Expression of Silencer of Death Domains and Death-Receptor-3 in Normal Human Kidney and in Rejecting Renal Transplants

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We have previously reported the pattern of cellular expression of tumor necrosis factor receptors (TNFR) in human kidney and their altered expression in transplant rejection. We have extended our studies to examine the expression of Silencer of Death Domains (SODD), a protein that binds to the cytoplasmic portion of TNFR1 to inhibit signaling in the absence of ligand. In normal human kidney SODD is expressed in glomerular endothelial cells where it colocalizes with TNFR1. During acute rejection both SODD and TNFR1 are lost from glomeruli, but we found strong expression of SODD on the luminal surface of tubular epithelial cells. This occurs in the absence of detectable TNFR1 expression, suggesting that SODD could interact with other proteins at these sites. Several other members of the TNF superfamily, including Fas and death receptors (DR)-3, -4, and -5, also contain intracellular death domains, but SODD only interacts with the death domain of DR3. We therefore studied the expression of DR3 in human kidney, and report that this death receptor is up-regulated in renal tubular epithelial cells and endothelial cells of some interlobular arteries, in parallel with SODD, during acute transplant rejection. In less severe rejection episodes, DR3 and SODD were more focally induced, generally at sites of mononuclear cell infiltrates. In ischemic allografts, eg, with acute tubular necrosis but no cellular rejection, DR3 was induced on tubular epithelial cells and on glomerular endothelial cells. These data confirm that TNF receptor family members are expressed in a regulated manner during renal transplant rejection, and identify DR3 as a potential inducible mediator of tubular inflammation and injury. (*Am J Pathol* 2003, 163:401–411)

Members of the tumor necrosis factor (TNF) receptor superfamily are type 1 transmembrane proteins that

share a common extracellular structural organization. At the cell surface, these receptors mediate responses to soluble cytokines or cell surface ligands that belong to the TNF superfamily. A subset of TNF receptor (TNFR) superfamily members, including Fas, TNFR1, TNF-related apoptosis inducing ligand (TRAIL) R1, TRAILR2, and death receptor-3 (DR3), contain a homologous intracellular region called a "death domain" (DD).¹ The DD is a protein-protein interaction domain that allows these receptors to interact with cytosolic adapter molecules that also contain a DD, such as Fas-associated DD protein (FADD), TNFR-associated DD protein (TRADD) and receptor interacting protein (RIP).² The DD acquired its name because these receptors, when engaged by their ligands, can initiate the formation of a death-inducing signaling complex (DISC) that catalyzes caspase activation and apoptotic cell death. Some of these receptors (eg, TNFR1, TRAIL1, and TRAIL2) also can initiate transcription of new genes, and the induced gene products may prevent cell death from occurring. In normal human endothelial cells (EC), TNFR-1 serves primarily as an initiator of activation rather than as a death receptor, leading to the expression of adhesion molecules and chemokines that trigger local inflammation as well as expression of anti-apoptotic proteins. For TNFR1, both death responses and activation responses are initiated by recruitment of TRADD to the DD of the occupied receptor. Since receptor density on the plasma membrane and the concentration of TRADD in the cytosol are not immediately affected by TNF binding, it is thought that ligand binding must induce either receptor clustering and/or a conformational change that favors TRADD binding. TRADD recruitment to ligand-unoccupied receptors may be further limited by interactions of the TNFR1 DD with another DD-containing cytosolic protein called Silencer of Death Domains (SODD). SODD also can bind to DR3, but not to the other death receptors. We have previously shown that in cultured human EC, most TNFR-1 molecules are sequestered in the Golgi rather

Supported by grants from the National Kidney Research Fund and Medical Research Council, United Kingdom, and from the National Institutes of Health, Bethesda, Maryland.

Accepted for publication March 18, 2003.

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than the cell surface,³ but that on addition of TNF, TRADD is recruited only to the cell surface receptors. Recently, we have studied the distribution of TNFR1 molecules in human kidney.⁴ In healthy kidney tissue, TNFR1 expression is confined primarily to glomerular EC, being largely absent from other cell types. As in cultured EC, most TNFR1 *in situ* is contained within the Golgi. During allograft rejection, this expression in EC is lost, and TNFR1 is almost exclusively found on infiltrating leukocytes. Here we extend these studies to examine the distribution of SODD. As expected, SODD colocalizes with TNFR1 in resting kidney, being found primarily in EC and concentrated in the Golgi of the EC. In allograft rejection, SODD, like TNFR1, is lost from the glomeruli, but is retained in some other microvascular cells that now lack TNFR1. SODD is also up-regulated in tubular epithelial cells, which remain TNFR1-negative. Coincidental with SODD expression, tubular cells and some microvascular cells acquire DR3, which is largely absent from normal kidney except for resident leukocytes. These data confirm that SODD, like TNFR1, shows a limited pattern of expression *in vivo* and further suggest that inducible death receptors, such as DR3, may play a role in renal allograft injury during rejection, providing a novel target for tissue protection.

