The Death Ligand TRAIL in Diabetic Nephropathy

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ABSTRACT

Apoptotic cell death contributes to diabetic nephropathy (DN), but its role is not well understood. The tubulointerstitium from DN biopsy specimens was microdissected, and expression profiles of genes related to apoptosis were analyzed. A total of 112 (25%) of 455 cell death–related genes were found to be significantly differentially regulated. Among those that showed the greatest changes in regulation were two death receptors, OPG (the gene encoding osteoprotegerin) and Fas, and the death ligand TRAIL. Glomerular and proximal tubular TRAIL expression, assessed by immunohistochemistry, was higher in DN kidneys than controls and was associated with clinical and histologic severity of disease. In vitro, proinflammatory cytokines but not glucose alone regulated TRAIL expression in the human proximal tubular cell line HK-2. TRAIL induced tubular cell apoptosis in a dosage-dependant manner, an effect that was more marked in the presence of high levels of glucose and proinflammatory cytokines. TRAIL also activated NF-κB, and inhibition of NF-κB sensitized cells to TRAIL-induced apoptosis. It is proposed that TRAIL-induced cell death could play an important role in the progression of human DN.


Diabetic nephropathy (DN) is one of the major complications of diabetes and the most common cause of ESRD. Cell death by apoptosis might contribute to the gradual loss of renal mass in DN. In fact, apoptosis has been detected in tubular epithelial, endothelial, and interstitial cells of renal biopsy samples from patients with DN.1,2

Cell death via apoptosis is an active response of cells to altered microenvironments and is characterized by the activation of specific intracellular pathways. Hyperglycemia is among the microenvironmental factors that may induce or facilitate apoptosis. A high glucose concentration, per se, promotes apoptosis in a variety of cell types, including renal tubular epithelium.3–5 Lethal cytokines from the TNF superfamily activate death receptors on the cell surface with subsequent activation of caspases, central activators, and effectors of apoptosis.6 The apoptotic process is modulated by a host of checks and balances with a multitude of positive and negative regulators.6

To display the apoptosis network that is active in human DN in a comprehensive manner, we have studied the molecular mechanisms underlying tubulointerstitial apoptosis in the course of DN by monitoring changes in the renal transcriptome of the diabetic kidney. The combination of unbiased

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gene expression profiling with focused data mining has proved to be a powerful tool to expand our knowledge of relevant pathways and players in human disease.6 Applying gene ontology category “cell death” followed by pathway mapping to the genome-wide data sets defined OPG and TRAIL as key nodes regulated in the transcriptomic profile. TNF-related apoptosis–inducing ligand (TRAIL/APO2L/TNFSF10)8 is an atypical member of the TNF gene superfamily because it is capable of binding a complex system of receptors. Two of them, TRAIL-R1 (DR4/TNFRSF10A) and TRAIL-R2 (DR5/TRICK2/KILLER/TNFSFR10B), contain a cytoplasmic death domain and transmit an apoptotic signal in response to TRAIL.9 Two others, TRAIL-R3 (TRID/DcR1/TNFRSF10C) and TRAIL-R4 (DcR2/TNFRSF10D), bind TRAIL without activation of the apoptotic machinery and seem to antagonize the death domain–containing TRAIL receptors.10 In addition, osteoprotegerin (OPG/TNFRSF11B), a regulator of osteoclastogenesis that binds to the TNF superfamily member RANKL,11 is a soluble decoy receptor for TRAIL.12

We have found that TRAIL expression is altered in the DN kidney. Tubulointerstitial TRAIL expression is increased in both mRNA and protein in DN samples. The functional relevance of these changes in TRAIL expression has been studied using cultured renal tubular cells that express all relevant elements of the TRAIL pathway. Because we identified a functionally significant activation of the NF-κB pathway in DN in a parallel study,7 we evaluated the interplay of the TRAIL pathway, NF-κB, and cell death in tubular cells. TRAIL was found to activate NF-κB, and inhibition of NF-κB activation sensitized cells to TRAIL–induced apoptosis.

