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Hepatocyte Nuclear Factor (HNF)-4α Induces Expression of Endothelial Fas Ligand (FasL) to Prevent Cancer Cell Transmigration: A Novel Defense Mechanism of Endothelium against Cancer Metastasis

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Endothelial Fas ligand (FasL) contributes to the "immune privilege" of tissues such as testis and eye, in which apoptosis is induced in infiltrating Fas-positive activated T cells and results in the inhibition of leukocyte extravasation. In this study, we examined the role of endothelial FasL in controlling cancer cell transmigration using rat lung endothelial (RLE) cell line bearing a doxycycline-inducible hepatocyte nuclear factor (HNF)-4α expression system. We showed that a detectable level of FasL was expressed in RLE cells and that this expression was markedly up-regulated and well correlated to the degree of HNF-4α expression in a time-dependent manner. When various cancer cells were overlaid on an RLE monolayer sheet, we examined the ability of endothelial FasL to induce massive apoptosis in Fas-expressing cancer cells and found a causal link to inhibition of the transmigration. Finally, we showed that FasL was expressed in capillaries of the rat brain by immunohistochemical staining, suggesting that FasL serves its functions not only *in vitro*, but also *in vivo*. These results raise the possibility that HNF-4α is involved in regulating cancer cell transmigration by modulating the Fas-FasL system.

Key words: Fas ligand — Hepatocyte nuclear factor-4α — Endothelial cell — Apoptosis — Transmigration

The sheet of vascular endothelial cells that coats the luminal surface of vessel walls has many physiological functions, including the control of vascular permeability to maintain tightly closed circulating homeostasis.[1)] Endothelial cells also play important roles in extravasation of fluids and cells during inflammation and in the metastatic process of cancer cells. Cancer cell extravasation, followed by adhesion to the endothelium, is believed to be a critical event in a potentially self-limiting step-by-step sequence, which includes migration from the primary lesion, invasion of the surrounding connective tissue, entrance into vascular structures, adhesion to the vascular endothelium, extravasation, and colonization and growth at a distant site.[2)] Although cancer cells are subjected to intense selective pressure by host immune defense mechanisms in the circulation, the inability of the immune system to react to the cancer cells may be implicated in immune escape and clinical tumor growth.[2, 3)]

Fas (also known as CD95 or APO-1) and its ligand Fas ligand (FasL) are cell surface molecules that regulate apoptosis within the immune system.[4–6)] A low level of Fas is normally expressed in resting T cells.[7)] It is well established that binding of FasL or activating antibodies to Fas triggers apoptosis.[8, 9)] The Fas-FasL system plays an important role in B- and T-lymphocyte development and maturation, which may be normally needed to terminate the immune response at the end of the inflammatory process,[10)] and in mediation of T-cell cytotoxicity.[11)] Mutations in either the Fas (*lpr*) or FasL (*gld*) loci in mice result in a syndrome of lymphoproliferative and autoimmune disease.[12, 13)] Similar human lymphoproliferative disorders have been reported in children with *Fas* gene mutation.[14)]

Fas is constitutively expressed in a wide range of cells such as T- and B-cells, natural killer (NK) cells, hepatocytes, and some types of carcinoma cells,[15)] whereas FasL expression has been reported in a limited number of cell types, including activated T cells, Sertoli cells in the testis, epithelial cells in the anterior chamber of the eye, Paneth cells of the small intestine, and Clara cells in the bronchus.[4, 16)] The "immune privilege" of tissues, such as testis and eye[17)] has been attributed to local expression of FasL inducing apoptosis when Fas-positive activated T cells infiltrate. In addition, some reports have suggested that FasL can prevent allograft rejection[18, 19)] and may contribute to evasion of immune surveillance for some types of tumors.[20, 21)] Recently, Sata and Walsh[22)] reported that endothelial cells constitutively express FasL and may have a function to inhibit leukocyte extravasation through inducing apoptosis in adherent monolayer cells. Notably,

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the regulatory elements controlling constitutive and organ or cell-type specific FasL expression have not been identified. An experimental system employing inducible FasL expression would help to elucidate the distinctive genetic regulatory mechanisms in certain cell types.

The hepatocyte nuclear factor (HNF)-4α belongs to the orphan steroid hormone nuclear receptor superfamily.[23] HNF-4α is expressed in organs containing epithelial tissues in the adult, including liver, kidney, and intestine, and activates diverse genes such as transthyretin, α1-antitrypsin, and apolipoprotein CIII.[23–26] It has been reported to restore liver-specific gene expression and to establish an appropriate cellular morphology, including epithelial cell adhesion.[27, 28] It has also been shown to be involved in early development, liver organogenesis, and mesenchymal-epithelial transitions during kidney formation.[28] We have previously demonstrated that enhanced paracellular barrier function in mesothelial and rat lung endothelial (RLE) cells induced by treatment with all-*trans*-retinoic acid leads to significant resistance to cancer cell invasion.[29, 30] Considering these results together with the fact that retinoid receptors directly regulate expression of the *HNF-4α* gene through binding to the retinoic acid response element (RARE),[31, 32] we hypothesized that expression of HNF-4α-target genes in endothelial cells might prevent invasion of cancer cells. Since our preliminary data suggested that HNF-4α induced FasL expression in endothelial cells *in vitro*, in the light of recent evidence implicating apoptosis and gene regulation in the control of tumor behavior, we were led to consider the potential role of HNF-4α in the endothelium for tumor responses to apoptosis-inducing factors in the microenvironment.