Materials and Methods

All human tissues were acquired under a protocol approved by the local Ethics Committee. Samples of normal human renal tissues were obtained from six nephrectomy specimens removed for renal tumors. Nine different samples of tissue from renal transplants undergoing severe acute rejection were obtained from six biopsies and from three allograft nephrectomy specimens. In addition, biopsies from three patients with mild to moderate rejection, and three patients with evidence of early ischemic injury but without rejection were examined. For nephrectomy specimens, slices of tissue <1-mm-thick were taken from the cortex through to the medulla. The tissue was divided into two portions. One portion was fixed by immersion in 2% or 4% formaldehyde (BDH Merck Ltd., Lutterworth, Leics, UK) in 0.1 mol/L PIPES buffer, pH 7.6 for 4 hours at 4°C for light microscopy and one portion was snap-frozen in liquid nitrogen cooled isopentane and stored at -70°C until use. Tissue selected for light microscopy was either embedded in paraffin wax or in ornithine carbamyl transferase (OCT). Paraffin sections from each batch of tissue were stained with hematoxylin and eosin for comparative histological examination. Additional paraffin sections (5- μ m-thick) were cut and placed on poly-L-lysine-coated glass slides (BDH Merck Ltd.) and used as indicated for immunohistochemistry. Cryostat sections (7- μ m-thick) of OCT-embedded specimens were collected on poly-L-lysine-coated glass slides and used for immunofluorescence microscopy and *in situ* hybridization.

Confocal Immunofluorescence Microscopy

Single Immunostaining for SODD or DR3

All incubations were carried out at room temperature in a humid chamber, unless otherwise stated. Paraffin sections were dewaxed in xylene and rehydrated through descending series of ethanol concentrations. They were then washed in running tap water and in deionized water before incubation with 50 μ g/ml Proteinase-K (Roche Diagnostics Ltd, East Sussex, UK) in 0.1 mol/L Tris-HCl buffered saline (TBS) at pH 7.6 for 10 minutes for antigen retrieval before immunostaining. Cryostat sections were briefly fixed in cold methanol at -20°C for 5 minutes and rinsed in TBS before immunostaining. Both types of sections were then incubated with blocking buffer containing 0.5% bovine serum albumin (BSA) in TBS to suppress non-specific antibody binding. Excess blocking buffer was removed and sections were incubated overnight at 4°C in primary antibody: rabbit polyclonal anti-hSODD (H-300; catalog no. sc-8980) or rabbit polyclonal anti-hDR3 (H-300; catalog no. sc-7909), both antibodies from Autogen Bioclear Ltd, Wiltshire, UK) at 1:50 dilution in blocking buffer at a working concentration of 4 μ g/ml each. As a negative control, the primary antibody was replaced by normal rabbit serum. Positive controls included staining of sections of human tonsils for DR3 and SODD. To confirm antibody specificity, a blocking peptide was incubated with the specific antibody overnight at 4°C before staining as above.

After rinsing extensively with TBS, bound anti-SODD was either detected by secondary antibody conjugated with Texas Red (Vector Laboratories, Peterborough, UK) or with alkaline-phosphatase (σ -Genosys Ltd, Pampisford, UK). Bound anti-DR3 was detected by secondary antibody conjugated with fluorescein isothiocyanate (FITC) (Vector) diluted 1:100 in blocking buffer. Slides were incubated with secondary antibody for 45 minutes. After extensive rinsing in TBS, sections labeled with FITC and Texas-Red-conjugated antibodies were further rinsed in deionized water and mounted in Vectashield Mounting Medium containing 1.5 μ g/ml 4',6-diamidino-2-phenylindole (DAPI; Vector) before viewing using a TCS-NT Confocal Laser Scanning Microscope (CLSM) (Leica Microsystems Ltd, Knowlhill, Milton Keynes, UK). Sections labeled with alkaline-phosphatase-conjugated antibodies were rinsed in 0.1 mol/L TBS, pH 8.4, for 10 minutes and antibody binding sites were visualized microscopically by incubation for 20 to 45 minutes with an alkaline phosphatase substrate kit (Vector) that uses Vector Red as its chromogen. The sections were then rinsed in deionized water and mounted either in Vectashield Mounting Medium as described above or rinsed in ascending series of ethanol solutions, cleared in xylene and mounted in DePeX before viewing as described above. The Vector Red reaction product is a bright red precipitate, which can be viewed by confocal microscopy as previously described using Texas Red excitation and emission filters.

