepared from the culture supernatants of the Fas^{H} and Fas^{L} cells and treated with a lysis buffer containing 1% Triton X-100 (Fas^{H}/Sup or Fas^{L}/Sup) or PBS (Fas^{H}/PBS or Fas^{L}/PBS) overnight. Jurkat cells (5x10^{5}) were incubated with medium as a negative control, 50 ng/ml Fas antibody as a positive control (Fas ab), or 0.5 μl microvesicle supernatants or PBS alone. The percentage of apoptotic cells was determined using an Annexin V assay. The figure shows a representative flow cytometric result. The numbers in the quadrants represent the percentage of cells. Apoptotic cells are in the lower and upper right quadrants. (B) Jurkat cells were incubated with medium as a control (C), 1% Triton X-100 lysis buffer (B), 50 ng/ml Fas antibody as a positive control (Fas Ab), and 1% Triton X-100-treated fractions isolated from the culture supernatants of Fas^{H} (Fas^{H}/Sup) and three representative Fas^{L} cholangiocarcinoma cell clones (Fas^{L}/Sup1, Fas^{L}/Sup2, Fas^{L}/Sup3) were incubated overnight and then apoptosis of Jurkat cells was determined by an Annexin V assay. The figure shows the mean ± SE of percentage of cell death in three individual experiments. **p<0.01 and ***p<0.001 versus the Fas^{H} cells in Student t-test. (C) Jurkat cells were incubated with the indicated volumes of supernatant prepared from the Fas^{L} cells overnight. Apoptotic cells were detected by Annexin V. The results are shown as the mean ± SE from three independent experiments. (D) Jurkat cells were preincubated with 2 ng/ml neutralizing Fas antibody (nFas Ab, black column) or mouse IgG (gray column) for 1 h and then incubated with medium (C), 50 ng/ml activating Fas Ab (Fas Ab), and supernatant fractions isolated from the cultured supernatants of Fas^{H} (Fas^{H}/Sup) and Fas^{L} (Fas^{L}/Sup) cholangiocarcinoma cells overnight. Cell death was determined using an Annexin V assay. The results are shown as the mean ± SE from three independent experiments. **p<0.01 and ***p<0.001 compared with the treatment of neutralizing Fas antibody in Student t-test.

activity in the Fas^{H} cells compared to the Fas^{L} cells. Luciferase activity driven by the FasL promoter in the Fas^{H} cells is significantly lower than in the Fas^{L} cells (p<0.001) showing a low FasL promoter activity in the Fas^{H} cells compared to the Fas^{L} cells. These results indicate that an inverse expression of Fas and FasL in the Fas^{L} and Fas^{H} cells is most likely due to reciprocal promoter activities of the Fas and FasL genes.

Reciprocal promoter activity of the Fas and FasL genes is regulated by NF-κB. To determine the mechanism whereby the activities of the Fas and FasL promoters are inversely regulated, we first determined the binding of nuclear extracts from the Fas^{L} and Fas^{H} cells to NF-κB oligonucleotides by EMSA analysis. As demonstrated in Fig. 5A, the Fas^{L} cells had higher NF-κB binding capacity compared to the Fas^{H} cells. The regulatory activity of the NF-κB transcription factor

