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ORIGINAL ARTICLE

Hiroaki Nishimatsu · Takumi Takeuchi · Tetsuo Ueki
Takahiro Kajiwara · Nobuo Moriyama
Toshimitsu Ishida · Baoxing Li
Tadao Kakizoe · Tadaichi Kitamura

CD95 Ligand expression enhances growth of murine renal cell carcinoma in vivo

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Abstract The CD95/CD95 ligand (CD95L) system plays an important role in the induction of lymphoid apoptosis and has been implicated in the suppression of immune responses. In this system, two murine CD95L-transfected renca clones and a control renca clone transfected only with the vector were implanted into the subcapsule of the left kidney of Balb/c and Balb/c nude mice. Both CD95L-expressing and control renca clones formed macroscopic tumors in all of the Balb/c and Balb/c nude hosts 14 days after implantation. Growth of tumors of murine CD95L-transfected renca cells was significantly better than that of control renca cells in Balb/c mice, while the growth advantage of CD95L transfectants was not observed in Balb/c nude mice. Lymphocytes underwent apoptosis mainly in the periphery of the CD95L-expressing tumors but not in control tumors grown in Balb/c mice, while lymphocytes undergoing apoptosis were not observed in CD95L-expressing tumors or in control tumors grown in Balb/c nude mice. Neutrophilic recruitment was rarely observed in CD95L-expressing or control tumors. CD95L expressed on renca cells possibly suppressed immune responses against renca tumors by inducing apoptosis of the infiltrating lymphocytes. However, CD95L-expressing renca cells did not form tumors in the renal subcapsule of allogeneic C3H/HeJ mice.

Key words CD95L · FasL · Renca · Apoptosis · Tumor growth

H. Nishimatsu (✉) · T. Takeuchi · T. Ueki · T. Kajiwara
N. Moriyama · T. Ishida · B. Li · T. Kitamura
Department of Urology, University of Tokyo,
Faculty of Medicine,
7-3-1 Hongo, Bunkyo-ku,
Tokyo 113-8655, Japan
Tel.: +81-3-5800-8662, Fax: +81-3-3622-7958

T. Kakizoe
National Cancer Center, Tokyo

Introduction

CD95 (Fas) is a type I membrane protein, a member of the tumor necrosis factor (TNF) receptor family, which mediates apoptosis. CD95 ligand (CD95L) is a 40-kDa type II membrane protein, a member of the TNF family, expressed mainly in cytotoxic T cells upon activation. When CD95L binds to CD95 on CD95-sensitive cells, target cells die by apoptosis [5]. A family of cysteine proteases is sequentially activated leading to CD95-induced apoptosis, and Bcl-2 inhibits the process. The CD95/CD95L (Fas/FasL) system plays an important role in the induction of lymphoid apoptosis and has been implicated in the suppression of immune responses. Recently, there has been renewed interest in immune privilege as it was shown that two privileged sites (the eye and testes) constitutively express CD95L, which functions in the killing of lymphoid cells that invade these areas [3].

The first important report on the in vivo action of CD95L-expressing organs in transplantation was by Bellgrau et al. When normal mouse testes expressing CD95L were transplanted under the kidney capsule of allogeneic animals, their survival was prolonged indefinitely by inducing apoptosis in infiltrating activated T cells whereas testis grafts derived from mutant gld mice, which express non-functional ligand, were rejected [2]. In contrast, Seino et al. reported that CD95L-transfected murine tumor cells were rejected in syngeneic mice mainly by neutrophils [9]. Kang et al. reported that when pancreatic islets infected with an adenoviral vector containing CD95L cDNA were transplanted into allogeneic diabetic mice, their diabetes relapsed more rapidly with accelerated neutrophilic rejection in a T- or B-cell-independent manner than when control islets cells were transplanted [4]. Moreover, in CD95L transgenic mice expressing CD95L in pancreatic β cells under the control of the insulin promoter these cells were destroyed and diabetes developed as a result of massive neutrophil recruitment [4]. In a more recent report, the gene transfer of CD95L by an adenoviral vector into the

