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Apoptosis and JNK activation are differentially regulated by Fas expression level in renal tubular epithelial cells

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Apoptosis and JNK activation are differentially regulated by Fas expression level in renal tubular epithelial cells.

Background. In chronic renal disease, renal tubular epithelial cell (RTC) Fas expression is up-regulated, leading to apoptotic RTC deletion and tubular atrophy. In vitro, cytokine- or hypoxia-induced up-regulation of Fas expression is associated with RTC apoptosis. In contrast, constitutively expressed, low level RTC Fas does not mediate apoptosis, suggesting that Fas may be coupled to expression level-dependent RTC signaling pathways. Fas is known to signal through JNK in many systems, but the requirement of JNK activation for apoptosis remains controversial.

Methods. To determine if RTC Fas regulates JNK activity and apoptosis, human RTC were transfected with graded concentrations of a eukaryotic expression vector for murine Fas. Apoptosis was measured by annexin V, TUNEL and PARP cleavage assays. JNK activity was determined by immune complex kinase assay and/or immunoblots with phospho-specific JNK antibodies, in the presence or absence of co-expressed dominant negative JNK constructs.

Results. Fas antibody stimulation of RTC with high Fas expression levels (to model RTC phenotype in renal disease) caused a tenfold increase in apoptosis, while RTC with low level Fas expression (to model normal RTC phenotype) were apoptosis-resistant. Fas ligation activated JNK in RTC expressing low levels of Fas, but not in apoptosis-sensitive RTC with increased Fas expression. Dominant negative JNK co-expression failed to inhibit apoptosis in RTC expressing high levels of Fas, suggesting that JNK is neither necessary, nor sufficient, for Fas-dependent apoptosis.

Conclusions. At high levels of expression, RTC Fas promotes apoptosis in a JNK-independent manner. At low basal expression, Fas induces JNK activation, but not apoptosis, consistent with novel roles for RTC Fas as a mediator of cell stress or chronic inflammation.

Key words: cell death, CD95, inflammatory mediators, signal transduction, protein kinases, progressive renal disease.

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Tubular atrophy has repeatedly been shown to be one of the best histologic predictors of renal disease progression in biopsy series that include specimens from heterogeneous glomerular diseases [1–3], and renal tubular epithelial cell (RTC) apoptosis has been proposed as one pathogenetic mechanism of tubular atrophy [4–7]. Multiple studies have demonstrated that RTC constitutively express low level Fas (CD95, APO-1) in normal kidney in vivo [6, 8], while RTC Fas expression is increased in animal models of acute and chronic renal disease [6, 9, 10]. RTC also constitutively express cell surface Fas in vitro [6, 7, 9, 11], which can be up-regulated with stimuli such as cytokines or hypoxia that model aspects of glomerular injury [6, 7, 9]. It is clear that in cells with abundant Fas expression, like Jurkat T cells or RTC with Fas expression induced by interleukin-1 (IL-1) or hypoxia, Fas activates pro-apoptotic signaling pathways [6, 7, 9, 12].

In contrast to the established role of RTC Fas following induced expression, RTC with constitutively low levels of Fas fail to undergo apoptosis after agonistic Fas antibody exposure [7, 11]. These data suggest that despite detectable Fas expression, anti-apoptotic molecules, which prevent Fas activation (soluble Fas or soluble Fas ligand) [13, 14] or inhibit pro-apoptotic signaling pathways (bcl-2, IAPs or FLIPs) [15], may prevent apoptosis when Fas, constitutively expressed at low density on the cell surface, interacts with its ligand. Predominance of such anti-apoptotic mechanisms in normal kidney is consistent with both low basal RTC turnover, and the paradigm that apoptosis is a default pathway that is averted by constant generation of survival signals [16]. Although these mechanisms provide an explanation for prevention of apoptosis in the presence of Fas expression, low levels of expressed Fas may also mediate functions that are apoptosis-independent. For example, Fas and the tumor necrosis factor (TNF) receptor share structural homology [17], and both have been linked to apoptosis-independent functions such as inflammation and fibrosis [18–21]. Furthermore,

Fas stimulates signaling molecules that are activated by inflammatory cytokines, such as c-Jun NH_2-terminal kinase (JNK) and nuclear factor-κB (NF-κB) [22, 23]. While NF-κB convincingly has been shown to regulate anti-apoptotic programs [22], the role of JNK in apoptosis is more controversial. Although multiple studies indicate that the JNK activation mediates apoptosis, an anti-apoptotic role for JNK has also been described in some circumstances [22, 24–26].

In contrast to conditions characterized by up-regulated RTC Fas expression, with RTC Fas functioning as a prototypical death receptor, which may facilitate tubular atrophy, we have hypothesized that constitutively-expressed low abundance RTC Fas functions like a cytokine receptor and may promote renal inflammation. To address this hypothesis, a model system was developed whereby murine Fas was expressed at various levels in human RTC, which permitted determination of the effects of different Fas expression levels on apoptosis and JNK activation. Our results demonstrate that at high levels of expression, Fas promotes apoptosis in a JNK-independent manner. At low expression levels, Fas induces JNK activation, but not apoptosis, which is consistent with a novel role for RTC Fas as a mediator of chronic inflammation.

METHODS

Antibodies

Antibodies used in this study included: rabbit anti-mouse Fas IgG, rabbit antihuman JNK IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA); peroxidase-conjugated goat antirabbit IgG, peroxidase-conjugated goat antimouse IgG (Amersham Pharmacia Biotech, Arlington Heights, IL, USA); agonistic hamster antimouse Fas IgG, mouse antihuman poly(ADP-ribose) polymerase (PARP) IgG (Pharmingen, San Diego, CA, USA); biotinylated goat antihamster IgG, Texas red-conjugated streptavidin, phycoerythrin (PE)-conjugated streptavidin (Vector Laboratories, Burlingame, CA, USA); antiphosphospecific JNK IgG (New England Biolabs, Beverly, MA, USA); mouse anti-HA IgG (Roche Molecular Biochemicals, Indianapolis, IN, USA); agonistic mouse antihuman Fas IgM (Kamiya Biomedical, Seattle, WA, USA).

Cell culture

Human renal proximal tubular (HRPT) cells (gift from Dr. L. Racusen) were derived from human proximal tubule and have been extensively characterized [6, 7, 27]. HRPT cells were maintained in Dulbecco's modified Eagle's medium (DMEM)-F12 (Gibco BRL, Rockville, MD, USA) + 10% fetal bovine serum (Hyclone, Logan, UT, USA) + 1% penicillin/streptomycin (Sigma, St. Louis, MO, USA).

