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TRAIL, DR4 and DR5 Are Upregulated in Kidneys from Patients with Lupus Nephritis and Exert Proliferative and Proinflammatory Effects

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Abstract

We have previously reported that TRAIL is upregulated on T cells from patients with lupus and that T-cell associated TRAIL enhances autoimmune parameters in a murine model of lupus. Whether TRAIL/TRAIL-R interaction plays a role in organ involvement such as lupus nephritis has not yet been assessed. We demonstrate here that TRAIL, DR4 and DR5 are upregulated in proximal and distal tubules of patients with proliferative lupus nephritis. In vitro, expression of TRAIL, DR4 and DR5 on primary proximal tubular epithelial cells (PTEC) was induced by TNF α and IFN γ . Functionally, TRAIL did not induce apoptosis but rather enhanced the proliferation of PTEC through activation of PI3 kinase/AKT and ERK1/2, increased IL-8 production and upregulated ICAM-1 expression. These data demonstrate that cytokine induced upregulation of TRAIL, DR4 and DR5 in tubules from patients with proliferative lupus nephritis may play a protective role by enhancing PTEC survival while also exerting a proinflammatory effect that may contribute to local inflammation and injury.

Keywords

Autoimmunity; Systemic lupus erythematosus; Lupus nephritis; TNF related apoptosis inducing ligand; Apoptosis

INTRODUCTION

Tumor necrosis factor apoptosis inducing ligand (TRAIL), a member of the TNF family, induces apoptosis in transformed and normal cells but also exerts mitogenic, immunoregulatory and proinflammatory functions [1–3]. TRAIL interacts with five different receptors [4]. Two agonistic receptors, TRAIL-R1/(DR4) and TRAIL-R2/(DR5) are capable of transmitting a death signal, while three other receptors TRAIL-R3 (DcR1), TRAIL-R4 (DcR2) and the soluble receptor, osteoprotegerin, act as decoy receptors blocking TRAIL induced apoptosis [5]. Like other members of the TNF superfamily, TRAIL has the dual capacity to induce cell death or promote cell survival and growth [6–8]. As a result of these opposing functions, TRAIL potentially may modulate or exacerbate autoimmune diseases.

In fact, such dual roles for TRAIL have been reported in rheumatoid arthritis and experimental autoimmune encephalomyelitis [9–12].

Recently, our group and others identified TRAIL as one of the IFN α -regulated genes detected in peripheral blood mononuclear cells from lupus patients and reported that active disease is associated with increase in T cell associated and soluble (s) TRAIL levels [13–15]. The role of TRAIL in systemic lupus erythematosus (SLE) pathogenesis is highly complex. Through effects on immune cells, it may attenuate disease by inducing apoptosis of activated autoreactive T and B cells or may contribute to disease exacerbation through sustained lymphocyte activation and proliferation [16–21]. However, given the wide tissue distribution of TRAIL and its signaling receptors, it is likely that TRAIL/TRAIL-R interactions could contribute to SLE pathogenesis through effects on non-immune cells as well.

Lupus glomerulonephritis (GN) is a key feature of SLE and one of the major prognostic factors. Immune complex deposition occurring predominantly in the glomerulus is accompanied by varying degrees of interstitial and tubular inflammation, atrophy and fibrosis. Constitutive expression of TRAIL and of its signaling receptors has been described in normal human kidneys and upregulation of TRAIL, DR4 and DR5 was reported in kidney transplant rejection [22]. However, the role of TRAIL/TRAIL-R interaction in normal kidney homeostasis or kidney disease pathogenesis has not yet been assessed. In this study we have investigated whether expression of TRAIL, DR4 and DR5 is altered in kidney biopsy samples of patients with proliferative lupus GN and characterized in vitro the role of TRAIL/TRAIL-R interaction on human primary proximal tubular epithelial cells (PTEC). Our data demonstrate increased expression of TRAIL, DR4 and DR5 in proximal and distal tubules in kidney biopsies from patients with proliferative lupus GN compared to non-SLE renal diseases. Our data also indicate that in vitro, expression of TRAIL, DR4 and DR5 is modulated by TNF α and IFN γ , key cytokines in the development of lupus GN. Interestingly, TRAIL does not induce apoptosis of PTEC, but rather induces a survival signal mediated through phosphatidylinositol 3-kinase (PI3K)/AKT and mitogen activated protein kinase (MAPK) ERK1/2 while also exerting a proinflammatory effect through upregulation of ICAM-1 and production of IL-8.