RESULTS

Cell Death Genes Are Differentially Expressed in DN

DN is a complex disease, and multiple mechanisms intervene in the development of kidney damage. We focused our analysis on the mechanisms leading to tubulointerstitial cell death, because they are likely to contribute to the loss of renal function.1,13 We performed oligonucleotide arrays using mRNA isolated from microdissected tubulointerstitium from human DN tissue (n = 13), and control kidneys (n = 3) and focused our analysis on apoptosis-associated genes as defined by the GO category “cell death” (GO ID: 0008219).

First we defined the set of differentially regulated mRNA in DN. Of the 455 cell death genes (according to ENTREZ_GENE_ID),14 112 were differentially expressed (46 downregulated, 66 upregulated) using a P < 0.05 (Supplementary Table 1). Next, we defined the association of cell death gene expression fingerprints with the clinical status of the patients. The expression profiles of the 112 regulated mRNA were used to group the patient samples according to the their apoptosis gene expression by unsupervised hierarchical clustering.15 The resulting dendrogram reflects the relationship among the samples (Figure 1). Gene expression is conserved within the three control samples. DN samples 1, 2, 7, 8, 11, and 15 cluster close to controls.

To focus our analysis further, we identified genes with major differential regulation (fold change >1.5) and therefore most likely associated with significant functional alterations (Table 1). OPG and TRAIL were the two genes with maximal expression differences, and a co-citation analysis displays TRAIL as a central link between key apoptosis pathways activated in DN (Supplementary Figure 1). The upregulation of TRAIL and OPG mRNA in DN was confirmed by real-time quantitative reverse transcription–PCR (qRT-PCR) in all samples (Figure 2). An association between transcriptional regula-
TRAIL and OPG mRNA and clinical and histologic severity of human DN could be detected (Figure 2).

**TRAIL Protein Expression Is Increased in DN**

Transcriptional alterations reach functional significance when translated into parallel protein expression changes in a relevant tissue compartment. Immunohistochemical studies performed in an independent set of samples showed that TRAIL displays a low level of expression in a few distal tubular sections in control kidneys. Glomeruli were negative for TRAIL staining (Figure 3, A and B; Table 2). In contrast to control kidney, glomerular staining for TRAIL was present in 13 of the 17 independent DN cases studied (Table 2, Figure 3C).

**TRAIL expression in proximal tubules was increased in DN** (Figure 3D, Table 2). Renal biopsies with high proximal tubular TRAIL expression displayed more severe tubular atrophy, interstitial fibrosis, and interstitial inflammation (Figure 3E).

**Table 1.** Cell death genes that show at least a 1.5-fold dysregulation (up- or downregulation) in the DN samples compared with control tissue

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<th>Gene Symbol</th>
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**Figure 2.** TRAIL and OPG array expression correlate with severity of human DN. The panels show the correlation between TRAIL and OPG array expression and diverse parameters of severity of DN. Top and bottom left panels show the correlation between TRAIL and OPG gene expression in arrays and real-time qRT-PCR. TRAIL and OPG array mRNA data are expressed as Log2 signal intensity values. Linear regression was used to perform the best fit lines.
We studied factors that could account for the increased TRAIL expression observed in the tubulointerstitium of diabetic kidneys. A high glucose environment did not regulate TRAIL expression for up to 72 h of culture (Figure 4C). By contrast, cytokines present in the kidney during DN, such as TNF-α and IFN-γ, increased TRAIL expression (Figure 4D). Thus, tubular TRAIL expression is regulated by proinflammatory cytokines.

**High Glucose Sensitizes Cells to TRAIL-Induced Cell Death**

Next, the functional role of the TRAIL system was evaluated. Tubular epithelial cells express more than one receptor for TRAIL. Thus, it is difficult to predict the effect of TRAIL on these cells. TRAIL weakly induced tubular cell apoptosis in a dosage-dependent manner. This effect was more marked in the presence of high glucose (Figure 5A). The combination of TRAIL and the proinflammatory cytokines TNF-α and IFN-γ in a high glucose microenvironment significantly increased apoptosis over cells grown in basal culture conditions or in the absence of TRAIL (Figure 5, B and C; Supplementary Figure 2).