Transient transfections

Transient, rather than stable, transfections were employed to avoid activation of signaling pathways in stable transfectants, which may reflect adaptive mechanisms, rather than direct activation of Fas. HRPT (0.25×10^6) were plated in six-well dishes, and cultured overnight in DMEM–F-12 + 10% fetal bovine serum, to achieve approximately 80% confluence. Cells were then washed in serum-free DMEM, and maintained in DMEM for two hours. HRPT were transiently transfected with the following constructs: murine Fas cDNA (gift from Dr. S. Nagata) [28] subcloned into pcDNA3 expression plasmid (Invitrogen, Carlsbad, CA, USA); green fluorescence protein (GFP) cDNA (Clontech, Palo Alto, CA) subcloned into pcDNA3; nonphosphorylatable, dominant negative, HA-tagged JNK cDNAs (JNK1-and JNK2-APF) subcloned into LNCX retroviral plasmid vector (gift from Dr. L. Heasley) [29]. HRPT cells were incubated with plasmid (2 μg/well) plus cationic liposome (10 μL/well; Superfect, Qiagen, Valencia, CA, USA) complexes in serum-free DMEM for three hours at 37°C. To enhance cDNA construct expression, transfected cells were maintained for an additional 24 hours in complete media containing DMEM-F12 + 10% fetal bovine serum. Transfection efficiency was determined in each experiment by calculating the percentage of cells expressing GFP. Values routinely ranged from 20% to 30%, and were identical between conditions within an individual experiment.

Immunofluorescence microscopy

Methods previously described in detail for tissue sections and cell suspensions have been modified for cell culture monolayers [6, 7]. HRPT cells maintained on sterile, glass coverslips within six-well plates were fixed in paraformaldehyde (4%, 10 minutes, room temperature). Fixed cells were washed with phosphate-buffered saline (PBS) and incubated with primary antibodies, followed by biotinylated secondary antibodies and Texas red-conjugated avidin. Negative controls were cells incubated with PBS alone or with isotype-identical IgG, which was immunoreactive with an irrelevant epitope. Coverslips were mounted in antifade, aqueous media (Vectashield, Vector Laboratories) containing 4′,6 diamidino-2-phenylindole (DAPI) on standard microscope slides. Random fields were viewed at ×40 magnification using an Olympus epifluorescence microscope (Tokyo, Japan) with appropriate fluorescence filters. Representative fields were photographed with a Spot Digital System camera and Image Pro software system (Diagnostic Instruments, Sterling Heights, MI, USA).

Immunoblot analysis

Methods have previously been described in detail [30]. Briefly, HRPT cell monolayers were lysed and denatured

in boiling sodium dodecyl saline-polyacrylamide gel electrophoresis (SDS-PAGE) buffer (125 mmol/L Tris, pH 6.8, 2% SDS, 5% glycerol, 1% β-mercaptoethanol, 0.003% bromphenol blue) for five minutes. Samples (20 μg/lane) were resolved by 12% SDS-PAGE and transferred to polyvinylidine difluoride (PVDF) membranes. Blots were blocked in 5% dried milk and 2% bovine serum albumin, probed with primary antibody (1 μg/mL, 1 hour, room temperature), and then peroxidase-conjugated IgG (1:5000, 1 hour, room temperature). Band intensity was detected by enhanced chemiluminescence according to the manufacturer's instructions (Amersham Pharmacia Biotech, Arlington Heights, IL, USA) and exposure to Kodak Biomax ML film (Eastman Kodak, Rochester, NY, USA).

Fas expression by dual color flow cytometry

Human renal proximal tubular cells were transfected with GFP and mouse Fas cDNAs as described above, fixed in paraformaldehyde (1%, 1 hour, 4°C) and lifted by incubation with ethylenediaminetetraacetic acid (EDTA; 5 mmol/L, 10 minutes, 37°C). Fas expression was determined by sequential incubation with hamster antimouse Fas IgG (2×10^6 cells/μg antibody, 1 hour, room temperature), biotinylated goat antihamster IgG and PE-conjugated streptavidin. All cells were resuspended in PBS and assayed for GFP and Fas expression by flow cytometry (FACScan; Becton Dickinson, San Jose, CA, USA) as previously described [6, 7], using appropriate filters for GFP and PE fluorescence. Analyses were performed with a Macintosh Power PC computer (7600/132; Apple Computer, Cupertino, CA, USA) and appropriate software (Becton Dickinson).

JNK activity by immune complex kinase assay

Methods have previously been described in detail [31]. Briefly, HRPT cells were lysed in RIPA buffer [150 mmol/L NaCl, 50 mmol/L Tris, pH 7.2, 5 mmol/L EDTA, 1 mmol/L Na_3VO_4, 1% (wt/wt) Triton X-100, 0.1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 10 μg/mL leupeptin, and 20 μg/mL aprotinin]. Cell lysates, containing equal amount of protein (400 μg), were sequentially precleared with purified non-immune rabbit IgG and goat anti-rabbit IgG-agarose beads at 4°C for 60 minutes. After centrifugation at 10,000 × *g*, supernatants were incubated overnight at 4°C on a rotating platform with 1 μg rabbit polyclonal anti-JNK IgG and 25 μL goat anti-rabbit IgG-agarose. After centrifugation, immunoprecipitates were washed twice with lysis buffer and once with kinase buffer (20 mmol/L HEPES, pH 7.6, 20 mmol/L $MgCl_2$, 25 mmol/L β-glycerol phosphate, 0.1 mmol/L Na_3VO_4, 2 mmol/L dithiothreitol). Immunoprecipitate-associated kinase activity was assayed at 30°C for 20 minutes in 30 μL kinase buffer containing recombinant ATF-2 or GST-c-Jun (0.5 mg) as the substrate, combined with 20 μmol/L adenosine 5′-triphosphate (ATP), and 5 μCi [γ-^{32}P] ATP. The kinase reaction was terminated with 40 μL Laemmli sample buffer (125 mmol/L Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 2% β-mercaptoethanol). After boiling for five minutes, samples were resolved by 10% SDS-PAGE. Gels were stained with Coomassie blue, de-stained with 20% methanol, 10% acetic acid and 70% H_2O, and dried. Incorporated [^{32}P] was visualized by autoradiography.

Apoptosis detection by annexin V and chromatin labeling

Apoptotic cells were identified by labeling externalized phosphatidylserine with annexin V according to the manufacturer's directions (Roche Molecular Biochemicals) as previously described [7]. Briefly, HRPT cells cultured on coverslips were washed in PBS and then incubated with biotin-conjugated annexin V in a buffer containing 140 mmol/L NaCl, 10 mmol/L HEPES/NaOH, pH 7.4, 5 mmol/L $CaCl_2$ for 15 minutes at room temperature. Coverslips were washed in the HEPES buffer, incubated with Texas red-conjugated avidin (1:300, 20 minutes, 4°C), and fixed in paraformaldehyde (4%, 10 minutes, room temperature). Monolayers were viewed by fluorescence microscopy as described above, and apoptotic cells were defined by fluorescent labeling of at least half of the plasma membrane perimeter. Nuclear morphology was simultaneously determined by chromatin labeling with DAPI. Morphology consistent with an apoptotic phenotype included apoptotic bodies or pyknosis, according to established criteria [32, 33]. The percentage of apoptotic cells was calculated from a total of 200 to 300 transfected cells per experimental condition, which were identified by GFP fluorescence from randomly selected fields. All observations were made in a blinded fashion with respect to experimental group by at least two observers.

Apoptosis detection by TUNEL

Apoptotic nuclei were detected by the terminal deoxyribonucleotide transferase (TdT)-catalyzed DNA end labeling (TUNEL) technique according to the manufacturer's instructions (Oncor, Gaithersburg, MD, USA) as previously described [6]. Briefly, 1% paraformaldehyde-fixed HRPT cultured on coverslips were incubated with TdT and digoxigen-labeled dUTP, followed by FITC-conjugated antidigoxigen IgG. Negative controls were cells incubated without TdT. Monolayers were viewed at ×40 magnification using an Olympus epifluorescence microscope with a fluorescein filter. The percentage of fluorescent, apoptotic nuclei was calculated from a total of 200 to 300 cells per condition, which were identified by DAPI staining of all nuclei, from four to six randomly selected fields. All observations were made in a blinded fashion with respect to experimental group.