PATIENTS AND METHODS

Patients and controls

Renal biopsy specimens from twenty patients with SLE who fulfilled the ACR criteria for diagnosis [23] and had class III or IV lupus GN diagnosed according to the International Society of Nephrology/Renal Pathology Society (ISN/RPS) 2003 classification [24] were included in the study. As non-SLE controls, 9 patients with minimal change disease (n=1), IgA nephropathy (n=3), acute tubular necrosis (ATN) (n=3) and antineutrophil cytoplasmic antibody-positive pauci-immune (ANCA+) GN (n=2) were selected. All patients underwent renal biopsy within 3 months of the onset of proteinuria and none were treated with corticosteroids and/or immunosuppressive agents at the time of biopsy. SLE activity was assessed by the SELENA-SLEDAI [25]. Demographic and clinical data including disease activity, proteinuria and baseline medications at the time of biopsy are presented in Table 1.

Antibodies and reagents

FITC-anti-TRAIL-R1, -R2, -R3, -R4 (Axxora, San Diego, CA), PE-anti-human TRAIL and PE anti-ICAM-1 Ab (BD Pharmingen, San Diego, CA) were used for immunofluorescence staining. Purified recombinant human IFN α , IFN γ , TGF β and IL-1 were obtained from PeproTech Inc. (Rocky Hill, NJ). Recombinant human soluble TRAIL was obtained from

Biomol International (Plymouth Meeting, PA). Antibodies for phosphorylated AKT, ERK1/2, p38 kinase and JNK were purchased from Cell Signaling Technology (Danvers, MA). For immunohistochemistry studies primary anti-TRAIL mouse monoclonal Ab (BD Pharmingen), anti-TRAIL-R1/(DR4) goat polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-TRAIL R2/(DR5) rabbit polyclonal antibody (Calbiochem, La Jolla, CA) were used. HRP conjugated goat anti-mouse IgG, donkey anti-goat and goat anti-rabbit (Santa Cruz Biotechnology) were used as secondary Abs.

Cell culture, proliferation and apoptosis analysis

Primary PTEC from three separate donors and renal epithelial cell growth medium system were obtained from Lonza Inc. (Allendale, NJ). All experiments were performed on cells of the second–fifth passage that had been grown to subconfluence and growth factor arrested for 18h. For proliferation assays, PTEC were plated onto 96 well plates at 5×10^3 cells/well for 24h, changed to basal renal medium (starvation medium) then treated with rhTRAIL. At 72 h, PTEC proliferation was measured using CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI) following manufacturer's instructions and by [3 H]-Thymidine incorporation. One μ Ci was added to each well during the last 18h of incubation and [3 H]-Thymidine labeled-DNA was then measured by liquid scintillation counting. For apoptosis, PTEC were harvested by trypsin treatment and pooled with floating cells to analyze the degree of apoptosis in the entire cell population. Cells were then double stained for Annexin V and PI (BD Pharmingen) and analyzed by flow cytometry.

Flow cytometric analysis

Cultured PTEC were trypsinized lightly, washed with standard buffer (PBS, 2% BSA, 0.1% sodium azide) incubated for 30 min at 4° C with 1 μ g anti TRAIL–PE, anti TRAIL R1-4 - FITC and anti-ICAM-1 PE antibodies. Cells were washed three times with standard buffer, then fixed in 1% paraformaldehyde and analyzed in a FACScan flow cytometer (BD Biosciences, Mountain View, CA).

Immunohistochemistry studies

Paraffin sections of renal biopsies were deparaffinized and rehydrated. For DR5 and TRAIL a standard heat-induced epitope retrieval procedure (20 min, 98°C) using Target Retrieval Solution (DAKO, Carpinteria, CA) was performed. For DR4 no antigen retrieval was preformed. Endogenous peroxidases were blocked with 3% H₂O₂ in PBS for 5 min. Slides were incubated overnight with primary antibodies diluted 1/25 for TRAIL and 1:50 for DR4 and DR5. After washing in PBS, slides were incubated for 1h at room temperature with HRP conjugated goat anti-mouse IgG at 1:100 dilution for TRAIL, donkey anti-goat IgG-HRP at 1:250 dilution for DR4 and goat anti-rabbit absorbed IgG at 1:100 dilution. Specific deposits were developed using Nova Red (Vector Labs, Burlinghome, CA). Counterstaining was performed with Harris hematoxylin (Sigma, St. Louis) for 1min. Negative controls (no primary antibody) were performed on each section. All sections were reviewed by two examiners (CD and HR) in a blinded fashion. Staining intensity was graded on a scale of 0 (no staining) to 3+ (intense staining).