Double-Immunostaining for SODD and TNFR1 or CD31 or DR3

Cryostat sections of normal kidney or rejecting allograft were incubated overnight with either polyclonal rabbit anti-human SODD antibody and mouse monoclonal anti-hTNFR-1 (1:20 dilution, 25 μ g/ml; R&D Systems, Abingdon, UK) or mouse anti-hCD31 (endothelial cell marker; Dakocytomation, Ely, Cambridgeshire, UK) or goat polyclonal anti-hDR3 (1:50 dilution, 4 μ g/ml) in blocking buffer. After thorough rinsing in Tris buffer, bound anti-SODD was detected using secondary antibody conjugated to Texas Red (Vector), and bound TNFR1 or CD31 or DR3 was detected using secondary antibody conjugated to FITC. All antibodies were diluted 1:100 in blocking buffer and incubated in the dark at room temperature for 45 minutes. Sections were then rinsed in Tris buffer, thoroughly washed in deionized water and mounted in Vectashield Mounting Medium before viewing using confocal fluorescence microscopy as previously described. Negative control specimens included replacing the primary antibody with isotype-specific serum, and sections of human tonsils were used as positive controls.

Double-Immunostaining for DR3 and Cytokeratin or Smooth Muscle Actin or CD31 or CD45 or CD68 or CD4 or CD8

The phenotype of the DR3-positive cells in allograft rejection was investigated using a panel of mouse monoclonal antibodies purchased from Dakocytomation. In these experiments, cryostat sections were first labeled with anti-hDR3 and further labeled with anti-human cyto-keratin (reacts with epithelial cells), anti- α -smooth muscle actin (α -SMA; reacts with smooth muscle cells and mesangial cells), an endothelial cell marker (CD31), a pan leukocyte marker (CD45), a macrophage/monocyte marker (CD68), or T-cell subset markers CD4 or CD8. The secondary reagent for detecting DR3 was FITC-conjugated anti-rabbit IgG (H+L) (Vector). Alexa-Fluor⁵⁶⁸ conjugated anti-mouse IgG (H+L) (Cambridge Bioscience, Cambridge, UK) was used to detect the various mouse antibodies.

In Situ Hybridization

For detection of specific mRNA in normal and allograft kidney, 60 mer single-stranded anti-sense DNA oligonucleotide probes 3' end-labeled with digoxigenin specific for human DR3 (5'-AGTCTAGGCA-TGCTTGGCAG-TAGAAG-GGTG-AACT GCTGAC ATTGGCTG-ACCTGGCACT-3' gb/U72763) and for human SODD (5'-ATGGCCTCCT-CCGC-CTTCTC-CCAGCCAGGT-GGTCTCCGCC GGCCGCCCC-CGCGCACCCG-3'; gb/AF111116) were purchased from MWG-Biotech, Milton Keynes, UK. Cryostat sections were brought to room temperature and fixed in freshly prepared 4% formaldehyde (σ -Genosys) in 0.1 mol/L PBS for 5 minutes at room temperature. After washing in two changes of PBS, sections were treated with 5 μ g/ml Proteinase-K (Roche Diagnostics, Lewes, East Sussex,