The pathophysiological significance and regulatory mechanism of endothelial FasL are poorly understood. In this study, we analyzed the function of HNF-4α to seek evidence of a role in the induction of FasL expression and to understand the role of endothelial FasL in controlling cancer cell transmigration. We demonstrated that FasL expression was markedly enhanced, with an increase in HNF-4α expression, in vascular endothelial cells in a time-dependent manner, eventually resulting in induction of apoptosis of Fas-expressing cancer cells and inhibition of transmigration through an RLE monolayer sheet.

MATERIALS AND METHODS

Construction of expression vector and transfection RLE cells expressing the doxycycline (Dox) (Sigma, St. Louis, MO)-controlled transactivator rtTA (*tet*-on system),[33] designated RLE:rtTA L20, and RLE:rtTA:HNF-4α L23 cells, which express both rtTA and the Dox-inducible HNF-4α, were established and will be described in detail elsewhere. In brief, the response plasmid (pUHD10-3) includes the coding sequence of tetracycline, which contains a reverse tetracycline-dependent promoter (rtTA coding sequence) under the control of the PGK promoter. The presence of Dox, an effector substance, in the culture medium makes this system active, and the cloned gene of interest is expressed rapidly and strongly within 24 h.

Rat HNF-4α cDNA was generated by PCR amplification using an *Eco*RI site-tagged forward primer (5′-GAATTCGCCGCCACCATGGACATGGCTGACTAAGT-3′) and *Xba*I site-tagged reverse primer (5′-TCTAGACTAGATGGCTTCCTGCTTGGT-3′) in the reaction mixture containing reverse-transcribed cDNA from 1 μg of total RNA extracted from rat kidney. The PCR product was subcloned into T-vector. The digested 1436 bp *Xba*I-*Eco*RI fragment was ligated in the sense orientation into the response plasmid, pUHD10-3, to afford pUHD10-3-HNF-4α plasmid. The sequence and the frame of the inserted HNF-4α were confirmed by direct sequencing.

RLE cell line was transfected with the pUHD10-3-HNF-4α plasmid by the lipofection method using "Lipofectoamine" 2000 reagent (Gibco BRL, Grand Island, NY). RLE cells were seeded onto 10 cm dishes at the density of 1×10^6 cells/dish and maintained at 37°C overnight. The plasmid mixture used for transfection contained 5 μg of pUHD10-3-HNF-4α+1 μg of pHTpuro, a selection plasmid containing a puromycin-resistant cassette. Selection by growth in the presence of puromycin (5 μg/ml) (Sigma) was started 48 h after transfection. Three weeks later, resistant clones were expanded as a monoclonal population and 96 colonies were picked up. A cell line that had rapid and strong expression of HNF-4α was selected to assess mRNA expression and immunofluorescence staining, and cultured on glass coverslips.

Cell line and culture Wild-type RLE, RLE:rtTA L20, RLE:rtTA:HNF-4α L23 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin in the presence or absence of various concentrations of Dox at 37°C in a humidified 5% CO_2 atmosphere, and media were renewed every 2 days. The plating time was defined as 0 h, and the cells after 6, 12, or 24 h of culture were subjected to the various experimental conditions described below. Both wild-type RLE and RLE:rtTA L20 cells were used as control cell lines.

Various cancer cell lines including HCCL4.2, HepG2, KATO III, LHK2, and LG2-MEL were also maintained under the same conditions as RLE cells. HCCL4.2 and LHK2 are unreported cell lines. HCCL4.2 was established from a serologically virus-free patient with a liver tumor diagnosed pathologically as moderately differentiated hepatocellular carcinoma. LHK2 was established from floating cells in pleural effusion in a patient with advanced lung cancer diagnosed as poorly differentiated adenocarcinoma. HepG2, KATO III, and LG2-MEL cells originated from hepatoblastoma with epithelial cell-like morphology,

signet-ring cell carcinoma of the stomach, and malignant melanoma, respectively.

Total RNA extraction and semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) Total RNA from each specimen was extracted using TRIzol (Gibco BRL) as the extraction medium according to the manufacturer's instructions, and purified by ethanol precipitation. RNA was then dissolved in RNase-free water, and quantitated spectrophotometrically. For RT-PCR, 1 μg of total RNA was reverse-transcribed using poly-T oligonucleotide and M-MuLV reverse transcriptase (TaKaRa, Kyoto) in 20 μl of reaction mixture. HNF-4α, FasL, and Fas were amplified using primers HNF4-sense (5′-AGTGCCCTGTGTGCCATCTGTG-3′) and HNF4-antisense (5′-AGATGATGGCTTTGAGGCAGGCG-3′), FasL-sense (5′-GTGAATTACCCATGTCCCCAGAT-3′) and FasL-antisense (5′-GTTCCTCATATAGACCTTGTGGC-3′), Fas-sense (5′-CGTCTGTTGCTAGATTATCGTCCAA-3′) and Fas-antisense (5′-CTTCATTGACACCATTCTTT-CGAAC-3′) in 20 μl of PCR reaction mixture to generate 689, 646, and 631 bp products, respectively.