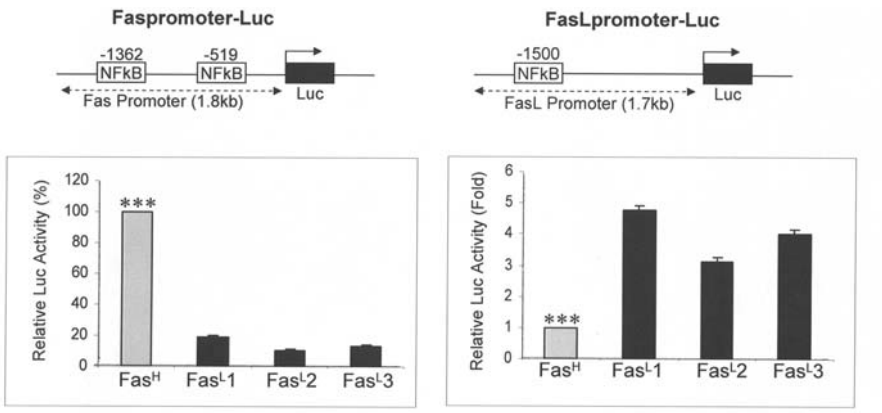

Figure 4. Schematic illustration of Faspromoter-Luc and FasLpromoter-Luc and their activities in the Fas^L and Fas^H cells. Two luciferase-expressing plasmids driven by the Fas promoter (1.8 kb, Faspromoter-Luc) or the FasL promoter (1.7kb, FasLpromoter-Luc) and the location of NF-κB binding motifs in the promoters are illustrated. The Fas^H and Fas^L cells were transiently transfected with these plasmids, respectively. At 48 h after transfection, luciferase activity in 50 μl cell lysate was measured as per manufacturer's protocol. The results are shown from three independent experiments and presented as the mean ± SE. $^{***}p<0.001$ versus the other cell clones in Student t-test.

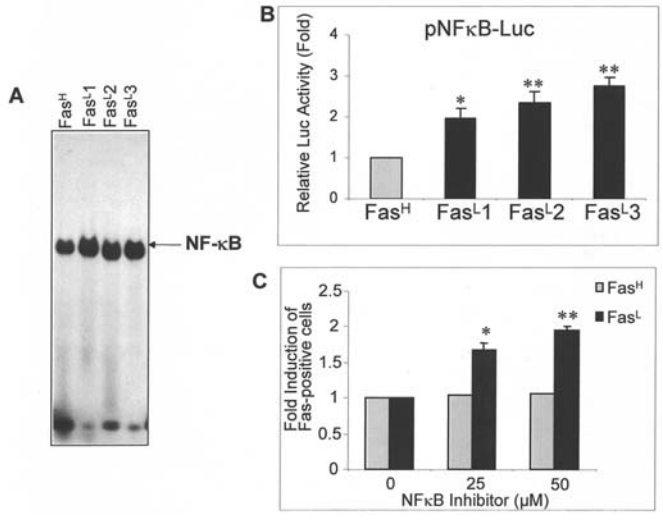

Figure 5. The intracellular NF-κB transcription factor is increased in the Fas^L cells. Nuclear extracts were prepared from the Fas^L and Fas^H cells. (A) EMSA was performed using 5 μg nuclear protein and ^{32}P-labeled NF-κB oligonucleotides. The DNA-protein complex was resolved on 4.5% native polyacrylamide gels and then dried gels were exposed to X-films. The signal of DNA-protein complex in the Fas^L and Fas^H cells is indicated. (B) Luciferase assay for the activity of transcription factors. Fas^H and Fas^L tumor cells were transiently transfected with luciferase expression plasmids, in which the luciferase activity is regulated by multiple NF-κB binding sites (pNF-κB-Luc). A plasmid lacking the transcription factor-binding site (pRF-Luc) was used as a negative control. Cells were harvested 48 h after transfection, lysates were prepared, and luciferase activity was quantified in a Packard luminometer. The results shown here are the mean ± SE from three independent experiments. $^{**}p<0.01$ and $^{*}p<0.05$ compared to the Fas^H cell clone in Student t-test. (C) Fas^H and Fas^L cells were cultured with the indicated concentrations of NF-κB inhibitor, ADPC, for 48 h and then stained for Fas using a PE-conjugated mouse anti-Fas antibody. The Y-axis represents fold induction of Fas-positive cells, calculated by dividing Fas-positive cell number in the presence of NF-κB inhibitor by Fas-positive cell number in the absence of the inhibitor. The data are presented as the mean ± SE of three independent experiments. $^{*}p<0.05$ and $^{**}p<0.01$ compared to the relative Fas^H cell clone in Student t-test.

in the Fas^L and Fas^H cells was then determined using a reporter construct for NF-κB (pNF-κB-Luc) following transient transfection for 48 h (Stratagene). The control construct, pRF-Luc was concurrently transfected to normalize for transfection efficiency. Fig. 5B shows that luciferase activity in the FasL cells was markedly increased compared to the Fas^H cells.