$CD95^+$ renca tumor cells (murine renal cell carcinoma) eliminated the tumor by inducing cell death, while the same strategy eliminated the $CD95^-$ CT26 colon carcinoma tumor by a mechanism mediated by inflammatory cells [1]. We have stably established renca clones transfected with murine CD95L cDNA and examined the in vivo tumor growth of those cells in order to determine whether CD95L expressed on tumor cells suppresses the immune response by introducing apoptosis in infiltrating lymphocytes or activates a local immune response by recruiting neutrophils.

Materials and methods

Transfection of murine CD95L cDNA into renca cells

Murine CD95 ligand cDNA (CD95L) was cut out with *Xba*I from pBL-MFLW4 provided by DR. S. Nagata [11]; it was then gel-purified, blunt-ended by Klenow fragment DNA polymerase, and ligated into the end-filled *Eco*RI site of the mammalian expression vector pCAGGS [6] (pCmCD95L). pCmCD95L was digested with the unique restriction enzyme, *Sal*I, to form linear DNA. Naive renca cells were co-transfected, using Lipofectamine (Gibco BRL, Gaithersburg, M., with *Sal*I-digested pCmCD95L or *Sal*I-digested pCAGGS as a control together with the neomycin-resistance gene [12] in a molar ratio of 10:1 and cloned in RPMI-1640 medium containing 10% fetal calf serum (FCS), 100 IU/ml penicillin, 100 μg/ml streptomycin, and 400 μg/ml G418 (Gibco BRL, Gaithersburg, Md.). Isolated renca clones were tested for chromosomal integration of the pCmCD95L gene by 30 cycles of polymerase chain reaction (PCR) amplification (95 °C denaturation for 1 min, 55 °C annealing for 1 min, and 72 °C extension for 1 min) of the sequence of the vector construct in genomic DNA using a 5′ sense primer (5′-GCAACGTGCTGGTTGTTGTG-3′) taken from the pCAGGS expression vector and a 3′ antisense primer (5′-CGGAATTCTTAAAGCTTATACAAGCCGA-3′) taken from murine CD95L cDNA, followed by electrophoresis in 1.5% agarose gels. Chromosomal integration of the pCAGGS vector in control renca clones was confirmed by PCR and gel electrophoresis as described above, using sense/antisense primers (5′-GCAACGTGCTGGTTGTTGTG-3′/5′-ACCACCTTCTGATAGGCAG-3′ respectively) taken from the pCAGGS vector sequence. To assess the functional activity of CD95L expressed on CD95L-transfectants, CD95-sensitive naive renca cells [1] and in the study described below) were cultured on cover slips and then CD95L-transfected (CD95-resistant in the study below) or control renca cells were added. Cells positive for terminal-deoxynucleotidyl-transferase-mediated dUTP nick-end labeling (TUNEL) were stained as described below.

RT-PCR/Southern hybridization

RNA was prepared as previously described [13] from renca cells cultured in RPMI-1640 medium supplemented with 10% FCS. Individual aliquots of each sample were resolved in denaturing formaldehyde gels to document the integrity of the RNA (data not shown), essentially as reported [13]. Reverse transcriptase/polymerase chain reaction (RT-PCR)/Southern hybridization was performed as described [13]. Briefly, the cDNA was synthesized at 42 °C from 10-μg aliquots of total RNA from renca cells in a total volume of 50 μl buffer supplied with avian myeloblastosis virus reverse transcriptase (RAV-2: Amersham Japan, Tokyo, Japan) and an oligo (dT) primer (Promega, Madison, Wis.). The quality of the cDNA was assessed by 30 cycles of PCR amplification of the β-actin sequence as described below and by examining the products following electrophoresis through 1.5% agarose gels containing ethidium bromide. Primers used for β-actin were as described previously [13]. Aliquots of 1 μl each cDNA synthesis product were amplified in a thermal cycler (Taitec, Koshigaya, Japan) for 30 cycles of denaturation (95 °C for 1 min), annealing (55 °C for 1 min) and extension (72 °C for 1 min). Sense/antisense oligonucleotide primers for murine CD95L and murine CD95 were 5′-GTGCCATGCAGCAGCCCATGA-3′/5′-TTAAAGCTTATACAAGCCGA-3′ [11] and 5′-GAGGCCCATTTTGCTGTCAA-3′/5′-TTCACAGCCAGGAGAATCGC-3′ [14] respectively. PCR-amplified sequences (10 μl) were resolved in 1.5% agarose gels containing ethidium bromide.