Dilutions of cDNA were amplified for 20, 25, 30, 35, and 40 cycles at 96°C for 10 s, 60°C for 5 s, and 72°C for 30 s to define optimal conditions for linearity to permit semiquantitative analysis of signal length. Agarose gel electrophoresis and ethidium bromide staining were used to analyze the PCR products of HNF-4α amplified for 25 cycles, FasL for 35 cycles, or Fas for 30 cycles. As a positive control, and to confirm that each RNA sample could yield products after RT-PCR, amplification was done using G3PDH primers 5′-TCCACCACCCTGTTGCT-GTA-3′ and 5′-ACCACAGTCCATGCCATCAC-3′, to yield a 581 bp product. To ensure the reproducibility of expression quantification, triplicate independent PCR reactions were carried out. The relative ratios of the PCR products were measured by densitometric analysis using NIH Image software.

Western blot analysis FasL protein levels were examined by western blot analysis. Total cell lysates, each with 20 μg of denatured proteins, were run on 12% polyacrylamide gels containing sodium dodecyl sulfate (SDS) and electroblotted onto nitrocellulose filters. The filters were then blocked with 5% nonfat dry milk in phosphate-buffered saline (PBS) with 0.5% Tween 20 (PBST) and incubated with a rabbit polyclonal anti-rat FasL (C-178) (diluted 1:200, Santa Cruz Biotechnology, Santa Cruz, CA) as the primary antibody diluted in PBST. After extensive washing in PBST, the filters were reacted with a horseradish peroxidase-conjugated secondary antibody (DAKO, Kyoto) in PBST and washed again. Finally, the immunoreaction was visualized by using an enhanced chemiluminescence (ECL) system (Amersham, Buckinghamshire, UK). Computer-assisted densitometric analysis was performed as described above.

Invasion assay Invasion assays were carried out using "Transwell" chambers (pore size 8 μm) (Becton Dickinson, Franklin Lakes, NJ) in a co-culture system using both RLE:rtTA:HNF-4α L23 and various human cancer cell lines, including HCCL4.2, HepG2, KATO III, LHK2, and LG2-MEL. First, RLE:rtTA:HNF-4α L23 cells were seeded at 5×10^4 cells/well in 12-well cell-culture plates at 0 h and cultured on the filter of the upper chamber to a confluent monolayer for 48 h with or without 1 μg/ml Dox. The medium was replaced with fresh medium and various tumor cells (1×10^4 cells/well) were seeded on the confluent RLE:rtTA:HNF-4α L23 cell monolayers. After 24 h incubation, the cells that penetrated through both the filter and cell monolayer to the collagen-coated coverslips located on the lower surface were stained with 4,6-diamidinophenylindole (DAPI) after being fixed in methanol:acetone (1:1), and the number of penetrating cells was counted.

Demonstration of DNA fragmentation by gel electrophoresis and quantification of DNA fragmentation These experiments were performed as described previously.[34]

Immunofluorescence microscopy RLE:rtTA:HNF-4α L23 cells grown on type I collagen-coated glass coverslips (Matsunami, Osaka) were fixed with a methanol:acetone 1:1 mixture solution for 10 min at −20°C. After rinsing with PBS, the coverslips were incubated with the primary antibody against HNF-4α (C-19) (diluted 1:40, Santa Cruz) for 1 h at room temperature. These specimens were then incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (DAKO) for 1 h at room temperature.

For immunohistochemistry of the capillaries in the brain, excised small pieces of cerebral cortex were put on slides directly, and the slides were pressed against each other, then immediately immersed in an ice-cold 1:1 methanol-acetone mixture for 30 min at −20°C for fixation. After rinsing with PBS, the slides were incubated with rabbit polyclonal anti-rat FasL antibody (diluted 1:40, Santa Cruz) for 1 h at room temperature. These specimens were then incubated with an Alexa Fluor 488 dye-conjugated secondary antibody (Molecular Probes, Eugene, OR) for 1 h at room temperature. All samples were examined with a Nikon Fx photomicroscope.

Statistical analysis Statistical significance of differences in this study was evaluated using the Mann-Whitney *U* test.