Soluble (s) TRAIL ELISA

The concentration of sTRAIL in cell-free supernatants of PTEC was measured using a commercial ELISA kit (Diaclone Research, Besancon, France) according to the manufacturer's instruction. The lower limit of detection of the assay is 64 pg/ml.

Western Blot

PTEC were cultured in renal epithelial cell growth medium, grown at subconfluence and subjected to complete growth factor withdrawal for 18 hours before the addition of TRAIL, TNF- α , IFN- γ , TGF- β or IL-1. Where indicated kinase inhibitors LY 294002 (10 μ M) and U0126 (10 μ M) were added 1h prior to TRAIL. Cells were harvested in RIPA lysis buffer (10mM Tris, pH 7.4, with 1mM EDTA, 1mM EGTA, 1mM NaF, 20mM Na₄P₂O₇, 1% Triton X-100, 0.1% SDS, 100mM NaCl, 10% glycerol, 0.5% sodium deoxycholate, 2mM Na₃VO₄). One tablet of complete mini protease inhibitor mixture (Roche Applied Science, Indianapolis, IN) was added just prior to use. Protein determination was performed by BCA assay (Pierce, Rockford, IL). Equal amounts of protein (50 μ g) for each sample were migrated in acrylamide gels and blotted onto nitrocellulose filters. Blotted filters were probed with primary antibodies for the phosphorylated AKT (1: 1000), ERK1/2 (1: 1000), p38 (1: 1000) or JNK (1:1000). After incubation with peroxidase-conjugated anti-rabbit (1:10000), anti-mouse (1:10000) or anti-goat IgG (1: 5000), specific reactions were revealed with the enhanced chemiluminescence Western blotting detection system. Membranes were stripped by incubation in Re-Blot 1X antibody stripping solution from PIERCE (Rockford, IL) and reprobed for the respective total protein kinase content or β -actin to verify loading evenness. The radiographic band density was measured using UN-SCAN-IT software (Silk Scientific, Orem, VT).

NF- κ B ELISA

Activation of NF- κ B p65 was determined from nuclear extracts of PTEC treated with TRAIL for 0, 30 min, 1, 2, 3 and 6h using a commercial ELISA kit (Panomics, Redwood City, CA) following the manufacturer's instruction.

Luminex Multiplex Assay

IL-8, RANTES, MCP-1 and IP-10 levels in cell free supernatants of PTEC were determined with the Luminex Multiplex Assay on a Luminex 100 instrument (Luminex Corp, Austin, TX) according to manufacturer's instructions.

Statistical analysis

The normality of the distribution was assessed for each variable. The difference in staining intensity of TRAIL, DR4 and DR5 between SLE and non-SLE specimens was compared with Student's unpaired t-test. Comparison of basal and cytokine stimulated concentrations of TRAIL and of basal and TRAIL induced apoptosis or proliferation was made with the Wilcoxon paired test. Spearman's rank correlation test was used to assess correlations of TRAIL, DR4 and DR5 staining intensity with activity and chronicity indexes, tubular atrophy and interstitial fibrosis. All statistical analysis were performed using Prism 4.0 software (Graphpad Software, San Diego, CA). P values below 0.05 were considered significant.