UK) in PBS for 8 minutes at room temperature. Enzyme activity was inhibited by incubation with 2 mg/ml glycine (σ -Genosys) in PBS for 5 minutes and rinsed in 2X sodium citrate-buffered saline (SSC) for 5 minutes. Slides were then pre-hybridized at 37°C for 1 hour in hybridization buffer consisting of the following components: 1 mol/L Tris-HCl, pH 7.4, 20X SSC, 0.05 mol/L EDTA, pH 8.0, 50% dextran sulfate, 50X Denhart's solution, 50% formamide and DEPC-H₂O. All reagents were of molecular biology grade from σ -Genosys. Excess solution was removed and 100 μ l of hybridization buffer containing digoxigenin-labeled anti-sense DR3 probe (4.6 μ g/ml) or anti-sense SODD probe (4.1 μ g/ml) was added to each section and incubated overnight in a humidified chamber at 37°C. Post-hybridization washings were performed as follows: 2X SSC for 5 minutes at 37°C; 0.2X SSC, 60% formamide for 5 minutes at 37°C with two changes; 2X SSC for 5 minutes at room temperature with two changes; and in 100 mmol/L Tris-HCl, 150 mmol/L NaCl, pH 7.5 (TBS) for 5 minutes at room temperature. The sections were then processed for immunological detection. Briefly, non-specific binding was blocked by incubating sections in TBS containing 1% acetylated bovine serum albumin (TBS-BSA) (σ -Genosys) for 30 minutes at room temperature. Excess blocking solution was removed and 100 μ l of 1:200 dilution in TBS-BSA of sheep anti-digoxigenin F(ab)₂ antibody conjugated to alkaline phosphatase (Roche Diagnostics) was added to sections and incubated in a humidified chamber at room temperature for 2 hours. Following washings in TBS for 5 minutes with two changes, sections were rinsed in 0.1 mol/L Tris-HCl, 0.1 mol/L NaCl, 50 mmol/L MgCl₂, pH 9.5 for 10 minutes at room temperature. Antibody binding sites were visualized using 100 μ l of color substrate (alkaline phosphatase ready-to-use substrate kit containing β -chloroindolyl phosphate and nitroblue tetrazolium; NBT-BCIP) (σ -Genosys). The sections were then washed thoroughly in deionized water and mounted in Glycergel Aqueous Mounting Medium (Dakocytomation). Additional controls to confirm specificity of probe-binding included using digoxigenin-labeled Oligo-dT or actin cocktail probes from R&D systems, and negative controls included incubation of parallel sections with a sense probe to either SODD or DR3 (MW Biotech). The sections were visualized directly with bright field light microscopy using Nikon OPTIPHOT-2 with a Polaroid DMC 2.0 Digital Microscope Camera (Nikon, Kingston on Thames, Surrey, UK).

Results

SODD Expression in Normal Kidney

In histologically normal adult human kidney tissue obtained from nephrectomy specimens for tumor, strong and homogenous expression of SODD was observed mainly in the glomerular capillary ECs and in peritubular capillaries of the renal cortex (Figure 1a). The glomeruli were uniformly positive for SODD expression. A high-

Figure 1. Top: Paraffin section of normal human kidney labeled with rabbit polyclonal anti-human SODD and secondary antibody conjugated with alkaline phosphatase and Vector Red as a substrate. A strong expression of SODD is present in the glomeruli (**arrows**) and in the peritubular capillaries (**arrowheads**) in the renal cortex. **b:** A high-power view shows positive reaction mainly confined to the glomeruli endothelial cells (EC), in peritubular capillaries (**arrowhead**), in epithelial cells of the Bowman's capsule (BC) and to a lesser extent on tubular epithelial cells (**arrows, inset**). **c:** No staining for SODD was detected on sections where primary antibody was replaced by pre-immune serum. **Bottom:** Cryostat section of normal human kidney hybridized with digoxigenin-labeled anti-sense oligonucleotide probe for SODD mRNA followed by anti-digoxigenin conjugated to alkaline phosphatase and developed using 5-bromo-4-chloro-3-indolyl-phosphate nitroblue tetrazolium (BCIP/NBT) substrate. **d:** A strong expression for SODD mRNA was demonstrated in the glomeruli (**arrows**) and in the Bowman's capsule in the renal cortex, and a moderate signal was observed in microvascular endothelial cells (**arrow, inset**). **e:** A high-power view shows the reaction product of BCIP/NBT mainly confined to the glomeruli endothelial cells (**arrows**). **f:** No signal was detected on parallel sections hybridized with a sense probe for SODD mRNA (negative control). Glom, glomerular. Original magnifications: **a**, $\times 10$; **b**, $\times 63$; **c**, $\times 40$; **d**, $\times 20$; **e**, $\times 40$; **f**, $\times 60$.