RESULTS

Establishment of doxycycline-inducible gene expression system in RLE cells We established RLE:rtTA:HNF-4α L23 cells, which exhibit Dox-inducible HNF-4α expression, allowing tight control of gene expression with both a

high level of tet-inducibility and low background activity. Time- and dose-dependent expression of HNF-4α transcripts and protein was confirmed by semiquantitative RT-PCR, immunohistochemical staining, and western blot analysis, as detailed elsewhere (our unpublished work, see also Fig. 1). Expression of HNF-4α mRNA was induced in the cells after 6 h of 1 μg/ml Dox treatment and the levels gradually increased to reach the maximum at 24 h after Dox treatment (Fig. 1A). We further confirmed the dose-dependence of HNF-4α expression after 24 h treatment with various concentrations of Dox (Fig. 1C). Immunofluorescence staining showed that both the number and the intensity of positive signals in the nucleus were gradually increased in a time- and dose-dependent manner, in good agreement with the levels of HNF-4α transcripts (Fig. 1, B and D).

HNF-4α-dependent expression of FasL in RLE:rtTA:HNF-4α L23 cells To investigate whether HNF-4α could induce FasL expression, we analyzed FasL mRNA expression by semiquantitative RT-PCR (Fig. 2A). Expression of FasL mRNA was detectable even in the absence of Dox; however, mRNA levels were significantly induced in the cells after 6 h of 1 μg/ml Dox treatment and the levels of these transcripts gradually increased with time to reach the maximum after 24 h of Dox treatment.

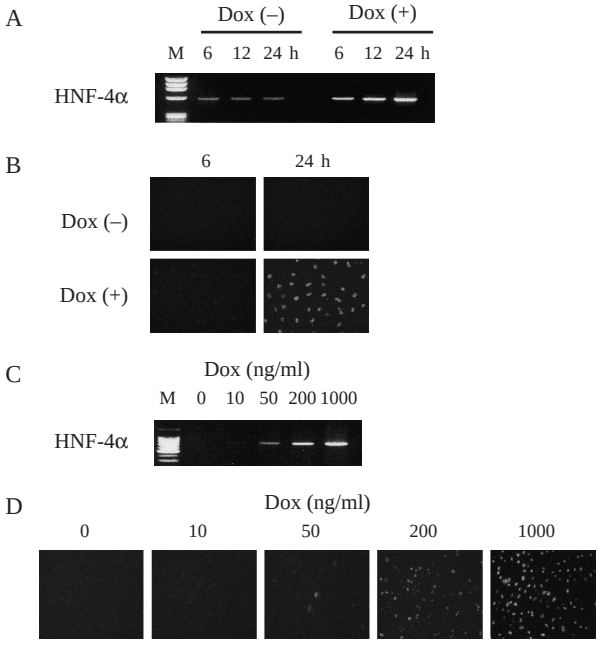

Fig. 1. Establishment of doxycycline (Dox)-inducible HNF-4α expression system in RLE cells. Semiquantitative RT-PCR (A, C) and immunofluorescence staining (B, D) were used to analyze inducible HNF-4α expression as described in "Materials and Methods." RLE:rtTA:HNF-4α L23 cells show time (A, B) and dose-dependent (C, D) expression of HNF-4α after Dox treatment. The cells were treated with 1 μg/ml Dox in the time-course study (A and B), and induced HNF-4α expression was assessed after 24 h treatments with various concentrations of Dox (C and D). Note that the maximal level of inducible HNF-4α was observed at 24 h after 1000 ng/ml Dox treatment. The size marker used is shown in the left-most lane (M). B, D: original magnification, ×100.

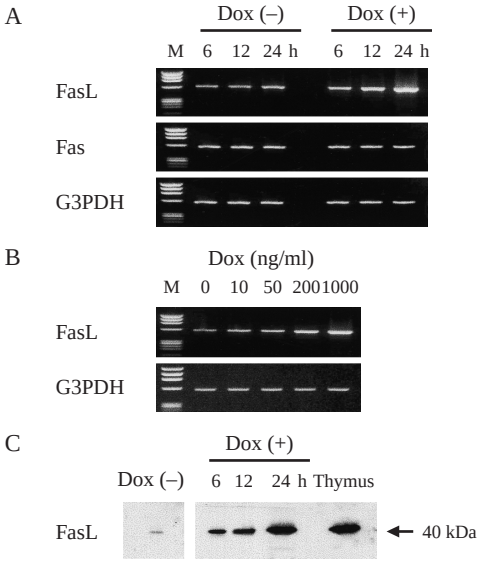

Fig. 2. HNF-4α-dependent expression of FasL in RLE:rtTA:HNF-4α L23 cells bearing a Dox-inducible HNF-4α expression system. (A), (B) Semiquantitative RT-PCR was used to determine the expression of FasL, and Fas transcripts. Total RNA was extracted from the cells treated with Dox at the indicated time points (A), or the indicated concentrations of Dox for 24 h (B), and subjected to RT-PCR. The PCR products were analyzed by FasL for 35 cycles, or Fas for 30 cycles. (A) Expression of the *FasL* gene, but not the *Fas* gene, was induced in the Dox-treated cells, in which HNF-4α expression was increased in a time-dependent manner. (B) The levels of FasL mRNA were increased by Dox in a dose-dependent manner. G3PDH was analyzed under identical conditions and served as an internal standard to ensure that equal amounts of total RNA were used. To ensure the reproducibility of expression quantification, triplicate independent PCR reactions were carried out. The size marker used was a ϕX174 *Hae*III digest, shown in the leftmost lane (M). (C) Western blot analysis for the expression of FasL protein in the RLE:rtTA:HNF-4α L23 cells grown with or without 1 μm/ml Dox for 6, 12, or 24 h. Total cell extracts were isolated from the cells, and 20 μg of denatured protein was loaded onto 12% SDS-polyacrylamide gel and analyzed by immunoblotting using an antibody specific for FasL (1:200). Detection was accomplished by using the ECL system according to the manufacturer's instructions. The FasL signal, a 40 kDa single band, was induced in the cells by Dox treatment time-dependently. Molecular weight is indicated as kDa. Rat thymus was used as a positive control.