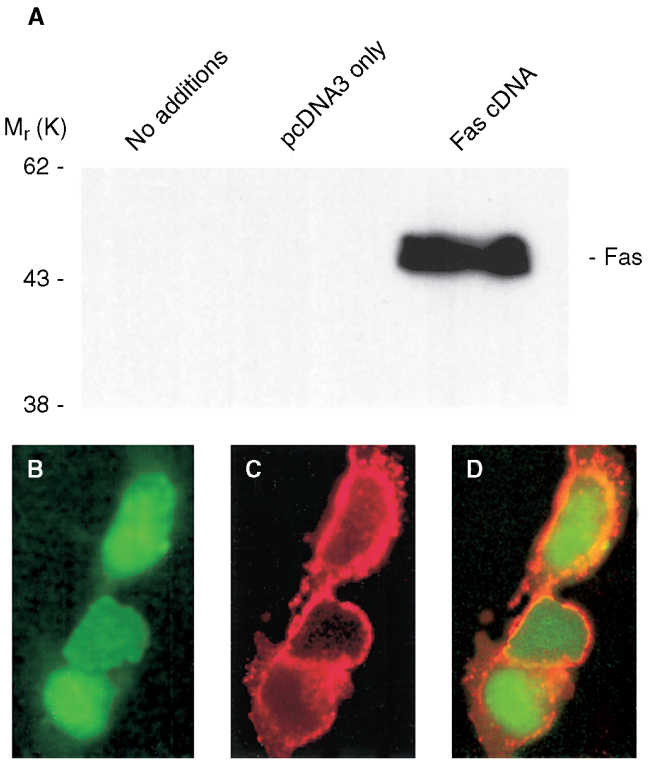

Fig. 1. Human renal tubular epithelial cells (RTC) express transfected murine Fas on the cell surface. (*A*) Human renal proximal tubule (HRPT) cells were transfected with mock vector (pcDNA3) or murine Fas cDNA (1 μg/well). Lysates (20 μg protein/lane) were resolved by SDS-PAGE and probed for Fas expression by immunoblot analysis with rabbit antimouse Fas IgG, as described in the **Methods** section. (*B–D*) Fas localization was determined in HRPT cells co-transfected with green fluorescence protein (GFP) and Fas cDNA reporter constructs by fluorescence microscopy. (B) Transfected cells were identified by GFP fluorescence. (C) Fas expression was determined following sequential incubation with hamster antimouse Fas IgG, biotinylated goat antihamster IgG and Texas Red-conjugated avidin. (D) merged images from B and C.

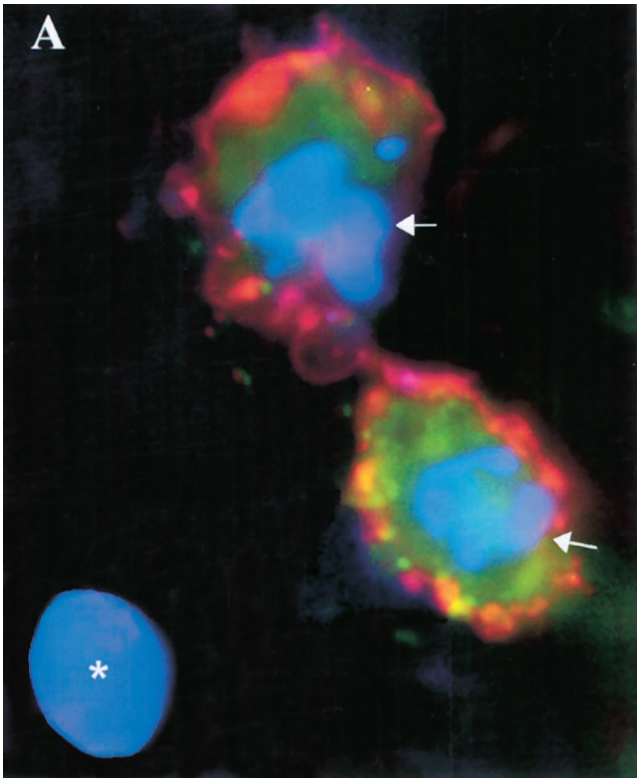

Fig. 2. Mouse Fas cDNA-transfected human RTC are susceptible to apoptosis. (*A*) HRPT cells were co-transfected with GFP cDNA (1 μg/well), to mark transfected cells, and murine Fas cDNA expression vector (1 μg/well). Fas cDNA-transfected RTC were then incubated with agonistic antimouse Fas antibodies (5 μg/ml, 14 hours, 37°C) and assayed for apoptosis by annexin V labeling of externalized phosphatidylserine and DAPI staining of chromatin, as described in the Methods. The transfected RTCs, indicated by GFP fluorescence (green), demonstrated an apoptotic morphology that included external plasma membrane labeling with annexin V (red) and chromatin fragmentation and condensation (arrows). The single untransfected cell in this field displayed a diffuse chromatin pattern (asterisk) and did not label with annexin V, characteristics of viable, nonapoptotic cells.

Apoptosis detection by PARP cleavage

SDS-PAGE and immunoblots were probed with mouse antihuman PARP IgG and peroxidase-conjugated goat antimouse IgG as described above. Apoptosis was defined by caspase-3-dependent cleavage of the 85 kD degradation product from the intact 116 kD parent PARP protein, which is a signature step in the Fas effector pathway.

Statistics

All experiments were performed a minimum of three times with duplicate samples for each experimental condition. Results are expressed as mean ± SE. Comparison between two groups was made by the unpaired two-tailed Student *t* test. Comparisons between more than two groups were made by one-way analysis of variance with the Bonferroni test for multiple comparisons. Statistical significance is defined as $P < 0.05$.

RESULTS

Human renal tubular epithelial cells express transfected murine Fas on the cell surface

To determine whether Fas function is regulated by Fas expression levels, human RTC were transfected with graded concentrations of a eukaryotic expression vector for murine Fas. This in vitro model has several advantages. First, human and mouse Fas require species-specific ligands, due to the heterogeneity of the respective extracellular domains. Mouse agonistic Fas antibodies do not activate human Fas, and the effect of murine Fas expression level on RTC apoptosis and JNK activity can thus be assessed without a contribution from endogenous Fas. Second, this model permits activation of low abundance RTC Fas, which models basal in vivo conditions. Third, it represents a design improvement for mimicking a pathologic state of up-regulated RTC Fas expression