**TRAIL Induces Activation of NF-κB in Tubular Epithelial Cells**

Like other TNF receptor family members, TRAIL-R1, -R2, or -R4 can also activate the transcription factor NF-κB upon ligand binding. We found that TRAIL increases NF-κB binding (Figure 6A) and transcriptional (Figure 7A) activity in tubular epithelial cells.

To define the functional interdependence of TRAIL and NF-κB pathways for apoptosis induction, we used parthenolide, a sesquiterpene lactone that inhibits NF-κB activation by preventing the degradation of IκBα. Inhibition of NF-κB in the presence of TRAIL resulted in sustained caspase-3 activation (Figure 6B) and increased apoptosis of tubular cells in response to TRAIL (Figure 6C).

**OPG Modulates TRAIL-Induced NF-κB Activation and Tubular Cell Survival In Vitro**

Because OPG expression is increased in the tubulointerstitium of diabetic kidneys, we explored the effects of this soluble TRAIL receptor on tubular epithelial cells. Addition of exogenous OPG or blocking endogenous OPG by means of anti-OPG blocking antibodies did not influence tubular cell death or proliferation in the absence of TRAIL (Figure 7B and data not shown); however, OPG interfered with both documented cell responses to TRAIL—that is, activation of NF-κB and induction of cell death (Figure 7). Anti-OPG blocking antibodies increased NF-κB activation in response to both endogenous and exogenous TRAIL (Figure 7A). It is unlikely that this effect was mediated by OPG potential to antagonize RANKL, because RANKL was not found to be expressed in tubular epithelial cells in culture (data not shown) and exogenous RANKL was unable to induce NF-κB activation on these cells (Figure 7A).

OPG prevented the mild decrease in cell survival induced by TRAIL alone, as well as the more severe loss of survival induced by TRAIL when NF-κB activation was blocked by parthenolide (Figure 7B). The protective action of OPG was related to inhibition of apoptosis as assessed by flow cytometry (control 2.1 ± 0.1,
Table 2. Histologic characteristics of the biopsies analyzed for TRAIL immunostaining

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*Histologic parameters of tubular injury (columns 2 through 4): 0, absent; 1, involving <30% tubular cross-sections; 2, 30 to 60% tubular cross-sections. Semiquantitative evaluation of glomerular TRAIL staining (column 5): 0, absent; 1, involving <5 cells/glomerular cross-section; 2, involving >5 cells/glomerular cross-section. Semiquantitative evaluation of tubular TRAIL staining (columns 6 and 7): 0, absent; 1, involving <30% tubular cross-sections; 2, 30 to 60% tubular cross-sections.

OPG 2.4 ± 0.1, TRAIL + parthenolide 39.4 ± 2.8, TRAIL + parthenolide + OPG 12.4 ± 3.2%; apoptosis at 24 h; *P 0.001 versus TRAIL + parthenolide). These observations are consistent with a role for OPG in antagonizing TRAIL actions on tubular cells as summarized in Figure 7, C through F.

DISCUSSION

Recent studies suggested a role for cell death in the progression of human DN.1,21 We have taken advantage of the microarray technology to study cell death genes in human DN kidney biopsies, comparing their gene expression profile with control kidneys. We found differential regulation of several apoptosis-related genes in the tubulointerstitium of human DN samples, with TRAIL and OPG showing the highest degree of regulation, and defined their action in tubular cells.