Induction of apoptosis by agonistic anti-(mouse CD95) mAb in CD95L transfectants

CD95L-transfected and control renca clones as well as na renca cells (1×10^6) were cultured in RPMI-1640 medium containing 10% FCS in flasks 3 cm in diameter on autoclaved glass converslips. After 24 h, 100 μg/ml agonistic anti-(mouse CD95) mAb (RK-8, MBL, Nagoya, Japan) or 100 μg/ml control hamster IgG1 (Dako Japan, Kyoto, Japan) was added to the cultures and coverslips were removed 24 h later. We assessed 300 cells on each coverslip, then TUNEL-positive cells, stained as described below, were counted.

Mice

Murine CD95L-expressing or control renca cells (1×10^5) were implanted into the subcapsule of the left kidney of syngeneic Balb/c and Balb/c nude mice as well as allogeneic C3H/HeJ mice at 6–8 weeks of age (purchased from Nisseizai; Tokyo, Japan). After 14 days, mice were sacrificed and tumor size [calculated by the formula: $V = 0.4\ W^2$ (nm^3)] was measured.

Histology

Hematoxylin/eosin staining

Cells on the coverslips, fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) for 10 min at room temperature, and paraffin-embedded sections were stained with hematoxylin and eosin.

Immunochemistry

Cryostat sections and cells on the coverslips were fixed in 4% paraformaldehyde for 10 min, rinsed with PBS (pH 7.4), then incubated with anti-(human CD95L) rabbit polyclonal antibody (Q-20, Santa Cruz Biotechnology Inc.) for 1 h at room temperature. Labeling was made visible by Envision labeled polymer reagent (Dako Japan, Kyoto, Japan).

Detection of apoptosis by the TUNEL method

Cells undergoing apoptosis were detected by the TUNEL method using an Apop Tag in situ apoptosis detection kit (Oncor, Gaithersburg, Md.) according to the manufacturer's instruction.

Statistics

The non-parametric Mann-Whitney *U*-test was applied to compare tumor volume.

Results

Cloning of murine CD95L-expressing renca cells

A number of renca clones with chromosomal integration of the murine CD95L cDNA expression vector construct

or of the control vector were isolated following transfection (data not shown). As shown in Fig. 1A, B, murine CD95L mRNA and protein were expressed in the CD95-transfected clones, but not in the control. Murine CD95 mRNA was expressed in the control and in CD95L transfectants as well as in naive renca cells (Fig. 1A). As shown in Fig. 2, apoptosis was induced with agonistic anti-(mouse CD95) mAb in about half of the naive and control renca cells (45.6 ± 1.5%, $n = 3$), but not in CD95L-transfected renca clones (0 ± 0%, $n = 3$, $P < 0.001$ compared with control renca cells by unpaired *t*-test). CD95L expressed on CD95L-transfected renca clones was functional (capable of inducing apoptosis), because CD95L-transfectants induced apoptosis in CD95-sensitive naive renca cells (data not shown).