Densitometric analysis revealed that this increase of FasL expression was 13-fold higher than that without Dox. Furthermore, induction of FasL transcripts in the cells after 24 h of Dox treatment occurred in a dose-dependence manner (Fig. 2B). In contrast, there was no induction of Fas mRNA expression in these cells after Dox treatment (Fig. 2A). Since a transcriptional hierarchy involving HNF-4α-mediated activation of the HNF-1 promoter has been proposed to be of crucial importance in regulating certain types of gene expression,[26] we also examined the HNF-1 signals. Unexpectedly, these were detectable but remained unchanged over the course of the experiment (data not shown).

We next determined the expression level of FasL protein in RLE:rtTA:HNF-4α L23 cells by western blot analysis using a polyclonal antibody against FasL (Fig. 2C). A faint signal was detected in the cells in the absence of Dox, but a strong single band of 40 kDa, which had the same motility as FasL, was detected in the cell lysate with Dox treatment. Expression of FasL protein was markedly induced after 1 μg/ml Dox treatment, and the maximum signal intensity, obtained after 24 h treatment, was 48-fold higher than that at 0 h without Dox.

These results strongly suggested that HNF-4α induced FasL expression in RLE:rtTA:HNF-4α L23 cells and that there are strong mutual correlations. The fatty acyl-CoA thioester palmitoyl CoA, a reported ligand of HNF-4α[35] did not affect these results.

Fas mRNA expression in cancer cells Five human cancer cell lines were analyzed for Fas expression by RT-PCR (Fig. 3). Strong Fas expression was detected in KATO III, LHK2, and LG2-MEL cells, whereas weak or negative expression for Fas was observed in HepG2 or HCCL4.2 cells, respectively. All cell lines gave negative results for FasL expression as determined by both RT-PCR and western blot analysis (data not shown).

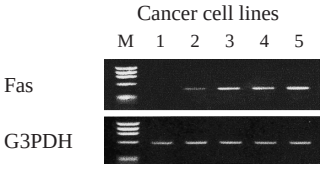

Fig. 3. RT-PCR analysis of Fas expression in five human cancer cell lines. Fas was expressed strongly in KATO III (lane 3), LHK2 (lane 4), and LG2-MEL (lane 5), but was weak and undetectable in HepG2 (lane 2) and HCCL4.2 (lane 1), respectively. G3PDH was analyzed under identical conditions and served as an internal standard to ensure that equal amounts of total RNA were used. To ensure the reproducibility of expression quantification, triplicate independent PCR reactions were carried out. The size marker used was a ϕX174 *Hae*III digest, shown in the leftmost lane (M).

Dox-treated RLE:rtTA:HNF-4α L23 cells inhibit cancer cell penetration through RLE monolayer To determine the invasive or penetrating capacity of the cancer cells through RLE:rtTA:HNF-4α L23 cell monolayer sheets grown for 48 h in the presence or absence of Dox, a co-culture method using "Transwell" chambers was employed in this study (Fig. 4). After 24 h of co-culture with or without Dox treatment, cancer cells that penetrated to the bottom of the lower surface of the lower chamber were positively immunostained by DAPI under fluorescence microscopy. All cancer cells used in this study could penetrate after 24 h of co-culture in the absence of Dox