Twenty-five percent of apoptosis-related genes were differentially regulated in renal biopsies from DN patients. Differentially expressed apoptosis-related genes include genes encoding proteins involved in death receptor interactions, such as Fas, TRAIL, and OPG. Renal cells are susceptible to Fas-induced cell death both in vitro22,23 and in vivo,24,25 and Fas upregulation has already been described in a variety of renal pathologies, including DN.26,27 By contrast, there is little information on the role of TRAIL in renal disease. Current studies on TRAIL and diabetes emphasize TRAIL’s role in immune response regulation.28,29 Thus, its relationship to kidney injury in DN is novel. In contrast to the scarce information on TRAIL in kidney disease, there is ample evidence that OPG is increased in serum of patients with renal dysfunction,30–32

Figure 4. Tubular epithelial TRAIL expression is regulated by proinflammatory cytokines. (A) Expression of TRAIL and OPG in HK-2 cells assessed by Western blot. (B) Semiquantitative RT-PCR. Expression plasmids for TRAIL-R1, -R2, and -R3 were used as controls. RT-PCR for TRAIL-R2 yielded two bands corresponding to its two transcript variants. (C and D) TRAIL protein expression in HK-2 cells. Cells were cultured in 5.5 or 25 mM glucose (C) or in 11 mM glucose and treated with a combination of cytokines (TNF-α and IFN-γ; D). Data are means ± SEM of three independent experiments. *P < 0.05. ADU, arbitrary densitometry units.
including DN. \textsuperscript{33,34} Increased OPG serum levels are also associated with increased coronary artery and aorta calcification.\textsuperscript{30,35}

Two different mRNA studies showed that TRAIL mRNA was upregulated in the tubulointerstitium of patients with DN. These data were validated at the protein level in an independent cohort of control and DN samples different from those used for the mRNA studies. Consistent with previous studies,\textsuperscript{36} we found that TRAIL is expressed in normal human kidney, where distal tubular cells are the main source of this cytokine. In DN, high TRAIL expression in proximal tubular epithelium was related to increased severity of tubulointerstitial injury.

Human TRAIL-R1 and TRAIL-R2 are expressed in almost all tissues. In human kidney, TRAIL-R1 and TRAIL-R2 are the main TRAIL receptors and are located in proximal convoluted tubules but not in normal glomeruli.\textsuperscript{36} Cultured tubular cells expressed TRAIL-R1, -R2, -R3, and -R4. Although the name TRAIL initially derives from its capacity to induce apoptosis,\textsuperscript{8} such a widespread distribution of its receptors argues against induction of normal parenchymal cell apoptosis being its main role in normal tissue physiology. In fact, depending on the cellular system, TRAIL can exert different functions that also

**Figure 5.** High glucose sensitizes cells to TRAIL-induced cell death. (A) Apoptosis induction. Dose-response at 24 h. ■ Cells cultured in 5.5 mM glucose; □, cells cultured in 25 mM glucose. \( *P < 0.05 \) versus absence of TRAIL 5.5 mM glucose; **\( P < 0.05 \) versus absence of TRAIL 25 mM glucose. Note the difference in the scale from B. (B) Apoptosis of HK2-cells treated with TRAIL 10 ng/ml and cytokines (TNF-\( \alpha \) and IFN-\( \gamma \)) for 24 h. ■ Cells cultured in 5.5 mM glucose; □, cells cultured in 25 mM glucose. Quantification of apoptosis by flow cytometry of DNA content. Experiments were performed five times, and each experiment consisted of triplicates. \( *P < 0.05 \). (C) Representative flow cytometry diagrams of cells cultured in 25 mM glucose. The line encompasses hypodiploid cells.

**Figure 6.** TRAIL induces activation of NF-\( \kappa B \) in tubular epithelial cells. (A) NF-\( \kappa B \) DNA binding is detected by electrophoretic mobility shift assay after TRAIL stimulation (10 ng/ml). (B) Western blot. Parthenolide (P) induces transient accumulation of I\( \kappa B \alpha \) and prevents TRAIL-induced I\( \kappa B \alpha \) degradation. The fragment corresponding to activated caspase-3 can be detected in cells treated with parthenolide and TRAIL. B, basal. (C) Parthenolide (P) enhances TRAIL-induced apoptosis. Apoptotic cells quantified by flow cytometry of cell DNA content. The pictures show apoptotic nuclei (white arrows) present in permeabilized, propidium iodide–stained cells treated with parthenolide and TRAIL for 24 h. Data are means \( \pm \) SEM of five independent experiments. \( *P < 0.05 \) versus other groups. Magnification, \( \times 40 \).
include survival, proliferation, and maturation. That tubular epithelial cells constitutively express low levels of TRAIL suggests that it may have a role in normal kidney physiology. Physiologic functions of TRAIL may include a role in tumor surveillance and immunologic responses; however, increased levels of TRAIL may be involved in pathology. In this context, TRAIL weakly induced death in tubular epithelial cells in a dosage-dependent manner. The low induction of apoptosis is consistent with a role in a nephropathy that progresses over years, such as DN. In addition, the combination of a high glucose microenvironment and proinflammatory cytokines further increases the susceptibility of tubular cells to TRAIL-induced apoptosis. The expression of proinflammatory cytokines is increased in DN and may increase further in the course of concurrent acute insults to the kidney.