RESULTS

In situ analysis of TRAIL, DR4 and DR5 expression in lupus GN and non-SLE renal disease

Previous reports have established that TRAIL and its signaling receptors DR4 and DR5 are constitutively expressed at low levels in proximal and distal convoluted tubules in normal human kidneys [22, 26]. TRAIL expression on various cells is upregulated by cytokines such as type I and II IFNs and TNF α [14, 27–29]. As these cytokines play a critical role in the pathogenesis of lupus nephritis [30–32], we set to determine whether expression of TRAIL, DR4 and DR5 is altered in kidney biopsies from lupus patients. To this end we performed immunohistochemistry on biopsy sections from 20 specimens of Class III and IV

lupus GN. For comparison, specimens from 9 non-SLE patients with minimal change disease, IgA nephropathy, ATN and ANCA positive GN were studied. Consistent with the staining distribution reported in normal kidneys, TRAIL, DR4 and DR5 immunostaining was predominant in proximal and distal tubules and was detected in cell membranes, cytoplasm or both, in a focal or diffuse pattern (Figure 1A–B). Staining intensity was variable within the same sample or between specimens and was noted both in areas of normal appearing as well as damaged tubules. In non-SLE renal disease, the staining pattern for TRAIL, DR4 and DR5 was similar to that detected in lupus GN but with a lower intensity. A semiquantitative analysis of staining intensity (Figure 1J) demonstrated higher expression for TRAIL, DR4 and DR5 in lupus GN than in non-SLE renal diseases. The difference was statistically significant for DR4 and DR5 (2.4 ± 0.6 vs. 1 ± 0.8 ; $p=0.0002$ for DR4 and 2.2 ± 0.7 vs. 0.6 ± 0.7 ; $p=0.0001$ for DR5). We did not find a significant correlation between tubular expression of TRAIL, DR4 or DR5 and pathological features of lupus GN such as activity or chronicity index, tubular atrophy or interstitial fibrosis nor with clinical parameters such as disease activity, proteinuria, anti-dsDNA antibody or C3, C4 level. Interestingly, TRAIL staining was also noted in a few interstitial infiltrating cells (Figure 1A insert) and in glomerular endothelial cells in four biopsies from lupus patients (not shown). DR4 and DR5 expression was also detected in glomerular endothelial cells (Figure 1D and 1G insert) in three, respectively five of the lupus nephritis biopsies. Interestingly, DR5 expression in glomerular endothelial cells was associated with endocapillary hypercellularity but not with subendothelial hyaline deposits or fibrinoid necrosis. Together, these results demonstrate that TRAIL and its signaling receptors are upregulated in proximal and distal tubules in proliferative lupus GN.

Upregulation of TRAIL, DR4 and DR5 on PTEC by nephritogenic cytokines TNF α and IFN γ

To verify in vitro the expression of TRAIL, DR4 and DR5 and characterize their modulation by nephritogenic cytokines, we used primary human PTEC from normal controls cultured in the presence or absence of TNF α , IFN γ , IL-1 and TGF β . In concordance with the immunohistochemistry data, TRAIL was constitutively expressed by PTEC as demonstrated by Western blot analysis (Figure 2A), ELISA from cell lysates (Figure 2B) and flow cytometry (Figure 2D). In addition, the soluble form of TRAIL was released in supernatants of PTEC in low amounts at baseline (Figure 2C). Among cytokines, TNF α upregulated TRAIL expression by approximately 2 fold by Western blot analysis and significantly increased TRAIL concentrations in cell lysates, while IFN γ increased TRAIL expression to a lesser extent. In addition, sTRAIL production increased from 435 ± 210 pg/ml in media alone to 2061 ± 396 and 2005 ± 260 pg/ml when incubated with TNF α and IFN γ , respectively ($p<0.05$ compared to media). By flow cytometry, surface expression of TRAIL increased from $6.25 \pm 2\%$ positive cells at baseline to $25.9 \pm 3\%$ (4.1 fold) in the presence of TNF α and to $34.5 \pm 8\%$ (5.5 fold) in the presence of IFN γ . IL-1 and TGF β had no effect on TRAIL expression (Figure 2A–C).

We determined next whether the expression of TRAIL receptors is also modulated by TNF α and IFN γ . By immunofluorescent staining, both DR4 and DR5 were expressed at baseline on $6.8 \pm 1.4\%$ and $23 \pm 5.3\%$ of primary PTEC, respectively (Figure 2D). Incubation with TNF α increased the surface expression of DR5 by 2 fold to $53 \pm 8.2\%$ while DR4 expression was only slightly increased to $9.6 \pm 3.2\%$ of the cells. In contrast, IFN γ downregulated the expression of both DR4 and DR5. We did not detect surface expression of DcR1 and DcR2 at baseline or after TNF α or IFN γ exposure.