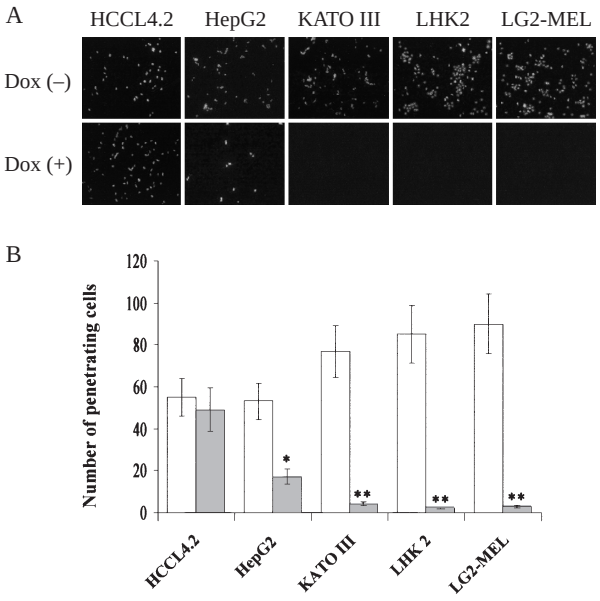

Fig. 4. Invasion assays using a Transwell chamber (pore size 8 μm) in a co-culture system using both RLE:rtTA:HNF-4α L23 cells and various cancer cell lines. RLE:rtTA:HNF-4α L23 cells were grown on the filter of the upper chamber for 48 h in the presence or absence of 1 μg/ml Dox, and cancer cells were overlaid on an RLE:rtTA:HNF-4α L23 cell monolayer. These cancer cells penetrated to the coverslips located on the lower surface of the chamber after 24 h of co-culture. The coverslips were processed for staining with DAPI (A), and the cells on the coverslips were collected and counted (B). (A) Penetration of KATO III, LHK2, and LG2-MEL cells, which expressed Fas mRNA at higher levels, through the Dox-treated RLE:rtTA:HNF-4α L23 cell monolayer was totally blocked, while that of HepG2 and HCCL4.2 cells was partially blocked, and not affected, respectively. (B) We found significant differences between cells with (hatched column) and without (open column) Dox for HepG2 (∗ $P<0.05$), KATO III, LHK2, and LG2-MEL cells (∗∗ $P<0.001$), but no significant differences were found for HCCL4.2 cells. Original magnification, ×40 (A). Data shown in (B) were confirmed in triplicate independent experiments and represent means; bars, SD.

(Fig. 4A). In the assay with Dox treatment, Fas-expressing cancer cells (HepG2, KATO III, LHK2, and LG2-MEL) were less invasive, while, Fas-undetectable cancer cells (HCCL4.2) were highly invasive, independently of Dox treatment (Fig. 4A).

Low magnification (×40) examination of DAPI-stained specimens was used to quantitate the numbers of attached or penetrating cancer cells (Fig. 4B). We clearly observed marked differences between the number of Fas-positive cancer cells co-cultured with RLE:rtTA:HNF-4α L23 cells grown in the presence of Dox and that in the absence of Dox. On the other hand, no differences were found in Fas-negative cancer cells. Moreover, KATO III, LHK2, and LG2-MEL cells, which expressed high levels of Fas, showed significant differences ($P<0.001$), whereas HepG2, which expressed lower levels of Fas, also showed significant but smaller differences ($P<0.05$) as compared with those with high Fas expression. Wild-type RLE and RLE:rtTA L20 cells treated with Dox could not inhibit Fas-positive cancer cell penetration.

FasL-positive RLE cells induce apoptosis in cancer cells If FasL-expressing endothelial cells can kill co-cultured cancer cells through Fas-FasL interaction, we can postulate that functional FasL in endothelial cells might inhibit cancer cell transmigration. Therefore, we examined whether apoptotic cell death occurred in these cancer cell lines when they were co-cultured with RLE:rtTA:HNF-4α L23 cells treated with 1 μm/mg Dox for 24 h, to investigate the functional significance of FasL expression in endothelial cells. As expected, significant target cell apoptosis characterized by a 180-bp chromosomal DNA ladder pattern was observed on agarose gel electrophoresis in KATO III, LHK2, and LG2-MEL cells after Dox treatment (Fig. 5A). HepG2 cells showed a significant but lower degree of apoptosis in the presence of Dox than the cells with high Fas expression, and HCCL4.2 cells showed little target cell DNA fragmentation, independently of Dox treatment. These findings showed that apoptotic cell death was closely correlated to the degree of Fas expression in cancer cells.

Furthermore, to confirm that endothelial FasL and Fas in cancer cells cooperate in generating apoptosis, the DNA fragmentation rates of all cancer cell lines used in this study were measured after 24 h co-culture with RLE:rtTA:HNF-4α L23 cells. The percentages of DNA fragmentation (Fig. 5B) were in good agreement with the data from agarose gel images; in the presence of Dox, KATO III, LHK2, and LG2-MEL cells showed significant differences ($P<0.001$), while HepG2 cells also showed significant but smaller differences ($P<0.05$) as compared with those with high Fas expression. Control cell lines treated with Dox gave negative results for inducing apoptosis even in Fas-positive cancer cells, and we could not observe significant apoptotic induction in any cancer cell line with RLE:rtTA:HNF-4α L23 cells in the absence of Dox. These data suggested that the cytotoxic activity of RLE:rtTA:HNF-4α L23 cells was mediated directly by Fas-FasL interaction. It should be noted that minimum levels of spontaneous DNA fragmentation were observed in all cancer cell lines even without Dox treatment. Although RLE:rtTA:HNF-4α L23 cells were confirmed to express Fas independently of their HNF-4α status, these phenomena might have been due in part to the lack of functional FasL expression in cancer cells. Co-culture experiments clearly revealed that FasL induced in endothelial cells had cytotoxic activity against cancer cells with Fas expression.