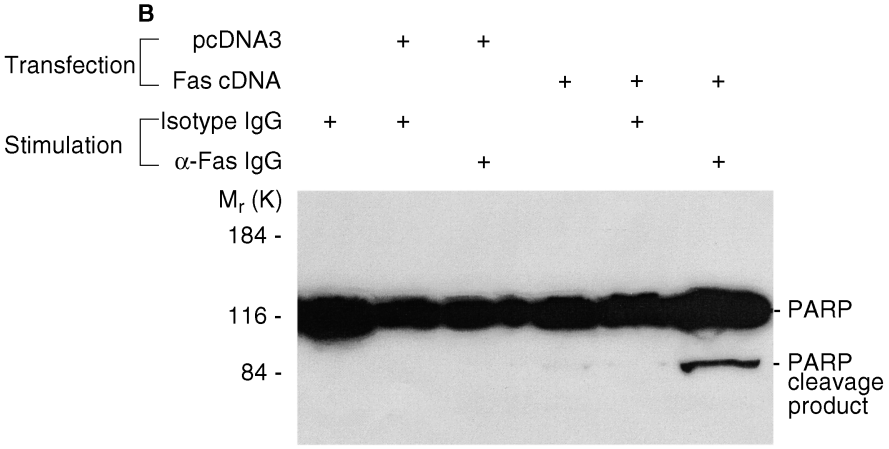

Fig. 2 (Continued). (*B*) HRPT cells were transfected with murine Fas cDNA expression vector (2 μg/well) or empty pcDNA3 vector (2 μg/well), followed by incubation with agonistic antimouse Fas IgG (5 μg/ml, 14 hours, 37°C) or isotype control IgG (IgG, 5 μg/ml, 14 hours, 37°C). Apoptosis was assessed by PARP cleavage product, as determined by immunoblot analysis (**Methods** section). Molecular weight markers ($\times 10^{-3}$) are shown at left.

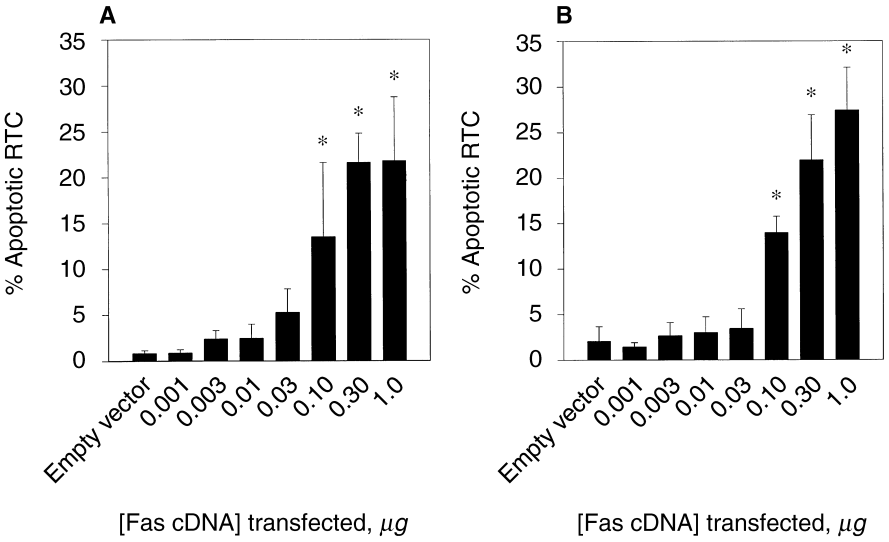

Fig. 3. Fas-mediated apoptosis is dependent upon transfected Fas expression level. HRPT cells were transfected with mouse Fas cDNA at indicated concentrations (per well) plus GFP cDNA (1 μg/well). Total transfected cDNA concentrations (2 μg/well) were kept constant by supplementation with pcDNA3. Transfection efficiency, as determined by GFP fluorescence, was 20 to 30% in each experimental group. All groups were exposed to agonistic antimouse Fas IgG (5 μg/ml, 14 hours, 37°C) and then quantitatively analyzed for apoptosis as described in the *Methods* section. (*A*) Apoptosis detection by annexin V labeling of GFP-positive cells. (*B*) Apoptosis detection by DAPI-labeled nuclear morphology in GFP-positive cells. $^*P < 0.05$ compared to groups transfected with 0 to 0.03 μg/well.

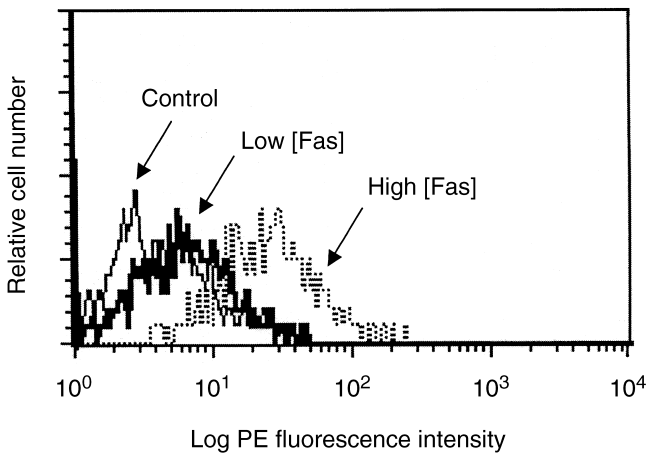

Fig. 4. Transfected murine Fas cDNA plasmid concentration correlates with surface Fas expression level. HRPT cells were transfected with 1.0 μg/well empty pcDNA3 vector (control; thin line), 0.1 μg/well mouse Fas cDNA + 0.9 μg/well pcDNA3 (low [Fas]; heavy line) or 1.0 μg/well mouse Fas cDNA (high [Fas]; dotted line) as described in the **Methods** section. All three groups were co-transfected with GFP cDNA (1.0 μg/well). Surface Fas expression was determined by indirect immunofluorescence with hamster antimouse IgG, biotinylated goat antihamster IgG and phycoerythrin (PE)-conjugated streptavidin. Cells were analyzed by dual color flow cytometry, as previously described [6, 7]. Phycoerythrin (PE) fluorescence in cells co-transfected with green fluorescence protein (GFP) is shown.

by avoiding exposure to cytokines or decreased oxygen tension used in previous studies [6, 7, 9], which may cause apoptosis in a Fas-independent manner. Figure 1 A demonstrates murine Fas expression in human RTC following transient transfection. Only transfected cells (indicated by green fluorescence in Fig. 1 B, D) expressed mouse Fas in a plasma membrane distribution (Fig. 1 C, D), indicating that transfected human RTC predominantly express the transmembrane mouse Fas isoform, rather than the alternatively-spliced soluble isoform [13]. Surface mouse Fas protein expression was readily detected up to 48 hours after transfection, with diminished expression levels thereafter. Fas expression was not observed in untransfected HRPT cells probed with anti-

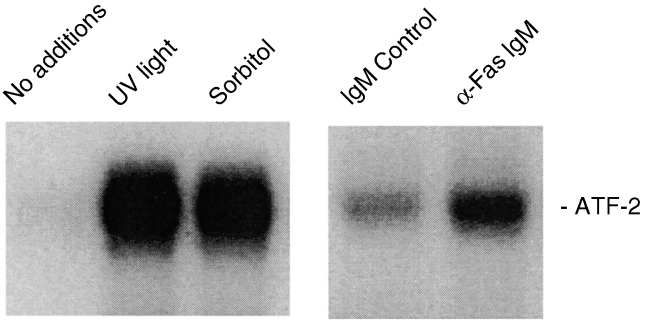

Fig. 5. Endogenous RTC Fas activates JNK but does not stimulate apoptosis. (Left panel) Untransfected HRPT cells were exposed to known, potent JNK activators UV-C light (3000 mW · cm^2, wavelength 254 nm) or hyperosmotic sorbitol (400 mmol/L, 30 min, 37°C) as positive controls. (Right panel) Untransfected RTC were incubated with agonistic anti-Fas IgM under conditions that do not stimulate apoptosis (150 ng/mL, 15 min, 37°C) or isotype control IgM (150 ng/mL, 15 min, 37°C). Cell lysates were assayed for JNK activity by immune complex kinase assay, using recombinant ATF-2 as the phosphorylation substrate, as described in the **Methods** section.