In vivo tumor formation of murine CD95L-expressing renca tumor

In the experiments on tumor formation in vivo, mCD95L renca 4 and 6 cells used as murine CD95L-transfected renca clones, because their levels of murine CD95L expression, determined by RT-PCR and immunocytochemical analysis, seemed sufficient. One renca clone transfected only with the pCGAAS vector was used as a control. Both the CD95L-transfected and control renca clones formed macroscopic tumors in all syngeneic Balb/c and Balb/c nude hosts 14 days after implantation. As shown in Table 1, the tumors derived from murine CD95L-transfected renca cells grew significantly better than tumors derived from control renca cells in the Balb/c mice. However, CD95L-transfected

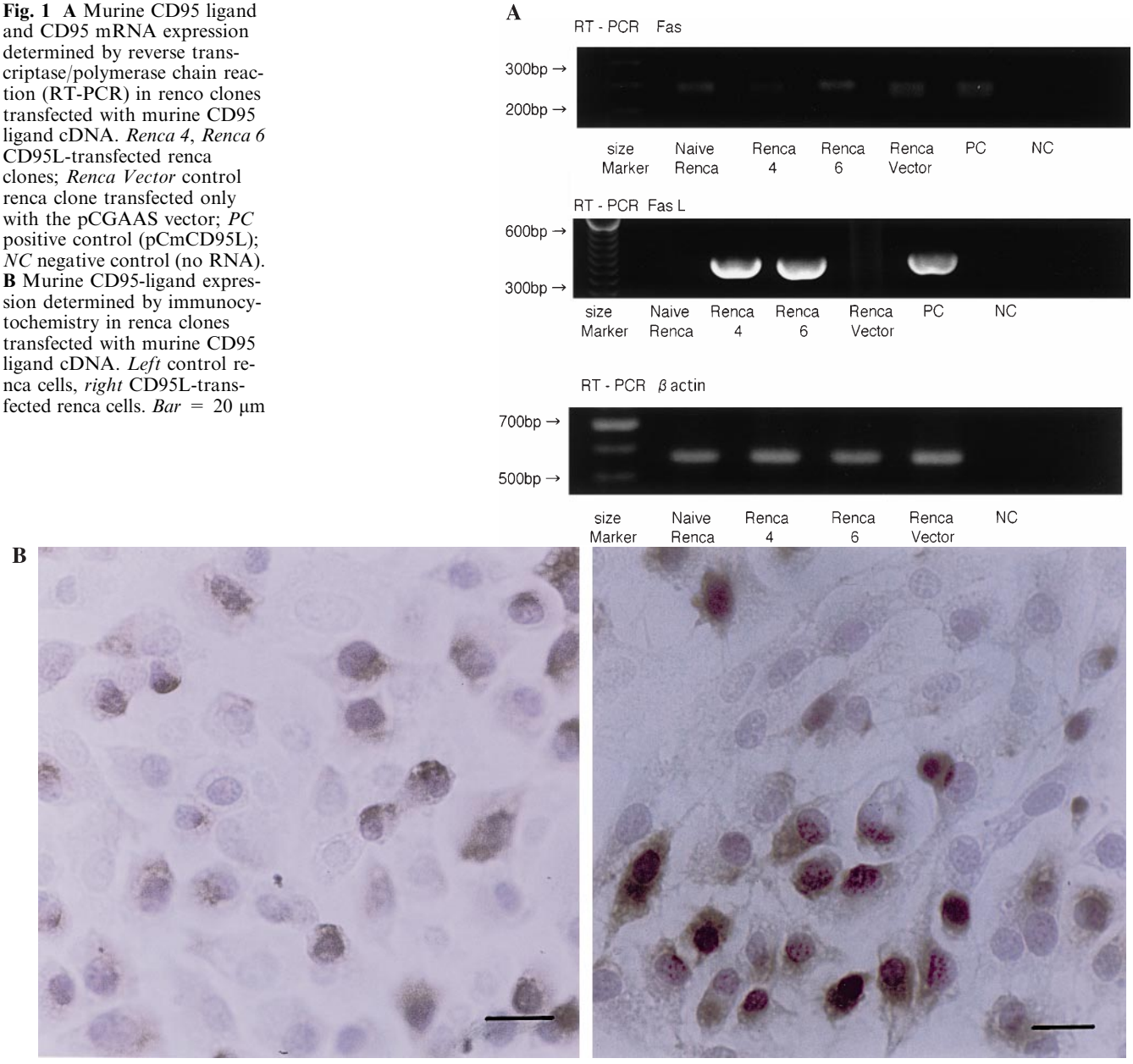

Fig. 1 **A** Murine CD95 ligand and CD95 mRNA expression determined by reverse transcriptase/polymerase chain reaction (RT-PCR) in renco clones transfected with murine CD95 ligand cDNA. *Renca 4*, *Renca 6* CD95L-transfected renca clones; *Renca Vector* control renca clone transfected only with the pCGAAS vector; *PC* positive control (pCmCD95L); *NC* negative control (no RNA). **B** Murine CD95-ligand expression determined by immunocytochemistry in renca clones transfected with murine CD95 ligand cDNA. *Left* control renca cells, *right* CD95L-transfected renca cells. *Bar* = 20 μm

and control renca cells did not form tumors in allogeneic C3H/HeJ mice ($n = 5$). Tumor size was much larger in Balb/c nude mice than in Balb/c mice for each renca clone. No growth advantage of CD95L-expressing renca cells over control renca cells was observed in Balb/c nude mice in contrast to Balb/c mice.

Histology of murine CD95L-expressing renca tumors

Tumors derived from CD95-transfected renca clones were shown to express CD95L protein by immunohistochemistry, whereas those of control renca clones did not (Fig. 3B). In Balb/c mice, lymphocytes underwent apoptosis in the periphery of CD95L-expressing tumors ($n = 10$) but not in control tumors ($n = 8$) as shown by TUNEL staining using as ApopTag kit (Fig. 3A, lower panel). Neutrophilic recruitment was rarely observed in either the CD95L-expressing or the control tumors by hematoxylin/eosin staining (Fig. 3A, upper panel). These peritumoral TUNEL-positive cells were not essentially observed in CD95L-expressing renca tumors grown in Balb/c nude mice (Fig. 3A, lower panel).