-power view shows fluorescence mainly confined to endothelial cells of glomerular and peritubular capillaries, on epithelial cells of the Bowman's capsule, and to a lesser extent on tubular epithelial cells (Figure 1b). No staining for SODD was seen in sections where primary antibody was replaced by pre-immune serum (Figure 1c). SODD mRNA was observed in endothelial cells of glomeruli, in the Bowman's capsule and interlobular arterioles (Figure 1, d and e). In contrast, no signal for SODD mRNA was observed in tubular epithelial cells. The specificity of the signal was validated by the absence of signal with a sense control probe (negative control) (Figure 1f). SODD expression was analyzed further by double-antibody staining. Precise colocalization of SODD and CD31 was seen constitutively in endothelial cells of glomeruli and in peritubular capillaries (Figure 2, a to c). There were a few cells within tubules that showed reactivity for SODD (Figure 2c). No apparent differences were seen among the various normal human kidneys studied. Sections of normal kidney double-labeled for SODD and TNFR-1 demonstrated co-staining in endothelial cells of glomeruli (Figure 2, d to f). There were a few cells that were SODD reactive but negative for TNFR1 (Figure 2f). In contrast, TNFR1 did not colocalize with the occasional tubular epithelial cells staining positive for SODD. These data show that SODD, like TNFR1, shows a restricted

distribution in human kidney *in vivo*. For the most part, SODD colocalizes with TNFR1 in glomerular and peritubular capillaries endothelial cells, identified as the primary site for synthesis by *in situ* hybridization. However, the presence of a subpopulation of SODD-positive, TNFR1-negative cells prompted us to examine the same tissue for expression of DR3, the only other protein known to interact with SODD.

DR3 Expression in Normal Human Kidney

Staining for DR3 was demonstrated in resident leukocytes in the interstitium and in scattered endothelial cells of occasional interlobular arteries (Figure 3a). No staining for DR3 was observed in glomeruli in normal kidney. Positive control sections of human tonsils showed strong expression of DR3 (Figure 3b). An identical pattern of staining was observed using either rabbit or goat polyclonal antibodies against human DR3 protein.

In situ hybridization analysis of normal kidney revealed absence of DR3 mRNA in glomeruli but a moderate signal was present in few (not all) epithelial cells in tubules and in interstitial resident leukocytes (Figure 3c). By contrast, no message for DR3 was present on sections of normal kidney hybridized with a sense probe for DR3 mRNA (negative control) (Figure 3d).

Figure 2. Cryostat section of normal human kidney double-immunolabeled for SODD and CD31 or TNFR1 shows colocalization for SODD and CD31 in the endothelial cells of glomeruli (Glom) and in peritubular capillaries (**a-c**). There are a few tubular epithelial cells, which are reactive for SODD (**arrows**). **d-f**: Colocalization for SODD and TNFR1 was mainly confined to the glomeruli endothelial cells, with the exception of a few glomerular resident cells that were reactive for SODD but negative for TNFR-1 (**arrows**). Nuclear-stained with DAPI; original magnifications: **a-c**, $\times 40$; **d-f**, $\times 63$.

SODD and DR3 Expression in Acute Cellular Rejection

Our previous studies showed that TNFR1 expression is altered by acute rejection.⁴ Therefore we examined the effect of rejection on the expression of SODD. The pattern of SODD expression in allograft rejection tissue differed from normal kidney. In severe allograft rejection SODD was barely detected in glomerular endothelium, but a strong signal was present in tubular epithelial cells, markedly accentuated on the luminal aspect (Figure 4, a and b). No staining for SODD was seen in sections where primary antibody was replaced by pre-immune serum (Figure 4c).

SODD mRNA expression was analyzed in the same tissue. SODD mRNA was no longer demonstrated in glomeruli but weak and infrequent expression was seen in epithelial cells of the Bowman's capsule and in mononuclear cell infiltrates in the interstitium, outside of the glomeruli (Figure 4d). In addition, a moderate expression of SODD mRNA was detected in endothelial cells of dilated interlobular arteries (Figure 4e). In contrast, no signal for SODD mRNA was demonstrated on sections of allograft

rejection hybridized with a sense probe (data not shown).