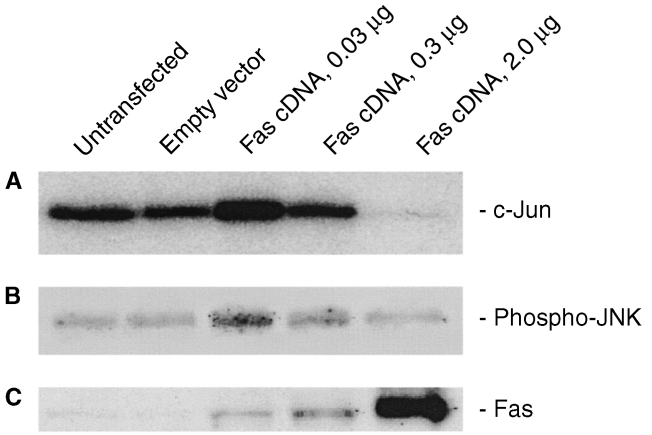

Fig. 6. Human RTC JNK activation correlates inversely with transfected murine Fas expression. HRPT cells were transfected with mouse Fas cDNA at indicated concentrations (per well). Total transfected cDNA concentrations (2 μg/well) were kept constant by supplementation with pcDNA3. All groups were exposed to agonistic antimouse Fas IgG (5 μg/ml, 15 min, 37°C) and lysates were analyzed for JNK activity in (*A*), by immune complex kinase assay using GST-c-Jun as substrate, and in (*B*) by immunoblotting with antiphosphospecific JNK antibodies. (*C*) Immunoblots were stripped by incubation with 100 mmol/L β-mercaptoethanol, 2% SDS, 62.5 mmol/L Tris-HCl, pH 6.7 (50°C, 30 min), and re-probed for Fas expression by immunoblotting with rabbit antimouse Fas IgG.

mouse Fas IgG and immunofluorescence microscopy (data not shown).

Mouse Fas cDNA-transfected human renal tubular epithelial cells are susceptible to apoptosis

To determine whether RTC undergo Fas-dependent apoptosis after activation by agonistic antibody in this heterologous expression system, human RTC were transiently transfected with the maximum murine Fas cDNA plasmid concentration that did not cause cell toxicity. Figure 2A depicts a typical field containing untransfected and transfected cells, as indicated by fluorescence of a GFP reporter. In each experiment, approximately 25% to 30% of cells were transfected. After incubation with agonistic antibody, transiently transfected RTC demonstrated an apoptotic morphology that included annexin V labeling of externalized phosphatidylserine (an early feature of apoptosis) and DAPI staining of condensed, fragmented chromatin (a late apoptotic feature, shown by arrows in Fig. 2A). In contrast, untransfected cells displayed a diffuse chromatin pattern (shown by an asterisk in Fig. 2A), and an absence of annexin V labeling. Apoptosis was also demonstrated in this induced Fas expression system by PARP cleavage (Fig. 2B) and TUNEL assay (not shown). No increase in apoptosis was observed in Fas cDNA-transfected RTC in the absence of agonistic antibody exposure, indicating that induced Fas expression did not result in self-oligomerization and ligand-independent cell death, as has been reported by others [34]. Taken together, results from these assays demonstrate that activated mouse Fas is capable of transducing apoptosis signals when abundantly expressed in human RTC.

Fas-mediated apoptosis is dependent upon Fas expression level

To determine whether Fas transduces apoptosis in an expression level-dependent fashion, HRPT cells were co-transfected with a GFP reporter at a fixed concentration and mouse Fas cDNA at concentrations that ranged from 1 ng to 1 μg per well. Fas protein expression varied directly with transfected Fas cDNA concentration, as measured by immunoblot analysis (Fig. 6C). RTC with differing Fas expression levels were subsequently exposed to agonistic antimouse Fas antibodies, and apoptotic cells were detected in the transfected cell population (indicated by GFP fluorescence) by annexin V labeling and nuclear morphology. As shown in Figure 3, apoptosis was induced only after Fas expression reached a threshold of 0.03 to 0.10 μg murine Fas expression vector per well. Maximum (8- to 10-fold) increase in apoptosis occurred in RTC transfected with 0.3 to 1.0 μg Fas cDNA per well. The results support previous observations obtained with cytokine- or hypoxia-induced Fas expression by demonstrating RTC Fas-mediated apoptosis is dependent on a threshold level of Fas expression [6, 7, 9].

Transfected murine Fas cDNA plasmid concentration correlates with surface Fas expression level

To determine the relationship between Fas cDNA plasmid concentration and surface Fas expression level, RTC were co-transfected with GFP cDNA and either high or low Fas cDNA concentrations. GFP and Fas