Taken together, these results demonstrate that TRAIL, DR4 and DR5 are coexpressed on PTEC and are upregulated in vitro, primarily by TNF α . This pattern of expression suggests that, when upregulated by nephritogenic cytokines, a functional interaction between TRAIL and its receptors is likely to occur in tubular cells.

TRAIL does not induce apoptosis in human PTEC cells but enhances their proliferation

Cell associated or sTRAIL may affect cells through either apoptotic or nonapoptotic pathways. To determine whether TRAIL/TRAIL-R interaction induces apoptosis, we cultured primary PTEC with concentrations of TRAIL known to induce apoptosis in TRAIL sensitive Jurkat cells. Apoptosis was measured by Annexin V staining. In agreement with previously reported data [33], at concentrations of 0.1, 1 or 10 ng/ml, TRAIL was able to induce apoptosis in Jurkat cells but not in PTEC (Figure 3A). Similar results were obtained by active caspase-3 intracellular staining (not shown). One of the mechanisms of resistance to TRAIL mediated apoptosis is low expression level of DR4 and DR5. To determine whether upregulation of DR4 and DR5 by TNF α sensitizes PTEC to TRAIL, we assessed the ability of TRAIL to induce apoptosis of PTEC pretreated with TNF α . As seen in Figure 3B, pretreatment of PTEC with TNF α for 24 hours did not alter the resistance of PTEC to TRAIL-mediated apoptosis.

On cells resistant to apoptosis, TRAIL can enhance cell survival and proliferation or promote inflammation [3, 34]. To determine whether TRAIL exerts such nonapoptotic effects, we initially assessed the proliferation of primary PTEC in the presence of TRAIL using two methods: the colorimetric CellTiter AQueous assay and [3 H]-Thymidine incorporation. As seen in Figure 3C and D, TRAIL enhanced the proliferation of PTEC compared to baseline in a dose dependent fashion. The difference was statistically significant at the higher doses of 1 and 10 ng/ml.

TRAIL activates AKT and ERK1/2 but not p38 kinase, JNK or NF-kB

To investigate the intracellular mechanisms involved in TRAIL-induced proliferation we tested the effect of TRAIL on signaling pathways that control cell proliferation, specifically PI3K/AKT, MAP kinases ERK1/2, p38 kinase and JNK. PTEC were treated with TRAIL and subsequently analyzed by Western blotting. As shown in Figure 4A, phosphorylation of AKT was rapidly induced by TRAIL and persisted up to 60 min. Treatment with TRAIL also induced activation of ERK1/2 phosphorylation in a time-dependent manner, with maximum activation occurring between 5 and 10 minutes (Figure 4B). In contrast, p38 kinase activation was minimal and JNK was not activated (Figure 5A and B)

To confirm that ERK1/2 and PI3K/AKT play a role in TRAIL-induced proliferation, we assessed whether inhibiting PI3K/AKT and ERK1/2 with specific pharmacological inhibitors affects TRAIL induced proliferation. PTEC were pretreated with LY294002 (PI3K inhibitor) and UO126 (MEK1/2 inhibitor) for 1h and then stimulated with TRAIL. Inhibition of PI3K and ERK1/2 pathways significantly diminished TRAIL-induced proliferation of PTEC to baseline levels (Figure 5C and D).

The transcription factor NF-kB plays a major role in cell survival. Therefore we assessed the activation of the p65 subunit in nuclear extracts from PTEC treated with TRAIL. TNF α was used as positive control. As expected, TNF α induced significant activation of NF-kB that peaked at 6h (2.3 fold increase). In contrast, TRAIL had no effect on NF-kB activation. These data suggest that the TRAIL/TRAIL-R interaction on PTEC induces their proliferation through activation of the PI3K/AKT and ERK1/2 pathway.