To further assess the role of the NF-κB transcription factor in reciprocally regulating the expression of Fas and FasL we examined the effect of the NF-κB inhibitor, Ammonium Pyrrolidinedithiocarbamate (APDC, Tocris, Elliscille, MO) on Fas expression (28,29). The percentages of Fas-positive cells prior to and after APDC treatment were detected by flow cytometry. The data are presented as the fold induction of Fas-positive cells in the presence of APDC compared to Fas-positive cells in the absence of APDC (Fig. 5C). Without APDC treatment, the number of Fas-positive cells was not changed before and after treatment in the Fas^L and Fas^H cells. APDC increased the number of Fas-positive cells in the Fas^L cells, but not in the Fas^H cells, by two-fold ($p<0.05$), suggesting that the inhibition of NF-κB stimulates Fas expression in the Fas^L cells increasing the numbers of Fas-positive cells.

Discussion

In this study we determined FasL expression in the Fas^H and Fas^L cells and demonstrated that the level of FasL expression in human cholangiocarcinoma cells is inversely related to that of Fas. A wide variety of malignancies, such as pancreas, liver, breast, and esophagus, have been reported to express decreased Fas as well as high FasL (30-32), whereas normal tissues from these organs do not express FasL. Strand *et al* analyzed the expression of both Fas and FasL in 22 hepatocellular carcinomas and suggested that tumor cells can evade immune attack by down-regulation of Fas and induce apoptosis of Fas-bearing lymphocytes through the elevated expression of FasL. The binding of FasL on tumor cells to the Fas molecule on lymphocytes triggered the lymphocytes to undergo apoptosis (33). Our results with cholangiocarcinoma confirm this inverse relationship of Fas and FasL expression in a cellular model and initiate the elucidation of the underlying molecular mechanisms.

The role of a deficiency of Fas expression or function in tumorigenesis has been extensively studied. It has been

reported that the potential for metastasis of osteosarcoma is inversely correlated with Fas expression (7). In cholangiocarcinoma, down-regulation of Fas significantly correlated with histological de-differentiation, vascular invasion, and the size of the tumor (22). However, dysregulation of Fas expression alone was reported to be insufficient to promote tumor progression, because blocking Fas expression in the Fas-expressing cells did not reduce tumor progression (8), implying that other genes may also contribute. Aberrant FasL expression in the Fas^L cells may contribute to its tumorigenic capacity.

The role of FasL expression in tumorigenesis remains controversial. For example, FasL-expressing carcinomas had significantly higher potential of lymph node and distant metastases than carcinomas that did not express FasL, supporting the hypothesis that aberrant FasL expression enhances tumor growth and metastasis (34). In contrast to this hypothesis, suppressing FasL expression in melanoma cells enhanced lung metastasis (35). In addition, other controversies concern the specificity of the reagents used for detection of FasL (antibodies, PCR assay, primers), the presence or absence of tumor-specific T lymphocytes infiltrating cancer lesions and whether or not tumors regress following delivery of FasL-expressing T cells into mice (19,36). Using heterogeneous cell lines may also cause varying results. The use of homogenous cloned cells in our system avoids these controversial factors providing an opportunity to simultaneously determine the roles of Fas and FasL in tumorigenesis. Our results indicate that increased FasL, either on the membrane of or released from the Fas^L cells, is capable of inducing apoptosis of Jurkat lymphocytes (Fig. 3) suggesting the function of FasL on tumor cells in protecting from immune attack by the host. It has been reported that the prognosis of patients with malignant tumors is dependent on the FasL expression of their tumors and the concomitant presence of apoptotic T cells in tumor lesions. Thus, the Fas^L cells not only have an acquired defensive mechanism (Fas resistance), but also provide an offensive mechanism (counterattack) through expressing FasL (37).