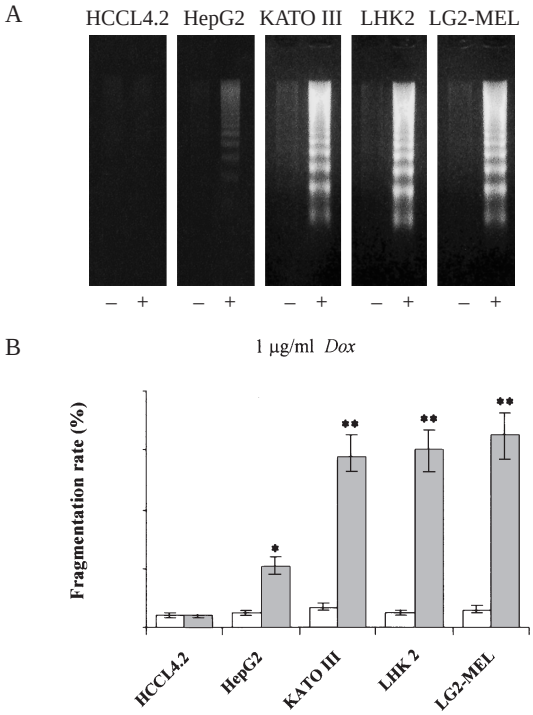

Fig. 5. Dox-treated RLE:rtTA:HNF-4α L23 cells induce apoptosis in Fas-expressing cancer cells. (A) Fragmented DNA from floating cells in the upper chamber was isolated and subjected to agarose gel electrophoresis. Obvious DNA ladder formation was observed for KATO III, LHK2, and LG2-MEL cells, and a low level of target cell apoptosis was found in HepG2 cells after 24 h co-culture with RLE:rtTA:HNF-4α L23 cells in the presence of 1 μg/ml Dox. However, significant apoptotic cell death was not observed in HCCL4.2 cells, irrespective of Dox treatment. (B) DNA fragmentation rates revealed significant differences between cells with (hatched column) and without (open column) Dox for HepG2 (* $P<0.05$), KATO III, LHK2, and LG2-MEL cells (** $P<0.001$), but no significant differences were found for HCCL4.2 cells. Note that the same trace levels of spontaneous DNA fragmentation were found in all cancer cell lines even without Dox. The reproducibility was confirmed in triplicate independent experiments and results represent means; bar, SD in (B).

FasL expression in the brain capillary endothelial cells Vascular endothelial cells in the brain cortex are known to form the blood-brain barrier, which plays an essential role in controlling vascular permeability in the central nervous system (CNS). Our preliminary data demonstrated that HNF-4α was expressed strongly in the brain capillary cells of the rat and might regulate the vascular permeability through controlling the paracellular barrier functions of endothelial cells. Therefore, we investigated the FasL expression, which is under the control of HNF-4α, in vascular endothelial cells in the CNS by using immunohistochemical staining. Our results clearly showed that FasL was preferentially expressed in capillaries of the rat brain cortex (Fig. 6), suggesting that FasL functions not only *in vitro*, but also *in vivo*.

DISCUSSION

We have provided the first evidence that transcriptional factor HNF-4α induces FasL expression in endothelial cells, and that Fas-FasL interactions between endothelium and cancer cells can suppress the transmigration of cancer cells through the endothelial monolayer sheet. It is feasible that endothelial cells are a defense line of the host against cancer metastasis, in terms of the inducibility of apoptosis in cancer cells. Although large numbers of cells may migrate from the primary tumor and gain access to the circulation, few of them will give rise to metastases. Several studies have suggested that the regulation of apoptosis must influence metastasis of tumor cells in the early stages after attachment of the vascular endothelium, and tumor cells that undergo less apoptosis in the target organs were more likely to successfully establish colonies.[36] This work further suggests the underlying mechanism of apoptosis in elimination of tumor cells in vascular structures, often termed as "metastatic inefficiency."[37, 38]

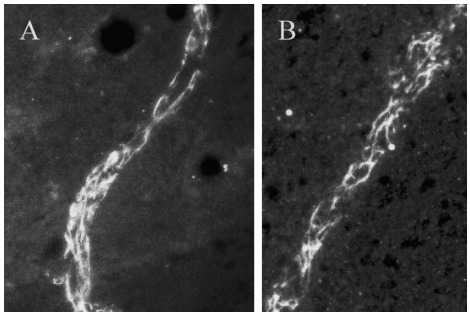

Fig. 6. Immunohistochemical staining of the capillaries in the rat cerebral cortex. Excised small pieces of cerebral cortex were subjected to immunohistochemistry using polyclonal anti-rat FasL antibody. Expression of FasL was clearly detected. Original magnification: A, ×100; B, ×200.