Discussion

Although renca cells have been reported to be $CD95^+$ and CD95-sensitive [1], the CD95L transfectants in this study were $CD95^+$ and CD95-resistant. CD95-expressing but CD95-resistant clones might have been selected by our experimental conditions, or $CD95^+$ CD95-sensitive cells might have been converted to $CD95^+$ CD95-resistant cells in the cloning step following the transfection of murine CD95L cDNA by lipofectamine in the experiments. In contrast to previous reports on CD95L-transfected tumors [1, 9], growth of renca tumors engineered to express CD95L was significantly better than that of control tumors. Our results indicated that CD95L expressed on renca cells induced apoptosis of the infiltrating lymphocytes and possibly suppressed immune responses against the tumor. In addition, acquisition or selection of CD95 resistance in the CD95L transfectants might have prevented the infiltrating $CD95L^+$ cytotoxic T lymphocytes (CTL) and natural killer cells (NK cells) from attacking CD95-resistant tumor cells via the CD95/CD95L mechanism. It is unclear that peritumoral TUNEL-positive cells are T cells. Nevertheless, it can at least be concluded that the growth advantage of CD95L-expressing renca cells and the appearance of peritumoral TUNEL-positive cells require T cells, because these were not observed in Balb/c nude mice lacking T cells. As neutrophilic infiltration was rarely observed in the CD95L-expressing renca tumors or in the control tumors, this did not seem to contribute to the tumor regression and rejection in our study. Whether tumor cells expressing CD95L show growth retardation as a result of the inflammatory response, with the recruitment of neutrophils or growth enhancement, by inducing apoptosis of the infiltrating lymphocytes in vivo, may depend on the level of CD95L expression on tumor cells, tumor cell types, methods of gene transfer, and the immune status of the host. Seino et al. reported that CD95L-transfected tumor cells were rejected when transplanted subcutaneously or intraperitoneally, but were maintained under the kidney capsule [8]. However, as CD95L-expressing renca cells did not form tumors in the renal subcapsule of allogeneic C3H/HeJ mice, CD95L expression itself was not sufficient to destroy the allogeneic barrier in this study, as previously reported [8].