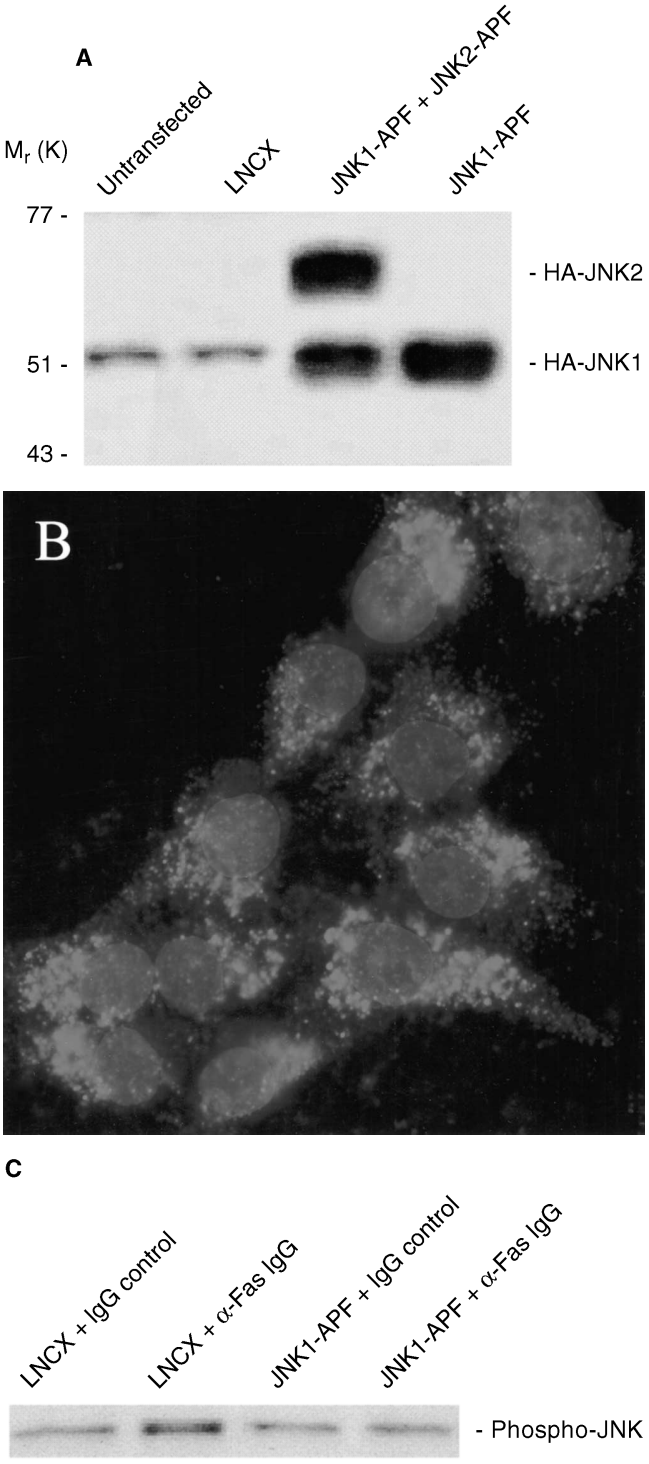

Fig. 7. Transfected RTC Fas activates JNK1. (*A*) HRPT cells were transiently transfected with HA-tagged, dominant negative JNK1 (JNK1-APF) and JNK2 (JNK2-APF) constructs (2 μg/well). Controls included untransfected cells and cells transfected with empty LNCX vector. Lysates were probed for dominant negative JNK isoform expression by immunoblotting with anti-HA antibodies. Molecular weight markers ($\times 10^{-3}$) are shown at left. Faint, nonspecific bands at ~55 kD, such as those seen in the two control lanes, have previously been recognized by the anti-HA antibodies in immunoblots from other tissues [29]. (*B*) HRPT cells cultured on coverslips were transiently transfected with HA-tagged JNK-APF constructs as described above, and then assessed for protein expression by immunofluorescence microscopy with anti-HA antibodies (shown in red, see cover), as described in the **Methods** section. Nuclei were counterstained with DAPI (shown in blue, see cover). (*C*) HRPT cells were transiently transfected with the murine Fas cDNA (0.03 μg/well) and dominant negative JNK (JNK1-APF, 2 μg/well) or empty vector (LNCX, 2 μg/well) and then stimulated with agonistic anti-Fas IgG (5 μg/ml, 15 min, 37°C) or isotype control IgG (5 μg/ml, 15 min, 37°C). Cell lysates were resolved by SDS-PAGE, and JNK activity was measured by immunoblot analysis with antiphosphospecific JNK antibodies.

expression (as determined by PE fluorescence) were then evaluated by dual color flow cytometry. Approximately 20% of cells were transfected in all groups, as determined by GFP fluorescence (not shown). Figure 4 shows that transfection with low concentration Fas cDNA resulted in increased surface Fas expression in cells co-transfected with GFP (mean 9.7 fluorescence units) compared to cells co-transfected with empty vector and GFP (mean 4.8 fluorescence units). Furthermore, mean and median PE fluorescence were not significantly different in the low concentration Fas cDNA group, indicating that increased PE fluorescence was not due to a small number of cells with extremely high Fas expression. As expected, Figure 4 demonstrates that cells transfected with higher Fas cDNA concentration displayed greater Fas expression levels (mean 26.9 fluorescence units). These data indicate that transient transfection with Fas cDNA caused dose-dependent Fas expression per cell.

Endogenous RTC Fas activates JNK but does not stimulate apoptosis

Renal tubular epithelial cells expressing low levels of Fas, a model of the normal kidney state, do not undergo apoptosis after incubation with agonistic antibody, suggesting that constitutively expressed RTC Fas could mediate apoptosis-independent functions. Previous studies have demonstrated that Fas stimulation triggers downstream JNK phosphorylation and activation, which is unrelated to apoptosis in many systems [22, 24–26, 35, 36]. To determine whether RTC Fas at low constitutive levels signals through JNK, untransfected human RTC were incubated with agonistic antihuman Fas IgM under conditions (150 ng/mL, 15 min) that do not induce apoptosis (not shown). Figure 5 demonstrates that RTC Fas stimulation caused JNK activation, as determined by immune complex kinase assays. These results indicate that RTC Fas activates JNK under conditions that are not associated with apoptosis.

Human RTC JNK activation correlates inversely with murine Fas expression

Agonistic Fas antibody activates JNK, but not apoptosis, in human RTC in which Fas expression has not been up-regulated. Since apoptosis only occurs in RTC with