TRAIL induces IL-8 production and ICAM-1 upregulation by PTEC

In certain cells, TRAIL exerts proinflammatory activities through increased production of proinflammatory cytokines and upregulation of adhesion molecules [3]. To determine whether TRAIL/TRAIL-R interaction affects the production of chemokines and expression of adhesion molecules by PTEC, we assessed the production of IL-8, MCP-1, RANTES and IP-10 by PTEC as well as surface expression of ICAM-1 in response to TRAIL. Using a

multiplex cytokine assay, we detected a significant increase in IL-8 production in supernatants of PTEC cultured in the presence of TRAIL (42 ± 9.2 ng/ml) compared to media alone (22 ± 4 ; $p < 0.05$). This response was less robust than that induced by TNF α , a strong proinflammatory cytokine (Figure 6A). We did not observe increased production of the other chemokines tested, i.e. MCP-1, RANTES or IP-10 (not shown). In addition, the surface expression of ICAM-1 on PTEC (Figure 6B) increased markedly both as percentage positive cells ($54\% \pm 9$ in the presence of TRAIL vs. $36\% \pm 6$ in media; $p < 0.05$) and as mean fluorescence intensity (1.9 fold increase). These results suggest that TRAIL/TRAIL-R interaction on PTEC may also exert a proinflammatory role by increasing IL-8 production and expression of ICAM-1.

DISCUSSION

This study provides the first evidence that TRAIL and its signaling receptors DR4 and DR5 are upregulated in kidney biopsy specimens from lupus patients with proliferative GN. Consistent with previous descriptions of staining distribution in normal kidneys [22, 26], our immunohistochemistry analysis indicate that TRAIL, DR4 and DR5 are mainly expressed in proximal and distal tubules of patients with proliferative lupus GN as well as other non-SLE renal disease. Importantly, DR4 and DR5 expression is significantly higher in proliferative lupus GN compared to non-SLE renal disease. However, the upregulation of TRAIL, DR4 and DR5 in renal tubular epithelial cells is not specific for lupus GN and was also reported in patients with renal transplant rejection [22]. We have confirmed the immunohistochemistry data by *in vitro* studies using primary human PTEC. Our data indicate that under basal conditions, TRAIL, DR4 and DR5 are constitutively expressed at low levels on primary human PTEC. Furthermore, nephritogenic cytokines such as TNF α upregulates the expression of both TRAIL and its signaling receptors, while IFN γ modestly upregulates TRAIL expression alone. These data support the idea that the upregulation of TRAIL and its receptors in proliferative lupus GN is triggered by the proinflammatory cytokines produced *in vivo* by infiltrating cells at sites of inflammation or by intrinsic resident cells. Intrarenal production of IFN γ and TNF α by inflammatory cells infiltrating the glomeruli and interstitium and by intrinsic renal cells is well documented in lupus nephritis [31, 32, 35, 36].

We then determined the functional significance of TRAIL/TRAIL-R interaction on PTEC. TRAIL/TRAIL-R signaling on a variety of cells induces various responses such as apoptosis, survival/proliferation and inflammation [2, 3, 8]. As a result of these opposing functions, TRAIL could be involved in renal injury by promoting parenchymal cell death and local inflammation or could participate in renal repair mechanisms by promoting inflammatory cell death and by generating survival signals for resident cells. We first considered the possibility that TRAIL might induce apoptosis of PTEC. Consistent with other reports [33, 37, 38], our data demonstrate that normal human PTEC are not sensitive to TRAIL induced apoptosis, suggesting that TRAIL/TRAIL-R interaction, unlike Fas-FasL interaction on PTEC, is not involved in the development of tubular atrophy in lupus GN. This finding is further supported by the lack of correlation between TRAIL, DR4 and DR5 staining intensity and tubular atrophy.

Multiple mechanisms could account for the resistance of PTEC to TRAIL mediated apoptosis. One possible mechanism entails variations in the expression level of the death signaling receptors DR4 and DR5 and/or of the inhibitory decoy receptors, dcR1 and dcR2. Our data demonstrate that the surface density of the death signaling or decoy receptors did not play a role in the resistance of PTEC to TRAIL induced apoptosis. Specifically, TRAIL could not induce apoptosis of PTEC regardless of whether DR4 and DR5 were expressed at low constitutive levels or were upregulated by preincubation with TNF α . These results are

in contrast to those reported for Fas/FasL, which at low levels of expression was not apoptotic but induced PTEC death when upregulated [39]. In addition, inhibition of apoptosis was not mediated by upregulation of inhibitory decoy receptors, as neither DcR1 nor DcR2 were detectable on PTEC at baseline or after incubation with cytokines.