The NFAT family transcriptional factors, NF-κB, and AP-1 have been reported to transcriptionally regulate FasL expression in activated human T cells.[39, 40] The transcriptional regulation of FasL, however, is not fully understood and is still controversial.[41] In the present experiments, we demonstrated the participation of HNF-4α in the expression of FasL in endothelial cells, suggesting that HNF-4α plays an important role in the gene regulation in not only epithelium, but also endothelium.[23–28]

Our results also revealed a discrepancy in the maximum induction of FasL expression between mRNA and protein levels in Dox-treated RLE:rtTA:HNF-4α L23 cells. This difference may reflect possible undefined regulatory mechanisms of FasL and/or HNF-4α. The regulation of gene expression within the cell depends on both transcriptional and posttranscriptional mechanisms. Posttranscriptional regulation may be particularly important for short-lived proteins such as cytokines and regulators of cell proliferation and differentiation, including FasL. Although transcriptional control has been the most commonly described mechanism for the regulation of HNF-4α-inducible genes,[23–26] there are a few instances in which posttranscriptional mechanisms are involved, and it is likely that other underlying mechanisms remain to be discovered. Another possible explanation of this discrepancy arises from our data showing that FasL protein stability may be markedly affected by HNF-4α transcripts directly or other transcripts of possible target genes for HNF-4α. Further studies will be needed to clarify in more detail the regulatory mechanisms of FasL, including posttranscriptional regulation, though our findings suggest that a unique mechanism(s) regulates FasL transcription in endothelial cells. Moreover, we will have to establish the specific mechanisms through which HNF-4α functions to increase FasL transcript levels in endothelial or other cells, and obtain evidence that HNF-4α has a critical role in regulating FasL expression in arguably more relevant *in vivo* settings.

Several studies have shown that FasL may help to maintain the integrity of immune privileged sites. In many of these sites, including the eye, testis, brain, and inner ear, constitutive FasL is involved in controlling infiltration of inflammatory cells with Fas.[17] The present study showed that HNF-4α was sufficient for induction of *FasL* gene expression in RLE:rtTA:HNF-4α L23 cells and FasL was able to induce apoptosis in functional Fas-expressing cancer cells. Consideration of the regulatory loop between HNF-4α and HNF-1[26] prompted us to test the possibility that the induced FasL resulted from the up-regulation of HNF-1. Unexpectedly, HNF-1 signal intensity was unchanged throughout the experiment. These data strongly suggested that transcriptional factor HNF-4α, but not HNF-1, could directly induce FasL mRNA expression. Alternatively, taken together with the observation that

an HNF-4α inhibitor is abundantly present in *Xenopus* embryos, in which the *HNF-1* gene is only marginally expressed,[42] we propose that similar kinds of inhibitors might be present in RLE cells to restrict the activity of the HNF-4α.

Fas is widely expressed in epithelial cells as well as subsets of leukocytes; however, many carcinomas have lost surface Fas protein.[15] *In vitro* experiments have shown that even cancer cell lines with residual Fas expression are frequently resistant to Fas-mediated cell killing by various means.[43, 44] Our results demonstrated that significant apoptotic cell death could not be induced in Fas-negative (HCCL4.2) or weakly expressing cells (HepG2). Moreover, even in Fas-positive cells, activation of certain oncogenes, for example *bcl-2*, may partially inhibit the apoptotic signal from Fas.[45, 46] In agreement with this concept, we recognized the importance of other defined mechanisms for evading FasL-mediated cytotoxicity via loss of function of the Fas-FasL system. In our co-culture system, although Fas-FasL interaction was sufficient for the induction of significant apoptosis in the majority of Fas-expressing cancer cells (<60%), almost 40% of the cells that were resistant to Fas-induced apoptosis in spite of exhibiting a significant degree of Fas expression could not penetrate through the FasL-positive endothelium. These findings support our previous observation that all-*trans*-retinoic acid-induced functional tight junctions in mesothelial and endothelial cells play a key role in giving rise to resistance to cancer cell invasion.[29, 30] This study suggests that HNF-4α may regulate cancer cell transmigration via not only activation of the Fas-FasL system, but also by modulation of the expression of previously undefined target genes. Alternatively, Tanaka *et al.* reported that FasL is rapidly cleaved from the membrane by a metalloproteinase to become a soluble form (sFasL),[47] and that sFasL inhibits Fas-mediated lymphocyte apoptosis.[48] The finding that sFasL is less cytotoxic than membrane-bound FasL (mFasL) and may act as an antiapoptotic molecule in Fas-expressing cells[49] suggests that conversion of mFasL to sFasL may prevent the killing of Fas-expressing cancer cells.

Vascular endothelial cells are resistant to Fas-mediated apoptosis under normal conditions[22]; however, these cells can be sensitized to Fas-mediated apoptosis under pathological conditions.[50] In this experiment, we could not address the significant apoptotic cell death in wild-type RLE, RLE:rtTA:L20, and RLE:rtTA:HNF-4α L23 cells.

Finally, we clearly demonstrated that FasL was strongly expressed in the capillary endothelial cells of the rat brain cortex. This may suggest that FasL functions not only *in vitro*, but also *in vivo*. Once the specific ligand for HNF-4α in the endothelium has been discovered, this factor will be a good candidate for controlling the behavior of endothelium against cell transmigration. Studies along these lines will be useful for the functional understanding of the maintenance of homeostasis established by endothelial cells and for clinical applications to prevent cancer extravasation. This study provides a foundation for the pursuit of HNF-4α-based therapeutic modalities for in cancer metastasis *in vivo*.

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