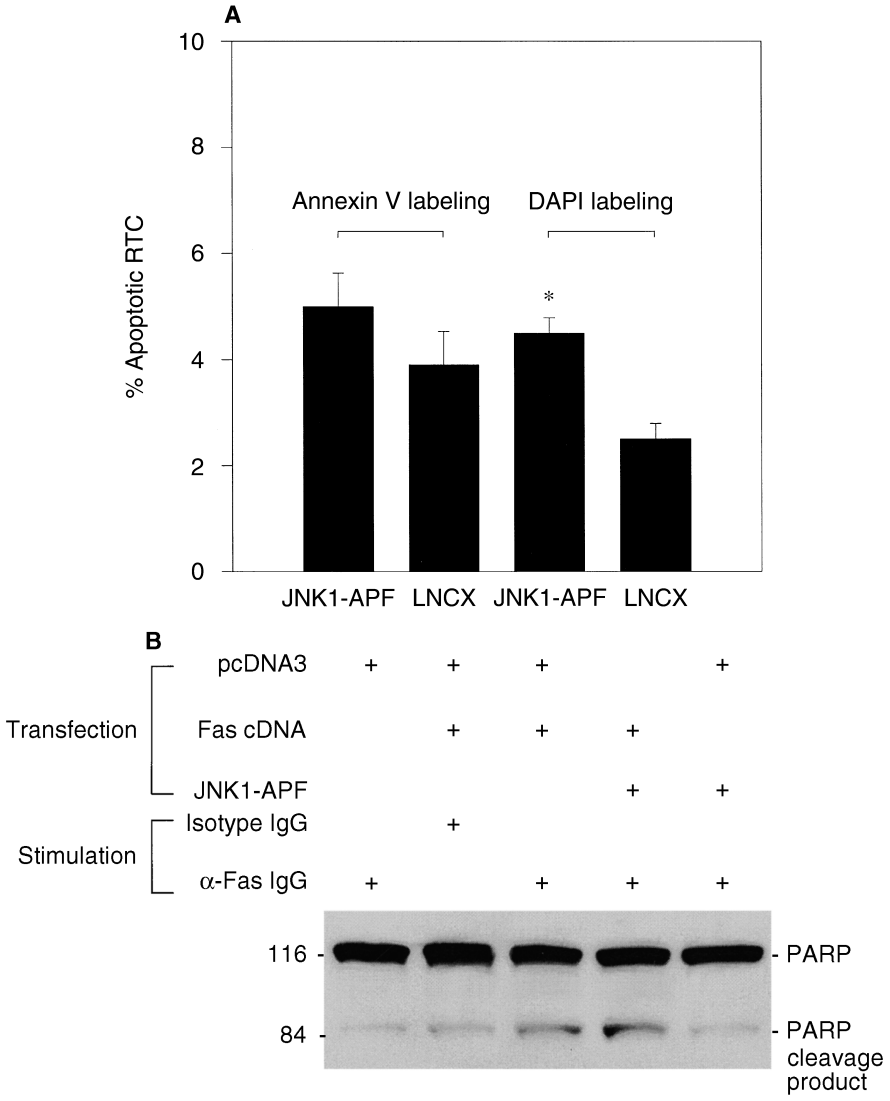

Fig. 8. JNK activity is dispensable for transfected Fas-dependent RTC apoptosis. (*A*) HRPT cells with endogenous, constitutive Fas expression were transfected with JNK1-APF or empty vector (LNCX) as described above, then incubated with agonistic antihuman Fas IgM (150 ng/mL, 14 h, 37°C), and assayed for apoptosis by annexin V labeling of externalized phosphatidylserine or DAPI labeling of condensed chromatin. $*P < 0.05$ compared to DAPI-labeled, LNCX-transfected cells. (*B*) HRPT cells were transiently transfected with murine Fas cDNA (1 μg/well) and/or JNK1-APF (1 μg/well) constructs. Total transfected cDNA concentrations (2 μg/well) were kept constant by supplementation with pcDNA3. Cells were exposed to agonistic antimouse Fas IgG or isotype control IgG (5 μg/mL, 14 h, 37°C), and lysates were probed for PARP cleavage by immunoblot analysis as described in the **Methods** section.

abundant Fas expression, activated Fas may stimulate JNK activity at all levels of surface expression, but Fas expressed at high levels may activate additional pathways required for apoptosis. To address the relationship between Fas expression level and JNK activation, RTC were transfected with graded Fas cDNA concentrations and JNK activity was determined by immune complex kinase assays and immunoblotting with phosphospecific JNK antibodies (Fig. 6). JNK was activated in RTC transfected with the lowest Fas cDNA concentration following stimulation with agonistic antibody (Fig. 6 A, B), despite only minimal Fas expression (Fig. 6C). Conversely, maximum RTC Fas expression was not associated with JNK activation in response to agonistic Fas antibody incubation (Fig. 6). In addition, apoptosis was not observed under these conditions, thereby demonstrating that lack of Fas-dependent JNK activation was not due to RTC death. These data indicate that RTC Fas expression level inversely correlates with Fas-induced JNK activation, and suggest that JNK activation through low density RTC Fas may be linked to apoptosis-independent cell functions.

RTC Fas activates JNK1

The specific JNK isoform regulated by Fas was tested by assaying Fas-dependent JNK activation in RTC expressing dominant negative JNK isoform constructs [29, 37]. Figure 7 A and B demonstrate expression of dominant negative JNKs in RTC by immunoblot and immunofluorescence analyses, respectively. JNK isoform activation was determined in human RTC co-transfected with murine Fas cDNA concentrations that permit JNK activation (Fig. 6), and dominant negative JNK constructs. Figure 7C demonstrates that Fas ligation with agonistic Fas antibody stimulated JNK activation, which was completely inhibited by JNK1-APF co-expression. Fas-depen-

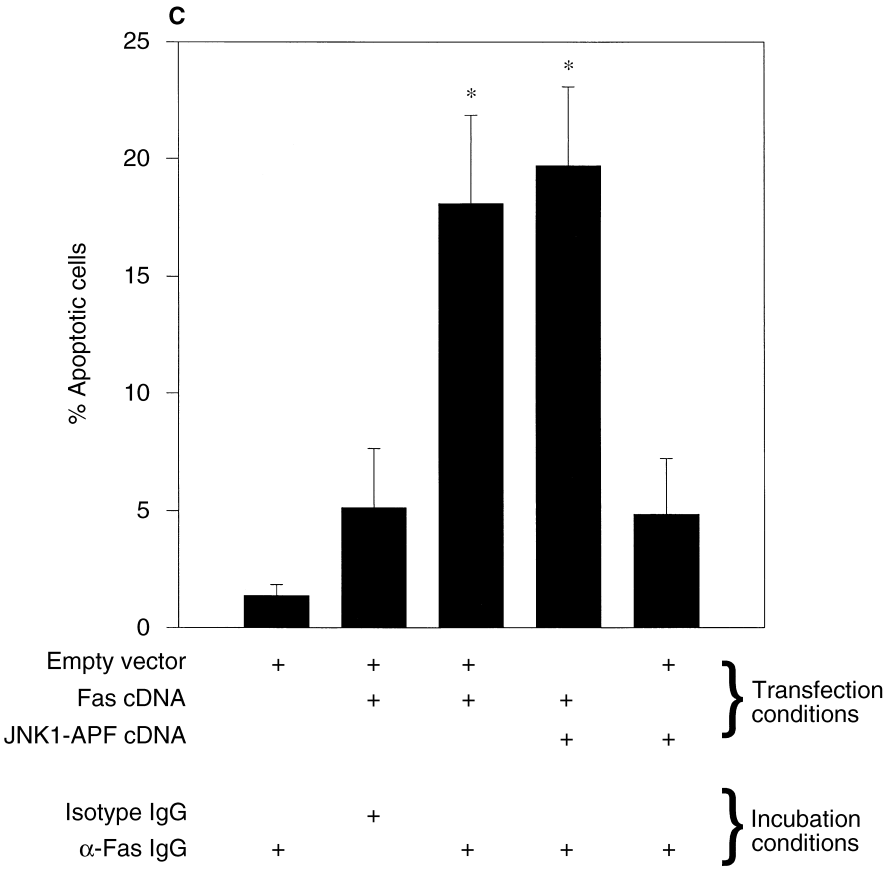

Fig. 8 (Continued). (*C*) HRPT cells were transiently transfected with GFP cDNA (0.67 μg/well) plus murine Fas cDNA (0.67 μg/well) and/or JNK1-APF (0.67 μg/well) constructs. Total transfected cDNA concentrations (2 μg/well) were kept constant by supplementation with pcDNA3. Cells were exposed to agonistic antimouse Fas IgG (5 μg/mL, 14 h, 37°C) or isotype control IgG (5 μg/mL, 14 h, 37°C), and assayed for apoptosis by annexin V labeling. Results are expressed as percentage transfected (GFP-fluorescent) cells that co-label with annexin V. $*P < 0.05$ compared to other three experimental groups.

dent JNK activation was not inhibited by JNK2-APF co-expression (data not shown). These observations indicate that RTC Fas signals through the JNK1 isoform, which is consistent with data in other cell types [38].

JNK activity is dispensable for Fas-dependent RTC apoptosis

Fas-regulated JNK activity and apoptosis were directly tested in RTC expressing dominant negative JNK to determine if Fas-dependent JNK activity is dispensable for RTC apoptosis. Transfection of basal human RTC (absence of Fas up-regulation) with JNK1-APF alone caused a slight increase in apoptosis in response to agonistic Fas antibody, suggesting that JNK may actually play an anti-apoptotic role in RTC (Fig. 8A). RTC were then cotransfected with Fas cDNA at concentrations that mediate apoptosis in the presence or absence of JNK1-APF cDNA, at concentrations that inhibit Fas-dependent JNK activity. Figure 8B is a representative immunoblot showing apoptosis by PARP cleavage. The appearance of the 85 kD PARP degradation product in RTC with induced Fas expression is evidence of caspase-3 activation, which is a critical step in the Fas apoptosis pathway. Transfection with dominant negative JNK did not inhibit PARP cleavage, and had no effect on Fas-regulated apoptosis, as assessed by annexin V assays (Fig. 8C) and nuclear morphology by DAPI staining (not shown). Taken together, the data indicate that JNK is neither necessary nor sufficient for RTC Fas-dependent apoptosis.