In tumors, FasL has been demonstrated to have two forms: surface and soluble. The surface FasL is responsible for transduction of the Fas apoptotic signal after cell-cell contact (38). The soluble FasL (sFasL) released from tumor cells by a proteolytic metalloproteinase is unable to transduce the apoptotic death signal and, therefore, presumably suppresses Fas-mediated apoptosis as a decoy receptor for Fas (39). Abrahams *et al* reported that epithelial ovarian cancer cells secrete functional FasL via microvesicles (26). We detected this microvesicle-associated active FasL in the Fas^L cholangiocarcinoma cells. The Fas^L cells, but not the Fas^H cells, secrete functional FasL in microvesicles capable of activating Fas-mediated apoptosis of Jurkat cells (Fig. 3). Blockage of apoptosis by a neutralizing Fas antibody indicates that the Fas/FasL interaction is responsible for the apoptosis of the Jurkat cells induced by secreted sFasL. Thus, the secreted form of microvesicle-associated FasL in the Fas^L cells is a potential mechanism for counterattack and immune surveillance.

Simultaneous analysis of both Fas and FasL expression in the Fas^H and Fas^L cells provides insight into the mechanisms of reciprocal co-expression of Fas and FasL. The alteration in the Fas promoter region might result in the lack of Fas expression (40). However, we analyzed the partial DNA sequences of the Fas and FasL promoter regions in the Fas^H and Fas^L cholangiocarcinoma cells. No mutations or other alterations in either the Fas or FasL promoter region were identified (data not shown). Su *et al* studied Jurkat T lymphocytes that were transfected with an hFasLpro/GFP plasmid, in which the GFP gene is controlled by FasL promoter. They found that GFP-positive cells expressed low Fas, whereas GFP-negative cells expressed higher levels of Fas protein, indicating an inverse relationship between Fas promoter and FasL promoter activity in tumors (41). Our results confirmed inverse activities of Fas and FasL promoters in the Fas^H and Fas^L cells (Fig. 4). This suggests that some common transcription factors may participate in the reciprocal regulation of Fas and FasL expression.

The transcriptional machinery regulating the expression of Fas and FasL is not yet fully known. Several transcription factors have been identified that regulate Fas and FasL expression. Watabe *et al* reported that transcription factor, NF-κB, down-regulates Fas activation and inhibits Fas-mediated apoptosis (42), whereas Lu *et al* reported that NF-κB up-regulates FasL expression and activates FasL-mediated apoptosis (43). Several studies identified the location of NF-κB binding motifs in the Fas (44,45) and FasL promoters (46,47) (Fig. 4). In this study, we simultaneously analyzed the effects of NF-κB on Fas and FasL and found that the Fas^L cells have higher activity of NF-κB compared to the Fas^H cells (Fig. 5). These observations confirm that NF-κB down-regulates Fas and up-regulates FasL, simultaneously. Furthermore, inhibiting this elevated NF-κB activity in the Fas^L cells by APDC, an NF-κB inhibitor, significantly increased Fas and decreased FasL expression. Lu *et al* demonstrated that FasL-induced apoptosis was increased and TNF-α expression decreased by a specific NF-κB inhibitor or dominant expression of IκB subunit (43).

In conclusion, human cholangiocarcinoma cells reciprocally express functional Fas and FasL, which may be caused by the inverse activity of the Fas and FasL promoter regulated by NF-κB. Reciprocal expression of Fas and FasL in human cholangiocarcinoma may contribute to the survival of tumor cells, the failure of immune surveillance, and growth and metastasis of tumors. Regulating a single mechanism responsible for this reciprocal expression may provide opportunities for new therapies.

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