High glucose per se did not modulate TRAIL expression; however, proinflammatory cytokines present in chronic kidney injury, including DN, such as IFN-γ and TNF-α, increased TRAIL expression. In fact, increased tubulointerstitial TRAIL/OPG may not be limited to DN. In this regard, TRAIL and OPG mRNA were also found to be increased, albeit to a lesser extent, in the tubulointerstitium of lupus nephritis (2.2x/1.5x), membranous nephropathy (1.6x/1.5x), and hypertensive nephropathy (NS/2.3x) compared with 3.1x/3.7x in DN (M.K. group, unpublished observation). Thus, proinflamma-

more times. Samples were prepared at least in triplicate. (B) Cell survival was assessed using MTS. *, parthenolide. **P < 0.05 versus all other groups; ***P < 0.05 versus basal control. Experiments were conducted six times. Samples were prepared at least in triplicate. Data are means ± SEM. (C through F). Proposed interaction between TRAIL and OPG. (C) TRAIL induces a low level of apoptosis in cultured tubular epithelial cells. The concurrent activation of NF-κB by TRAIL contributes to the low level of apoptosis, because the NF-κB inhibitor parthenolide sensitizes to TRAIL-induced apoptosis (D). (E) OPG is a soluble decoy receptor for TRAIL and is able to block both apoptosis and NF-κB activation in response to TRAIL. (F) OPG limits the increased apoptosis induced by TRAIL when NF-κB is inhibited. We propose that in vivo the relative local concentrations of TRAIL versus OPG in the cell microenvironment determines the outcome of the interaction.
tory cytokines increased TRAIL expression as well as sensitivity to TRAIL in a high glucose microenvironment (Figure 8).

The TRAIL pathway is intimately linked to the stress and survival regulation by transcriptional master regulator NF-κB, which has been shown to be a key driving force of the inflammatory stress response in the same gene expression data set from DN biopsies. Besides its proinflammatory role, NF-κB is a key regulator of cell survival. Concurrent activation of NF-κB in TRAIL-treated tubular cells seems to account for the relative resistance to apoptosis induction, because inhibition of NF-κB sensitized cells to TRAIL-induced apoptosis. This has been reported to be the case in other cell types. OPG interferes both with TRAIL-induced NF-κB activation and with TRAIL-induced loss of tubular cell survival (Figure 7, C through F), suggesting that the interplay between TRAIL and OPG might be one of the pathways for OPG to modulate diabetic tissue injury. The end result of the TRAIL/OPG interaction in vivo will probably depend on the relative local tissue levels of both molecules in the cell microenvironment, as it is the case for other cytokine/soluble receptor systems. As an example, both TNF-α and soluble TNF receptor levels are increased in serum and synovial fluid in patients with rheumatoid arthritis; however, the local soluble TNF receptor levels, although elevated, are not enough to block the nocice actions of TNF. Thus, the therapeutic use of soluble TNF receptors to increase further their concentration in vivo leads to inhibition of TNF-α activity and has been remarkably successful in this disease.

