Cardiovascular, Pulmonary, and Renal Pathology

DR3 Signaling Protects against Cisplatin Nephrotoxicity Mediated by Tumor Necrosis Factor

Rafia S. Al-Lamki,*† WanHua Lu,‡§ Sarah Finlay,* Jason P. Twohig,‡ Eddie C.Y. Wang,‡ Aviva M. Tolkovsky,§¶ and John R. Bradley*†

From the Department of Medicine* and the National Institute for Health Research,† Biomedical Research Center, Addenbrooke’s Hospital, the Cambridge Center for Brain Repair‡ and the Department of Biochemistry,§ University of Cambridge, Cambridge; and the Institute of Infection & Immunity,¶ School of Medicine, Cardiff University, Cardiff, United Kingdom

The expression of death receptor 3 (DR3), a member of the tumor necrosis factor (TNF) receptor superfamily, is up-regulated in human tubular epithelial cells (TECs) during renal injury, but its function in this setting remains unknown. We used cisplatin to induce renal injury in wild-type (DR3+/+) or congenitally deficient DR3−/− mice to examine the in vivo role of DR3. Cisplatin increased the expression of DR3, its ligand, TNF-like ligand 1A (TL1A), and TNF in TECs, as observed in human renal injury. Cisplatin increased apoptotic death of DR3−/− TECs by two-fold compared with DR3+/+ TECs, whereas it reduced the number of tubules expressing phospho-NF-κBp65Ser276 by 50% at 72 hours. Similar degrees of induction of DR3, TL1A, and TNF, and changes in apoptosis and phospho-NF-κBp65Ser276, were obtained in mouse kidney organ cultures treated with cisplatin for 3 hours, suggesting a direct effect on TECs. TNF was implicated in mediating cisplatin-induced tubular damage given that the in vivo co-administration of GM6001, an inhibitor of TNF maturation and release, significantly reduced TNF production and tubular damage. Moreover, TNF exacerbated, whereas TL1A reduced, cisplatin-induced apoptosis in the DR3+/+ mouse proximal tubule cell line, TKPTS. Our data demonstrate that cisplatin-induced nephrotoxicity is mitigated by DR3 signaling, suggesting that this occurs by antagonizing pro-apoptotic signals induced by TNF. Therefore, activating DR3 may be beneficial in reducing acute kidney injury. (Am J Pathol 2012, 180:1454–1464; DOI: 10.1016/j.ajpath.2012.01.003)

Death receptor 3 (DR3; also known as Wsl-1, Apo3, LARD, TRAMP, TNFRSF25, and TR3)1–6 is a death domain-containing member of the tumor necrosis factor receptor (TNFR) superfamily that is important for regulating both apoptosis and cell protection through activation of NF-κB.7,8 DR3 and its principal ligand, TNF-like factor 1A (TL1A), have been mainly described as being pro-inflammatory in human tissues9–11 and some mouse tissues,12–14 but other effects of DR3 have also been noted.1,15 We reported that DR3 induction and up-regulation occur in tubular epithelial cells (TECs) in human renal biopsy specimens, with evidence of acute allograft rejection and ischemic injury,16 and suggested that TL1A can both promote and protect against tubule cell injury immediately treated mouse kidney organ cultures.17 The importance of DR3 during pathogenic injury is unknown.

Cis-Diamminedichloroplatinum (II) (cisplatin or CDDP) is widely used for the treatment of solid tumors, but nephrotoxicity is the most common adverse effect limiting its clinical use.18,19 Renal toxicity induced by cisplatin is primarily associated with proximal tubular injury and decreased glomerular filtration, which progresses to acute renal failure.20 TNF-α is a key player in the inflammatory response during cisplatin nephrotoxicity and the ensuing kidney tissue damage and acute renal failure.21,22 Cisplatin also stimulates TNF production by increasing TNF mRNA stability in kidney proximal tubule cells.23–25 Moreover, direct involvement of Fas- and TNFR-mediated apoptotic pathways in cisplatin-induced renal tubular cell death has been reported.26 However, it remains unclear whether DR3 and its ligand, TL1A, are also implicated in cisplatin-induced nephrotoxicity.