DISCUSSION

Of the known apoptosis pathways, signaling through Fas has probably been the most comprehensively studied. Data regarding Fas biology has been generated primarily in lymphocytes and cell lines with abundant endogenous Fas expression. In RTC, we and others have previously shown that prior exposure of RTC to endotoxin, TNF, IL-1, or hypoxia increased cell surface Fas expression, which mediated apoptosis upon agonistic Fas antibody stimulation [6, 7, 9]. The current study strengthens these observations by demonstrating that RTC transfected with Fas cDNA, which is a method that minimizes unrelated effects of cytokines or hypoxia on cell viability and signal transduction, resulted in Fas-stimulated apoptosis only after a threshold level of expression was achieved. Untransfected RTC or RTC transfected with lower concentrations of murine Fas expression vector did not undergo apoptosis in response to agonistic Fas antibody stimulation, suggesting that RTC, which consti-

tutively express low levels of Fas, must be primed through up-regulated expression of Fas to function as a death receptor. Furthermore, our studies are consistent with the concept that increased Fas density enhances the probability that Fas ligand or agonistic Fas antibody binding promotes Fas oligomerization, which is required for apoptosis [12].

Previous studies from our laboratory indicate that Fas functions as a classical death receptor after RTC Fas expression is up-regulated. However, a role for constitutively expressed Fas has not been described, and we now tested whether constitutively expressed RTC Fas stimulates signaling. Fas-induced JNK activation was examined because JNK has been linked to cytokine-induced inflammation, and in some instances, shown to be dispensable for apoptosis following Fas stimulation [24, 25, 39]. Cell type specificity, JNK isoform specificity, or concomitant activation of modifying signaling pathways may determine if JNK activity is necessary for Fas-dependent apoptosis [40]. Our data demonstrate that RTC undergo apoptosis in the setting of up-regulated Fas expression [6, 7], but under these conditions, RTC Fas stimulation does not activate JNK. On the other hand, stimulation of low abundance Fas led to JNK activation in the absence of apoptosis. Based upon these observations, we propose that RTC Fas expression levels modulate the cellular response to Fas, and JNK activation is not necessary for RTC Fas-stimulated apoptosis. One possible explanation for the absence of JNK activity in RTC with induced Fas expression is that cells were apoptotic, and therefore not signaling. This is unlikely, however, since significant apoptosis does not result from only 15 minutes of exposure to agonistic Fas antibodies. Furthermore, robust Fas expression and decreased JNK activation were observed in the same lysate (Fig. 6), indicating that the protein synthesis machinery was intact. Another potential explanation for the disparate effects of JNK on cell death is derived from studies demonstrating that JNK activity may be regulated by more than one upstream pathway. In the setting of stress or inflammatory stimuli, membrane-bound MEKK1 is rapidly stimulated, which catalyzes SEK1 and JNK activation as a "stress response" [39, 41]. Alternatively, Fas-dependent JNK activation during apoptosis is initiated by docking of the adapter molecule FADD with activated Fas, which triggers caspase-8-mediated cleavage of MEKK1 at a plasma membrane site. Truncated MEKK1 then translocates to the cytosol and catalyzes SEK1 and JNK activation [35, 39]. In contrast to inflammatory stimuli, which rapidly activate JNK, the kinetics of JNK activation in apoptosis are relatively slow (1 to 6 hours) [25, 39]. Consistent with this "kinetic model," our data demonstrate early (15 min) but not late (14 hours, not shown) JNK activation following Fas stimulation, suggesting that RTC Fas may mediate stress responses or inflammatory pathways through JNK.

Fas may also direct JNK activity through binding of different docking molecules to selective cytoplasmic Fas domains. For example, Wajant et al have shown that overexpression of dominant negative FADD inhibits Fas-dependent apoptosis without affecting JNK activation [26], suggesting divergence of these two Fas functions and that the FADD binding domain is not required for docking of upstream JNK activators. Deletion studies by Chang, Yang and Baltimore identified amino acids 192–200 within the Fas cytoplasmic domain as necessary for FADD binding and apoptosis, but not JNK activation [42]. Although these studies have provided a better understanding of Fas signaling as it relates to apoptosis, the Fas domain(s) required for apoptosis-independent signaling remain unclear. Recent reports have generated controversy regarding whether agonistic Fas antibodies represent an adequate stimulus for apoptosis compared to multimerized Fas ligand in some cell types [43]. However, Fas clustering with pentameric anti-Fas IgM or anti-Fas IgG plus protein A have been shown to faithfully mimic effects of multimerized Fas ligand [44]. Since previous studies from our laboratory have shown that human RTC incubated with anti-Fas IgM [7] and mouse RTC incubated with anti-Fas IgG plus protein G (unpublished observation) do not undergo apoptosis, we conclude that absence of apoptosis in RTC under basal conditions is not due to inadequate stimulation of Fas, but more likely results from insufficient Fas clustering due to low expression levels.

Based on our results, we propose the following model for expression-dependent Fas functions in RTC. Under basal conditions, RTC are resistant to Fas-dependent apoptosis, despite constitutive, low level RTC Fas expression. Since RTC are not actively dividing in vivo, programmed or accidental death is therefore not advantageous. In contrast, RTC in an environment of chronic renal disease, with constant exposure to inflammatory cytokines and hypoxia, are induced to express Fas, and can undergo Fas-dependent apoptosis upon encountering Fas ligand-bearing lymphocytes or RTC [6]. Apoptosis of RTC may comprise an adaptive response, by promoting deletion of cells that are secreting pro-inflammatory cytokines or scar matrix proteins. Alternatively, RTC apoptosis may be a maladaptive process, by causing hypertrophy of remnant RTC, which has been associated with eventual tubular atrophy and renal disease progression. Our results demonstrating JNK activity in RTC with low level Fas expression raises intriguing questions regarding the role of Fas-dependent JNK activation in the transition of RTC from an apoptosis-resistant to apoptosis-sensitive phenotype. Although Fas-dependent JNK activation may merely serve an anti-apoptotic function, Fas-generated signals may also promote apoptosis-independent manifestations of progressive renal disease, such as inflammation and fibrosis [19–21]. We speculate that interrup-

tion of RTC Fas-dependent JNK activation may block propagation of tubular injury. Inhibition of RTC Fas upregulation, which contributes to tubular atrophy, may slow or abrogate the progression of chronic renal disease.

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APPENDIX

Abbreviations used in this study are: ATP, adenosine 5′-triphosphate; DAPI, 4′,6-diamidino-2-phenylindole; GFP, green fluorescence protein; HRPT, human renal proximal tubule; IL-1, interleukin-1; JNK, c-Jun NH_2-terminal kinase; PARP, poly(ADP-ribose) polymerase; PE, phycoerythrin; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidine difluoride; RTC, renal tubular epithelial cells; TNF, tumor necrosis factor; TUNEL, terminal deoxyribonucleotide transferase (TdT)-catalyzed DNA end labeling.

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