Other mechanisms involved in resistance to TRAIL induced apoptosis include the altered expression levels of inhibitory intracellular proteins that either block the apoptotic signaling or activate a proliferative signaling pathway. A major finding in our study is that TRAIL/TRAIL-R interaction enhanced the proliferation of PTEC. This observation suggests that TRAIL/TRAIL-R interaction may mediate an autocrine survival pathway that maintains PTEC turnover. However, given the low basal expression of TRAIL, DR4 and DR5 and the absence of renal defects in TRAIL KO or DR5 KO mice, it is unlikely that TRAIL mediated survival plays a major role in normal kidney homeostasis. In contrast, in lupus nephritis and other nephropathies associated with upregulation of TRAIL, DR4 and DR5, TRAIL/TRAIL-R interaction may represent an essential survival mechanisms for cells with a low basal turnover and constant threat of apoptosis.

We further characterized the mechanisms involved in TRAIL induced PTEC proliferation. As previously reported, TRAIL can enhance the survival and proliferation of different cells through the activation of MAPKs as well as PI3K/AKT and NF- κ B [2, 8, 40–44]. In this study we demonstrate that TRAIL promotes PTEC proliferation by activating the AKT and ERK1/2 pathway. Other MAPKs, specifically p38 and JNK, were not activated in response to TRAIL. The inhibition of ERK1/2 and AKT abrogated TRAIL-mediated PTEC proliferation, confirming that both pathways are essential for their survival/proliferation. AKT phosphorylation was dependent on the activation of PI3K as demonstrated by the selective inhibitor LY294002. These results suggest that in PTEC, TRAIL mediated AKT and ERK1/2 activation exerts a rapid effect that does not require protein synthesis, which protects against apoptotic signaling from death receptors. Similar to results reported in other cells, we could not detect activation of NF- κ B in response to TRAIL suggesting that TRAIL promotes PTEC survival through an NF- κ B independent mechanism.

Interstitial inflammation is a major determinant of outcome in renal diseases [45]. Upregulation of chemokines and adhesion molecules by PTEC may be involved in renal infiltration by lymphocytes, monocytes and granulocytes, thus amplifying inflammatory responses [46]. We investigated the influence of TRAIL on markers of inflammatory activation of PTEC such as IL-8, MCP-1, RANTES, IP-10 and surface expression of ICAM-1. Our results indicate that TRAIL induced increased production of IL-8 and upregulated ICAM-1 expression on PTEC. The value of ICAM-1 expression on tubular cells as a poor prognostic indicator for lupus nephritis has been reported [45]. In view of previous reports indicating a proliferative effect of TRAIL on endothelial cells, the detection of TRAIL, DR4 and DR5 in glomerular endothelial cells in association with endocapillary hypercellularity is not surprising and points toward a potential role for TRAIL in glomerular response to immune mediated injury [8]. The full significance of glomerular expression of TRAIL and its receptors is under investigation.

In conclusion, we provide evidence that proximal and distal tubules are the site of robust expression of TRAIL, DR4 and DR5 in proliferative lupus GN. Furthermore, cultured PTEC upregulate TRAIL, DR4 and DR5 in the presence of nephritogenic cytokines TNF α and IFN γ . Finally, TRAIL/TRAIL-R interaction on PTEC plays an important role in PTEC survival while at the same time exerting proinflammatory activities.

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Figure 1. TRAIL, DR4 and DR5 expression in kidney biopsies from patients with proliferative lupus nephritis

In situ expression of TRAIL (A–C), DR4 (D–F) and DR5 (G–I) was determined by indirect immunoperoxidase on archived paraffin kidney biopsy specimens from 20 patients with class III and IV lupus GN (A, D, G) and 9 non-lupus renal disease (minimal change disease in B and E and ATN in H). Immunostaining is seen as the reddish-brown deposits in proximal and distal tubules. TRAIL expression in infiltrating mononuclear interstitial cell (panel A insert arrow) and DR4 and DR5 staining in glomerular endothelial cells (panel D and G inserts). Negative staining controls were performed without the addition of the primary antibody (C, F, I). Magnification $\times 100$. J. Mean TRAIL, DR4 and DR5 tubular

staining intensity in SLE and non-SLE renal diseases. Staining intensity was graded on a scale of 0 (no staining) to 3+ (intense staining). Data represent mean $\pm$ SE. * = $p < 0.05$.