To evaluate the physiological role of DR3 in renal injury induced by cisplatin nephrotoxicity, we used mice that are congenitally deficient in DR3 (DR3−/−). We show that signaling via DR3 is protective rather than prodeath, because the absence of DR3 exacerbates cisplatin-in-

Supplemental material for this article can be found on http://ajp.amjpathol.org or at doi:10.1016/j.ajpath.2012.01.003.
duced nephrotoxicity in mice and in an in vitro organ culture model. Moreover, the addition of TL1A to cultured kidney tubular cells inhibits cisplatin-induced apoptosis. In contrast, TNF is implicated in mediating cisplatin-induced kidney damage, because inhibition of its production in vivo using GM6001 reduces the extent of apoptotic death, and its addition to kidney tubular cells exacerbates cisplatin-induced cell damage. Given that TL1A can induce mild apoptosis when added to kidney organ cultures, we propose a novel role for DR3 in the context of kidney damage, by which TL1A acts as a partial agonist, thereby protecting against a more severe signal induced by TNF. Thus, although DR3 is a member of the death receptor family, DR3 agonists may protect against TNF-mediated kidney damage, and its stimulation may be of benefit in vivo.

Materials and Methods

Breeding and Maintenance of Mouse Strains

DR3−/− mice1 were bred into a CD1 background over >10 generations. DR3+/+, DR3−/−, and DR3−/− animals used in this study were bred from heterozygous parents. Experiments were subject to local ethical review and conducted according to personal, project, and institutional licenses under the UK Animals (Scientific Procedures) Act 1986. Mice (aged 8 to 13 weeks) received a single i.p. injection of cisplatin (20 mg/kg body weight) and additional licenses under the UK Animals (Scientific Procedures) Act 1986. Mice (aged 8 to 13 weeks) received a single i.p. injection of cisplatin (20 mg/kg body weight) using GM6001 reduces the extent of apoptotic death, and its addition to kidney tubular cells exacerbates cisplatin-induced cell damage. Given that TL1A can induce mild apoptosis when added to kidney organ cultures, we propose a novel role for DR3 in the context of kidney damage, by which TL1A acts as a partial agonist, thereby protecting against a more severe signal induced by TNF. Thus, although DR3 is a member of the death receptor family, DR3 agonists may protect against TNF-mediated kidney damage, and its stimulation may be of benefit in vivo.

Organ Cultures

Fragments of fresh kidney tissue from DR3+/+, DR3−/−, or DR3−/− mice (approximately 1 mm², in duplicate) were placed in flat-bottomed 96-well tissue culture plates and immediately immersed in medium 199 containing 10% heat-inactivated fetal calf serum and 2 mM/L L-glutamine. Tissue was incubated in 5% CO₂ incubator in either medium alone or medium containing 80 μmol/L cisplatin for 3 hours at 37°C. Tissue was fixed in 4% paraformaldehyde and paraffin embedded, as previously described.14,27

Cell Cultures

TKPTS cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 10 μg/mL insulin, 5.5 μg/mL transferrin, 5 ng/mL sodium selenite, and one times nonessential amino acids (Sigma-Aldrich, Poole, UK). Cells were grown to confluence in six-well plates and either left in medium alone (untreated) or treated with 100 ng/mL TL1A or 10 ng/mL TNF for 24 hours (concentrations that gave maximal responses). TKPTS were harvested on ice by scraping and washed twice with ice-cold PBS before analysis by immunoblotting.

Histological Analysis

Dewaxed sections of kidney tissues from the various mice were stained with H&E, as previously described.17 Sections were viewed using ×400 magnification on a Nikon Optiphot-2 microscope with a Pixera Pro 15 ES camera (Nikon, Kingston, UK). Tubular damage was scored for each animal according to whether damage was minimal (<10%) or areas of TEC degeneration were mild (<25%), moderate (25% to 50%), or severe (50% to 100%). Those conducting the histopathological examinations were blinded to the study treatments.