Increased expression of DR3 was seen in sections of allograft rejection, with high levels detected on intact and on damaged tubular epithelial cells and interstitial mononuclear cell infiltrate (Figure 5a). The signal was detected to a variable degree in all biopsies of allograft rejection examined. Double-staining identified many of the DR3-positive mononuclear cells within glomeruli as CD45-positive leukocytes or smooth muscle actin-positive mesangial cells. The DR3-positive mononuclear cells within the interstitium were found to be CD68-positive macrophages. No colocalization for DR3 was observed in CD4- or CD8-positive T lymphocytes in allograft rejection (data not shown). DR3 expression was also markedly accentuated in microvascular endothelial cells, some with damaged endothelial lining (Figure 5b), and in a majority of infiltrating CD45-positive mononuclear cells within glomeruli (Figure 5c). The tubular distribution of DR3 staining during rejection was confirmed by colocalization with monoclonal anti-human cytokeratin antibody (Figure 5, d to f) and the vascular distribution with monoclonal anti-human CD31 (Figure 5, g to i). DR3 expression was detected in approximately 20% of CD31-positive endo-

Figure 3. Top: Cryostat section of histological normal human kidney immunolabeled for DR3 shows (a) moderate DR3 expression in scattered endothelial cells of occasional interlobular arteries (Bv) and in resident leukocytes (arrows). DR3 staining was not detected in the glomerular (Glom) and peritubular capillary endothelium. **b:** A strong expression for DR3 was observed in positive control sections of human tonsils. **Bottom:** Cryostat section of histological normal human kidney hybridized with digoxigenin-labeled anti-sense oligonucleotide-probe for DR3 mRNA followed by anti-digoxigenin conjugated to alkaline phosphatase and developed using BCIP/NBT substrate. **c:** DR3 mRNA expression was detected in a few tubular epithelial cells (arrowheads) and in resident leukocytes (arrows). No signal for DR3 mRNA was present in the glomeruli (Glom) and peritubular capillaries. **d:** No signal was observed on parallel sections following hybridization with a sense probe to DR3 mRNA (negative control). t, tubules. Original magnifications: a, $\times 40$; b and c, $\times 20$; d, $\times 40$.

thelial cells of small arteries. No colocalization for DR3 and CD31 was detected either in glomerular endothelium or peritubular capillary endothelium (Figure 5, g to i). Pre-immune serum alone produced no staining and staining was not abolished by pre-incubation of sections with a blocking peptide (data not shown). Colocalization for DR3 and SODD was observed on a few (not all) tubular epithelial cells in allograft rejection (Figure 6, a to c).

In situ hybridization on sections of allograft rejection revealed strong expression for DR3 mRNA on the mononuclear cell infiltrate within glomeruli and in the interstitium, outside the glomeruli (Figure 7a). DR3 mRNA was also seen in endothelial cells of interlobular arteries, in tubular epithelial cells and in the interstitial mononuclear cell infiltrate (Figure 7, b to d). A strong expression for DR3 mRNA was also demonstrated in positive control sections of human tonsils following hybridization with anti-sense, and no reaction was observed in allograft sections hybridized with a sense probe for DR3 mRNA (data not shown).

To determine whether up-regulation of DR3 tends to occur at sites of mononuclear cell infiltration we have examined DR3 and SODD expression in biopsies from patients with mild to moderate rejection. Sections with mild to moderate rejection showed a focal up-regulation of DR3 in approximately 5% of tubular cells (Figure 8, a to c) with only a minimal expression for SODD (data not shown). DR3 signal was also demonstrated on interstitial mononuclear cell infiltrates, on isolated cells within glomeruli, and on vascular endothelial cells of few arterioles. We have also examined DR3 and SODD expression in biopsies with early ischemic injury (acute tubular necrosis; ATN) without rejection (Figure 8, d to f). Sections with ATN demonstrated a focal DR3 expression on intact tubular cells and a diffused pattern on damaged tubular cells. In the presence of ATN DR3 expression was also seen on glomerular endothelial cells. No significant staining for SODD was demonstrated in the biopsies with ATN that were examined.

Figure 4. Top: Paraffin section of severe acute cellular rejection labeled with rabbit polyclonal antibody to human SODD and secondary antibody conjugated with alkaline phosphatase and, developed using Vector Red substrate kit. **a** and **b:** A strong expression for SODD was demonstrated in the luminal aspect of tubular epithelial cells (**arrows**) and a weak and infrequent signal was detected in the glomerular endothelial cells (EC). **c:** No staining for SODD was present in sections where primary antibody was replaced by pre-immune serum. **Bottom:** Cryostat section of severe acute cellular rejection hybridized with SODD mRNA using digoxigenin-labeled anti-sense oligonucleotide probe followed by anti-digoxigenin conjugated to alkaline phosphatase and, developed using BCIP/NBT substrate. **d:** No signal for SODD mRNA was evident in the glomeruli but a weak and infrequent expression was observed in epithelial cells of the Bowman's capsule (**arrow**) and interstitial mononuclear cells (**arrowheads**) outside the glomerular. **e:** SODD mRNA was also observed in interlobular arteries (**arrows**). Original magnification, $\times 40$.