CD95L expression may contribute to the enhanced growth of human tumors under some conditions. Strand et al. reported that human hepatocellular carcinomas partially or completely lost CD95 constitutively expressed by normal liver cells and might thus evade CD95-mediated cell killing by CTL. On the other hand, CD95L-expressing HepG2 hepatoblastoma cells killed $CD95^+$ Jurkat T cells [10]. The colon cancer cell line SW620, expressing both CD95 and CD95L, killed Jurkat T cells but did not undergo apoptosis after treatment with anti-CD95 agonistic monoclonal antibody [7]. These reports suggested that those cancer cells are resistant to CD95-mediated T cell cytotoxicity but express

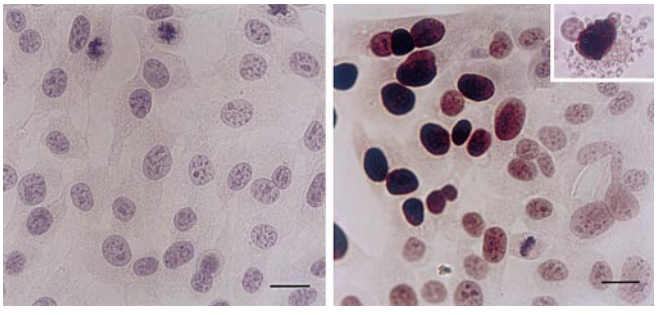

Fig. 2 Detection of apoptosis of renca clones following treatment with agonistic anti-(mouse CD95) mAb (24 h later) by the TUNEL method. *Left* CD95L-transfected renca cells, *right* control renca cells (apoptotic body is shown in the small figure). *Bar* = 20 μm

Table 1 Volume of tumours derived from murine renca cells transfected with CD95 ligand cDNA. *NS* not significant

Cells	Tumour volume, mean ± SE (mm^3)	
	Balb/c	Balb/c nude
Control renca	43 ± 19*,** ($n = 8$)	2937 ± 412 NS ($n = 4$)
mCD95L renca 4	317 ± 109* ($n = 10$)	2198 ± 459 NS ($n = 6$)
mCD95L renca 6	278 ± 91** ($n = 11$)	2842 ± 539 NS ($n = 5$)