Figure 2. TRAIL and TRAIL receptors expression in human PTEC

A–C. Monolayers of primary PTEC grown to subconfluency were cultured in the presence or absence of TNF α (30ng/ml), IFN γ (300U/ml), IL-1 (20 ng/ml) or TGF β (2ng/ml). After 48h the expression of TRAIL was determined in cell lysates (A and B) and supernatants (C) by Western blot and ELISA, respectively. Values represent mean plus SD of triplicate samples. * = $p < 0.05$ compared to media. D. Representative histogram of TRAIL, DR4, DR5, DcR1 and DcR2 surface expression on PTEC cultured in the presence or absence of TNF α or IFN γ determined by flow cytometry and expressed as % positive cells. Control antibody is shown (thin line). Values represent mean percentage $\pm$ SE of three different donors.

Figure 3. Recombinant TRAIL induces proliferation but not apoptosis of PTEC

A. Primary PTEC were incubated with 0.1, 1 or 10 ng/ml of TRAIL for 18h and apoptotic cells were measured by Annexin V staining. Jurkat cells, which are sensitive to TRAIL-mediated apoptosis, were used as positive controls. B. PTEC were preincubated for 24h with or without TNF α (30 ng/ml) then incubated for 18h with 0.1, 1 or 10 ng/ml TRAIL. C and D. PTEC were incubated with 0.1, 1 or 10 ng/ml TRAIL and proliferation was determined by CellTiter AQueous assay (Promega) and by [3 H]-Thymidine incorporation. Results represent means $\pm$ SE of three separate experiments. * = $p < 0.05$; ** = $p < 0.005$ versus media.

Figure 4. Activation of AKT and ERK1/2 in response to TRAIL

PTEC cells were stimulated with 2 ng/ml TRAIL for 0–60 minutes. Cell lysates were analyzed for activation of AKT (A) and ERK1/2 (B) by Western blot analysis of total and phosphorylated (P)-AKT and -ERK1/2 using specific antibodies. Protein bands were quantified by densitometry and levels of P-AKT and P-ERK1/2 were calculated for each time point after normalization to AKT and ERK1/2 in the same sample. Unstimulated basal expression was set as unity. C and D. PTEC cells were preincubated for 1 hour with PI3K inhibitor LY 294002 (C), MEK1/2 inhibitor UO126 (D) or vehicle then treated with TRAIL for 72h. Cell proliferation was assessed by [³H]-Thymidine incorporation. Results represent means ± SE of three separate experiments.

Figure 5. Lack of p38 kinase, JNK and NF-kB activation in PTEC in response to TRAIL
PTEC were stimulated with 2 ng/ml TRAIL for 0–60 minutes. Cell lysates were analyzed for activation of p38 kinase (A) and JNK (B) by Western blot analysis of total and phosphorylated (P)-p38 and -JNK using specific antibodies. Protein bands were quantified by densitometry and levels of P-p38 and P-JNK were calculated for each time point after normalization to p-38 and JNK in the same sample. Unstimulated basal expression was set as unity. C. Activation of NF-kB p65 was measured in nuclear extracts of TRAIL treated PTEC cells by ELISA. TNF α stimulation was used as positive control. Results represent means $\pm$ SE of three separate experiments.

Figure 6. TRAIL induces IL-8 production and ICAM-1 upregulation by PTEC
PTEC were stimulated with 2 ng/ml TRAIL for 48h. (A). IL-8 was measured in supernatant by Luminex assay. Results represent means $\pm$ SE of triplicate values and are representative of 3 separate experiments. * = $p < 0.05$; ** = $p < 0.005$ versus media. (B). Surface expression of ICAM-1 was determined by flow cytometry. The filled curve represents the isotype control.

Table 1

Demographic characteristics, clinical features and medications in the studied subjects

Characteristic	SLE patients (n=20)	Controls (n=9)
No female/no male	17/3	5/4
Age mean (range) years	28 (14–49)	44 (15–60)
Renal disease		
Class III Lupus GN	9	
Class IV Lupus GN	11	
ATN		3
IgA nephropathy		3
ANCA GN		2
Minimal change		1
SLEDAI Score	8 (4–16)	
Proteinuria mean ^{\$} (range)	2.9 (1.4–4.8)	1.2 (0–2.5)
Treatment		
None	2	12
Prednison *	2	0
Hydroxychloroquine	16	0
Immunosuppressors	0	0

^{\$} g/day;

* = less than 20 mg/day