Discussion

We have recently reported that the expression of TNFR-1 and TNFR-2 is restricted to different cell types in human kidney.⁴ In normal human kidney TNFR-1 is strongly expressed in glomerular endothelial cells, whereas TNFR2 is only weakly expressed on the luminal surface of tubular cells. During rejection TNFR1 expression diminishes, whereas TNFR-2 is up-regulated in tubular cells. We have extended our studies to examine the expression and distribution of SODD in human kidney, and find that it is expressed in glomerular endothelial cells where it colocalizes with TNFR1 in normal kidney. During severe acute rejection we have found strong expression of SODD on the luminal surface of tubular cells. This occurs in the absence of TNFR1 expression, suggesting that SODD could interact with other proteins at this site.⁵ The cytoplasmic tail of TNFR2 does not contain a death domain, and does not interact with SODD or TRADD. In addition to TNFR1, SODD is only known to interact with the death domain of DR3. We therefore studied the expression of DR3 in human kidney and found that DR3 is coordinately up-regulated with SODD in renal tubular cells and can be

demonstrated in approximately 20% of interlobular arteries in allograft rejection. DR3 was not demonstrated in glomerular endothelial cells or peritubular capillaries in allograft rejection, as evidenced by co-localization with endothelial marker CD31. For the most part, those cells which synthesize and express SODD but not TNFR1 also synthesize and express DR3, but occasional single positive cells expressing SODD or DR3 were found, especially in normal kidney. This may reflect difficulties in detecting low levels of synthesis or protein expression.

Our studies of mild to moderate rejection and ischemic injury indicate that up-regulation of DR3 occurs at sites of lymphocyte infiltration and inflammation. In addition, glomerular endothelial cells in ischemic allograft injury (ATN) can also express DR3, although the signals for this are not provided by allograft rejection. Further study will be needed to clarify the differences between glomerular and peritubular capillary endothelial cell responses.

The precise function of SODD is unknown, but it is likely to be a key regulator of TNFR1 signaling. SODD contains a protein-binding motif known as a BAG domain that can interact with heat shock protein 70 (Hsp 70) and

Figure 6. Cryostat section of severe acute cellular rejection double-immunolabeled by simultaneous incubation with goat polyclonal anti-human DR3 antibody followed by anti-goat antibody conjugated to FITC (green) and rabbit polyclonal anti-human SODD antibody followed by anti-rabbit secondary antibody conjugated with Texas Red (red) (**a–c**). Colocalization was detected in tubular epithelial cells (**arrows**). Nuclear-stained with DAPI. Original magnification, $\times 63$.

Figure 7. Cryostat section of severe acute cellular rejection hybridized with DR3 mRNA using digoxigenin-labeled anti-sense oligonucleotide probe followed by anti-digoxigenin conjugated to alkaline phosphatase and developed with BCIP/NBT substrate. **a:** A strong expression for DR3 mRNA was observed in the glomeruli resident cells (**arrows**) and interstitial mononuclear cell infiltrate outside the glomeruli (**arrowheads**). **b:** DR3 mRNA was also evident in endothelial cells of interlobular arteries (**arrows**) and interstitial mononuclear cell infiltrate (**arrowheads**). **c:** DR3 mRNA expression was also observed in tubular epithelial cells (t) and in interstitial mononuclear cell infiltrates (**d, arrows**). Original magnification, $\times 40$.

Figure 5. Confocal image of section of severe acute cellular rejection immunolabeled for DR3 shows (**a**) a strong expression for DR3 in tubular epithelial cells (t) and in mononuclear cell infiltrates (**arrowheads**). **b:** DR3 expression was also observed in endothelial cells of interlobular arteries (Bv) and in mononuclear cells within glomeruli (**c, arrows**). Tubular DR3 expression was confirmed by colocalization with antibody to cytokeratin (**d–f**) and vascular DR3 expression (**arrows**) was confirmed by colocalization with endothelial marker, CD31 (**g–i**). No DR3 expression was detected in the glomeruli endothelium and peritubular capillaries. Glom, glomeruli; t, tubules; I, interstitium. Nuclear-stained with DAPI. Original magnification, $\times 40$.