* $P < 0.01$; ** $P < 0.02$

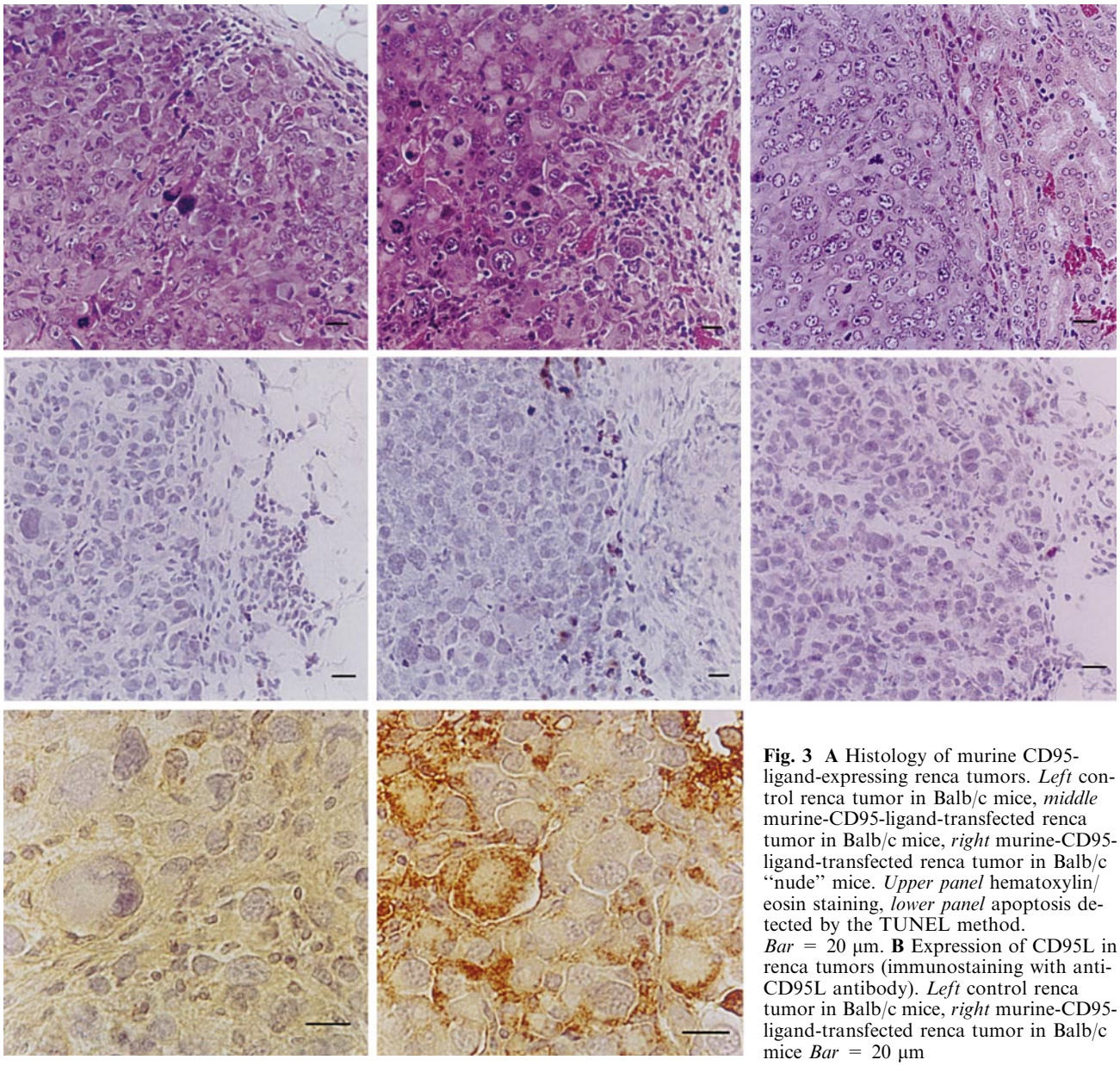

Fig. 3 **A** Histology of murine CD95-ligand-expressing renca tumors. *Left* control renca tumor in Balb/c mice, *middle* murine-CD95-ligand-transfected renca tumor in Balb/c mice, *right* murine-CD95-ligand-transfected renca tumor in Balb/c "nude" mice. *Upper panel* hematoxylin/eosin staining, *lower panel* apoptosis detected by the TUNEL method. *Bar* = 20 μm. **B** Expression of CD95L in renca tumors (immunostaining with anti-CD95L antibody). *Left* control renca tumor in Balb/c mice, *right* murine-CD95-ligand-transfected renca tumor in Balb/c mice *Bar* = 20 μm

functional CD95L, and apoptotic death signal to activated T cells. Zeytun et al. reported a mutual killing model in which $CD95L^+$, $CD95^+$ tumor cells, LSA and EL-4, killed $CD95^+$ tumor-specific CTL and were also killed by tumor-specific $CD95L^+$ CTL. They concluded that the survival of the tumor or the host may depend on which cells can accomplish CD95L-based killing more efficiently [15]. In the present study as well, CD95 resistance of the transduced tumor may be a more important factor in tumor growth than the demise of immune cells. We showed here that orthotopically implanted $CD95L^+$ and CD95-resistant murine renal carcinoma cells killed infiltrating lymphocytes by CD95L-based apoptosis, leading to the enhancement of tumor growth in vivo.

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