In summary, a significant number of apoptosis-related genes are differentially expressed in DN. These results are in agreement with histologic evidence of the involvement of tubular cell apoptosis in DN and might be related to the susceptibility of diabetic kidneys to acute tubular necrosis. Among them, TRAIL is expressed by tubular epithelium, where it modulates both NF-κB activation and cell death. OPG expression is also increased in DN, and it may modulate the biologic response to TRAIL. TRAIL could play an important role in modifying the progression of DN.

**CONCISE METHODS**

**Human Renal Biopsy Samples for Oligonucleotide Array-Based Gene Expression Profiling**

Human renal biopsies were collected in a multicenter study, the European Renal cDNA Bank (ERCB). The protocol was approved by the local ethical committees. Informed consent was obtained from patients according to local guidelines, and samples were processed according to the ERCB protocol. For oligonucleotide array–based gene expression profiling, a total of 16 kidney biopsies from individual patients were included: Peritubularation kidney biopsies from living donors used as control (n = 3, age 47 ± 11.6, F:M 1:2, serum creatinine <1 mg/dl, no proteinuria) and biopsies with histologic diagnosis of DN (n = 13, age 58.3 ± 3.4, F:M 4:9, diabetes duration 9.8 ± 3.5 yr, glycosylated hemoglobin 7.1 ± 0.3%, serum creatinine 2.2 ± 0.4 mg/dl, proteinuria 3.3 ± 0.7 g/dl). Biopsies were stratified by the reference pathologist of the ERCB according to their histologic diagnosis. Histology reports and clinical data were stored anonymously. The tubulointerstitial of the DN samples was evaluated using a semiquantitative grading score system. Detailed clinical and histologic patient characteristics have been recently published (Supplementary Table 2).

**Microdissection and RNA Isolation**

Tubulointerstitial compartments were manually microdissected from cortical tissue segments. Total RNA was isolated using a commercially available isolation protocol. For probe labeling, a modification of the Eberwein protocol was used.

**Microarray Data Analysis**

Affymetrix microarray analysis following the manufacturer’s protocol has been described. Image files were initially obtained through Affymetrix GeneChip software (MAS5). Subsequently, robust multi-chip analysis (RMA) was performed using RMAexpress (http://stat-www.berkeley.edu/users/bolstad/RMAExpress). Starting from the normalized RMA, the Significance Analysis of Microarrays (version 1.21; http://www-stat.stanford.edu/~tibs/SAM/) software was applied using a false discovery rate of 1% to identify genes that were significantly differentially regulated between the analyzed groups. For visualization of the results, unsupervised average linkage hierarchical cluster analysis was used to develop self-organizing maps. The clustering algorithm applied the Pearson correlation coefficient for the distance metric and the average-linkage method using unsupervised hierarchical cluster analysis. HG-U133A Affymetrix microarray chips display 22,283 probe sets. According to GeneOntologyChart in DAVID (Database for Annotation, Visualization, and Integrated Discovery), version 2.0 database, 791 of these probe sets (455 genes) belong to cell death genes (GO term ID: 0008219), and they were the ones considered for further analyses.

**Reverse Transcription and qRT-PCR**

Reverse transcription of RNA was performed as described previously. TaqMan reagents were used for TRAIL (Hs00234356_m1; NM_003810) and OPG (Hs 00171068_m1; MN_002546; Applied Biosystems, Darmstadt, Germany). As reference genes served 18S ribosomal RNA and cyclophilin A, both predeveloped TaqMan assay reagents, yielding comparable results. The data presented are normalized to ribosomal RNA. Quantification of the given templates was performed according to the standard curve method. Serial dilutions of kidney cDNA were included in all PCR runs and served as standard curve. All measurements were performed in duplicate. Controls consisting of bidistilled H2O were negative in all runs.

**Immunohistochemistry**

For immunohistochemistry studies, an independent cohort of three controls (histologically verified unaffected regions from tumor nephrectomies, n = 3, age 59.7 ± 1.5, F:M 0:3, serum creatinine 0.9 ± 0.1 mg/dl, proteinuria absent) and 17 type 2 DN cases (n = 17, age 59.1 ± 2.3, F:M 2:15, diabetes duration 12.5 ± 2.6 yr, glycosylated hemoglobin level 7.9 ± 0.3%, proteinuria 3.3 ± 1.2 g/d) were studied.
(Supplementary Table 3). Tissue samples were fixed in 4% buffered paraformaldehyde and embedded in paraffin. Routine stainings were performed according to standard techniques.