Figure 8. Confocal micrographs of paraffin-embedded sections immunostained for DR3. **Top:** Section with mild to moderate acute cellular rejection without ATN show focal DR3 staining on tubular epithelial cells (t) in **a**, on interstitial mononuclear cell infiltrates (arrows) in **b**, and on vascular endothelial cells (Bv) in **c**. **Bottom:** Section with ATN without rejection show focal DR3 expression on intact tubular epithelial cells (arrows) in **d**, and a diffused pattern on damaged tubular cells (t) in **e** and **f** and on glomerular endothelial cells (Glom). it, intact tubule. Original magnification, $\times 40$.

the Hsp 70 cellular homolog Hsc 70. The BAG domain of SODD has been shown to bind competitively to TNFR1 and the ATPase domain of Hsp 70. It has been proposed that TNFR1 may contain an ATPase and that the conformational state and function of TNFR1 may be modulated by SODD, binding this ATPase domain.⁶

A surprising finding of this study is the identification of DR3 expression by non-lymphoid cells in rejecting renal allografts. This observation conflicts with the notion that DR3 is restricted to lymphoid cells^{7,8,9,10} and supports reports of DR3 expression in other tissues such as neuroectodermal tumors of the central and peripheral nervous system,¹¹ human and rat brain,¹² and human lens epithelial cells.¹³ DR3 was expressed by infiltrating mononuclear cells, and double-immunostaining identified many of the cells as CD45-positive leukocytes or SMA-positive mesangial cells within glomeruli and CD68-positive macrophages in the interstitium. DR3 was not observed in CD4- or CD8-positive T-lymphocytes in allograft rejection.

The factors that result in the preferential expression of DR3 in tubules of allograft rejection and its pathogenic importance are at present not known. It is unclear whether it is a cause or effect of the rejection process, but inflammatory cells invading the graft may release cytokines¹⁴ or other related products into the microenviron-

ment leading to the expression of DR3 in tubules and interlobular arteries. In preliminary studies we have demonstrated that TNF, which is found in rejecting allografts, can induce DR3 on the surface of endothelial cells (unpublished observations). Renal tubular cells are a principal target for injury in allograft rejection, and the ability to induce cell death is a well-recognized feature of TNF receptor family members.^{11,12,14-16} One biological role of DR3, like TNFR-1, Fas, and TRAIL, may be to activate the conventional death domain-mediated pathways on endothelial cells and tubular epithelial cells on binding of its cognate ligand. Microscopic examination of DR3/APO-3-transfected HEK 293 cells 36 hours after transfection revealed a substantial loss of cell viability, membrane blebbing, and loss of cell volume.¹⁷ Indeed, ectopic expression of DR3 in MCK breast carcinoma cells and HEK 293 cells have been shown to induce rapid apoptosis.¹ *In vitro* studies have shown DR3-induced apoptosis to be blocked by two inhibitors of interleukin-1 β converting enzyme-like proteases CrmA and z-VAD-fmk.¹

In view of the evidence that DR3 stimulates cell death *in vitro*, we hypothesize that DR3 in allograft rejection may be an important effector pathway that sets a balance between susceptibility and protection from apoptosis.¹⁰ DR3, like TNF receptors containing death domains such as TNFR-1 and Fas, may transduce death signals by

interaction with death domains of FADD, a death domain-containing cytoplasmic protein capable of initiating DISC cascade.¹⁸ There have been many conflicting reports on possible ligands for DR3.^{7,9,14,17,19,20} Recently, an endothelial cell-derived TNF-like ligand for DR3, TL1A, has been described,¹⁹ and its expression is inducible by TNF and IL-1 α .¹⁹ Transcripts for TL1A have been shown to be present in human umbilical vein endothelial cells (HUVEC) and human kidney.¹⁹

These studies extend our observations that the expression of TNF receptor family members is regulated in human kidney. We further identify DR3 as a potentially important mediator of tubular inflammation and injury in renal transplant rejection. Although the interaction between SODD and DR3 has not been fully characterized, it is likely that SODD plays an important role in regulating both TNFR1 and DR3 signaling *in vivo*. The expression and localization of TL1A¹⁹ in allograft injury and other forms of renal inflammation will be an interesting subject for future studies.

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