Tissues were deparaffinized and rehydrated, and an avidin-biotin technique in which a biotinylated secondary antibody reacts with several peroxidase-conjugated streptavidin molecules was applied.47 Mouse anti-human TRAIL mAb (BD Pharmingen, Milan, Italy) was used as primary antibody and biotinylated goat anti-mouse as secondary antibody (HistoLine; Zymed, Milan, Italy). Specificity of antibody labeling was demonstrated by the lack of staining after substituting the primary antibody for PBS and proper control Ig (mouse IgG2b; Zymed).

Cell Culture

HK-2 human proximal tubular epithelial cells (ATCC, Rockville, MD) were grown on RPMI 1640 (Life Technologies, Grand Island, NY), 10% heat-inactivated FCS, 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin, 5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml sodium selenite, and 5 ng/ml hydrocortisone in 5% CO₂ at 37°C. For experiments, cells were cultured in serum-free medium 24 h before the addition of the stimuli and throughout the experiment. For 37°C. For experiments, cells were cultured in serum-free medium 24 h before the addition of the stimuli and throughout the experiment. For assessment of the effect of different concentrations of glucose, cells were grown in DMEM 5.5 mM glucose (Cambrex, Walkersville, MD) with the same additives. For experiments, cells were cultured serum-free in either 5.5 mM glucose plus 19.5 mM mannitol (to ensure equal osmolarity) or high glucose DMEM containing 25 mM glucose.3

Cytokines and Reagents

Recombinant cross-linked TRAIL was obtained from Alexis Corp. (Läufelfingen, Switzerland). Cross-linked TRAIL mimics the action of the native cytokine, and it is 100 to 1000 times more potent than the soluble form, inducing cell death at concentrations >1 ng/ml.48 TRAIL was used at a concentration of 10 ng/ml except when otherwise indicated. Human TNF-α (50 ng/ml) and human IFN-γ (300 U/ml) were from PreproTech EC (London, UK). For experiments on NF-κB inhibition, cells were pretreated for 90 min with 10 µM parthenolide (Sigma, Tres Cantos, Madrid, Spain) and then with the stimuli in the absence of the drug. Recombinant human OPG was used at a final concentration of 5 µg/ml (Ampen, Thousand Oaks, CA). A neutralizing anti-OPG antibody (R&D Systems, Minneapolis, MN) was used to block endogenous OPG at a final concentration of 1 µg/ml (ND50 = 0.25 to 0.7 µg/ml). This concentration of anti-OPG did not have any effect on HK-2 cell death or proliferation. Recombinant RANKL (Santa Cruz Biotechnology, Santa Cruz, CA) was used at 100 ng/ml. A concentration of 50 ng/ml RANKL is enough to elicit a biologic response.19

Semi-quantitative RT-PCR

Total RNA from cultured cells was extracted with a TRIzol Reagent (Invitrogen, Carlsbad, CA). RNA was reverse-transcribed by oligo(dT) primer and SuperScript reverse transcriptase (Invitrogen). The resulting cDNA was subjected to PCR using the following primer sets: TRAIL-R1, 5’-CAGAAGGTCTCGAGGCTGTAAC-3’ and 5’-AGTCCATTGCGATTCTTG-3’; TRAIL-R2, 5’-GGGAGAAGATCTCGAGATGTG-3’ and 5’-ACATGGTCCTCAGC-CCCAGGTCG-3’; TRAIL-R3, 5’-GGATGATATCCAGTGTT-GAAG-3’ and 5’-ACATGGTCCTCGAGATGTG-3’; TRAIL-R4, 5’-CTCCCTCCTACCTGGGCTTTG-3’ and 5’-CCACATGGTGCTCGTAATTG-3’; and glyceraldehyde-3-phosphate dehydrogenase, 5’-CTTGGATCTGCGAGGACTC-3’ and 5’-GTCTCATGAGCACAAGTGGAGA-3’.

PCR reactions were performed under the following conditions: 95°C for 5 min; 95°C for 60 s; 60°C for 60 s; and 72°C for 2 min for 25, 30, and 35 cycles. PCR products were analyzed by DNA electrophoresis in 1% agarose gel and visualized by ethidium bromide staining. Fragments were the expected size. Plasmids with inserts encoding for TRAIL-R1, -R2, and -R3 were used as controls.

Studies on Cell Death

Cells were seeded at a density of 25,000 cells/ml (in 96-well plates for MTS/PMS, 12-well plates for flow cytometry, and coverslips for cytokfluorescence) and grown for 24 h. Thereafter, they were placed in serum-free medium for 24 h, and stimuli were added to subconfluent cells. Cell viability was quantified by the MTS/PMS test following the manufacturer’s information (Promega, Madison, WI), and absorbance at 490 nm was monitored after suitable color development (usually 1 to 4 h). Apoptosis was characterized by morphologic (propidium iodide nuclear staining of permeabilized cells grown in coverslips) and functional criteria (detection of hypodiploid cells by flow cytometry) as described previously.50

Western Blot

Western blots were performed as described previously.51 Primary antibodies used were: Mouse monoclonal anti-human TRAIL (1:1000; Pharmingen), rabbit polyclonal anti-OPG (1:500; Santa Cruz Biotechnology), rabbit polyclonal anti-cleaved caspase-3 (1:1000; Cell Signaling), and rabbit polyclonal anti-IκBα (1:500; Santa Cruz Biotechnology).

Electrophoretic Mobility Shift Assays

Transcription factor activities were determined as described previously.52 Briefly, NF-κB consensus oligonucleotides (5’-AGTT-GAGGGGACTTTCCCCAGGC-3’) were (32P)end-labeled with γ-(32P)ATP and T4 polynucleotide kinase (Promega). Nuclear extracts (5 to 10 µg) were equilibrated for 10 min in binding buffer (4% glycerol, 1 mmol/L MgCl₂, 0.5 mmol/L EDTA, 0.5 mmol/L dithiothreitol, 50 mmol/L NaCl, 10 mmol/L Tris-HCl [pH 7.5], and 50 µg/ml poly(dI-dC); Pharmacia), and then the labeled probe (0.35 pmol) was added and incubated for 20 min at room temperature. The reaction was stopped by addition of gel loading buffer (250 mmol/L Tris-HCl, 0.2% bromophenol blue, 0.2% xylene cyanol, and 40% glycerol) and run on a nondenaturing 4% acrylamide gel in Tris-Borate. The gel was dried and exposed to x-ray film.

NF-κB Luciferase Reporter Gene Assay

Cells were plated at a density of 8 × 10⁴ cells in six-well plates (Costar, Cambridge, MA) 24 h before transfection with FuGENE 6 (Roche, Indianapolis, IN) according to the manufacturer’s instructions. Cells were transfected with the pNFκB-Luc Vector (Stratagen, La Jolla, CA) and the pRLTK vector, which contained the luciferase gene Renilla.
(Promega) in a ratio of 10:1. At 24 h after transfection, the medium was replaced with RPMI without serum. Then, cells were treated with the indicated stimuli for 24 h. Cells were harvested, and luciferase activity was determined from cell extracts by means of a luciferase assay system (Promega) and a luminometer (Berthold Analytical Instrument, Nashua, NH). Luciferase activity was normalized to Renilla activity to control for differences in transfection efficiency.

Statistical Analyses
Statistical analysis was performed using the SPSS software (SPSS Inc., Chicago, IL). Data were expressed as absolute values, mean ± SEM. Independent sample t test (assuming unequal variances), one-way ANOVA, and Mann-Whitney test were used to compare means. Pearson correlation was applied to examine nominal and ordinal variables. P < 0.05 was taken as significant.

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DISCLOSURES
None.

REFERENCES


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