Immunostaining

Paraffin wax or frozen sections of kidney tissue from DR3+/+, DR3−/−, or DR3−/− mice or organ cultures were immunostained using previously described protocols.17,27 Antigen retrieval of cleaved caspase-3 involved an incubation in 50 μg/mL Proteinase-K (Sigma-Aldrich, Gillingham, UK) for 5 minutes in 0.1 mol/L Tris-HCl and 0.01% Tween-20; and for phospho-NF-κBp65Ser276, DR3, TNF, and TL1A, it involved 2 minutes in a pressure cooker containing 0.01 mol/L sodium citrate buffer, pH 6.0. Rabbit anti-cleaved caspase-3 (catalogue number 9661) or anti-phospho-NF-κBp65Ser276 (catalogue number 3037; both from New England Biolabs, Hitchin, UK), anti-rabbit-DR3 (catalogue number sc-7909; Insight Biotechnology, Wemley, UK) and mouse anti-cytokeratin (catalogue number M0821, 1:100; Dakocytomation, Ely, UK), or anti-TNF (ab6671; Abcam, Cambridge, UK) or mouse anti-hTL1A (IgG2α; provided by Human Genome Sciences, Rockville, MD) was incubated at 1:50 dilution in blocking buffer (0.1 mol/L Tris-HCl and 0.01% Tween-20 containing 10% fetal calf serum) overnight at 4°C. Sections labeled with DR3 or TL1A were further incubated in secondary antibodies (anti-mouse Alexa Fluor®680 or anti-rabbit Alexa Fluor®488, Invitrogen, Paisley, UK), which were applied at 1:100 for 1 hour at room temperature, and nuclei were visualized using a 1:100 dilution of To-Pro-3’-iodide and mounted using Vectashield Mounting Medium (Vector Laboratories, Peterborough, UK). Slides were viewed using a Leica TCS SPE laser-scanning confocal microscope (Leica Microsystems Ltd, Knowlhill, UK). Sections for active caspase-3, TNF, and phospho-NF-κBp65Ser276 were incubated with anti-rabbit horseradish peroxidase–conjugated secondary antibody (Dakocytomation) at 1:100 for 1 hour at room temperature. Antibody binding sites were visualized using 3,3’-diaminobenzidine solution (Sigma-Aldrich, Dorset, UK) containing 0.01% H₂O₂, followed by counterstaining in Mayer’s hematoxylin, and viewed using a Nikon Optiphot-2 microscope. To further quantify the intensity of
immunostaining, images of dianmibenidine-stained sections were converted to cyan, magenta, yellow, and black; and the yellow panel was converted to monochrome. Images were imported into ImageJ version 1.46f (NIH, Bethesda, MD), and the average intensity of the entire field was determined.

**TUNEL Staining**

Apoptotic cells were detected on paraffin wax sections of kidney tissue from DR3<sup>+/+</sup> and DR3<sup>−/−</sup> mice or organ cultures by TUNEL assay, as previously described. Briefly, sections were incubated for 30 minutes at 37°C with a fluorescein isothiocyanate–conjugated TUNEL label mixture and 0.8 U/μL TdT enzyme (Roche Diagnostics Ltd, Manheim, Germany). Negative controls included omission of the TdT enzyme in the TUNEL mixture. After washing in MilliQ water, sections were labeled with Hoechst 33342 ( Molecular Probes, Eugene, OR), mounted in Vector mounting medium (Molecular Probes), and viewed on a laser-scanning confocal microscope. TUNEL-positive TECs (presented as the apoptotic index) were counted in 10 fields at a high power (×40 magnification) in three different samples of kidney organ culture or in vivo mouse tissue from each of the three treatments to determine the percentage of TUNEL-positive cells with apoptotic morphological characteristics. TUNEL-positive cells within glomeruli were not counted.

**Immunoblotting**

Proteins were extracted from organ cultures stored in RNAlater or from TKPTS cell lines in a 1% NP40 lysis buffer containing protease and phosphatase inhibitor cocktails (Sigma-Aldrich), and supernatant protein was quantified using a BCA kit (Sigma-Aldrich) and separated by SDS-PAGE, loading 50 μg of total protein per lane. After transfer to a nitrocellulose membrane, proteins were probed with rabbit anti-cleaved caspase-3<sub>Asp175</sub> (1:1000) and signals were detected using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Hemel Hempstead, UK), according to the manufacturer’s instructions. Protein loading was normalized by probing the membrane with an anti-actin antibody (1:2000, Sigma-Aldrich).

**Statistical Analysis**

Multiple comparisons were analyzed using one-way analysis of variance, followed by Bonferroni post hoc test using GraphPad Prism version 5.02 (LaJolla, CA). Two-group comparisons were analyzed by Mann-Whitney U-test.

**Results**

**Cisplatin-Induced Apoptotic Death of TECs Is Exacerbated in DR3<sup>−/−</sup> Kidneys**

To examine the roles of DR3 in cisplatin-induced toxicity, wild-type (DR3<sup>+/+</sup>) and DR3-deficient (DR3<sup>−/−</sup>) mice were injected with 20 mg/mL cisplatin, and kidney damage was evaluated after 48 and 72 hours by histological analysis (Figure 1) and blood urea content. The onset of pathological features was apparent at 48 hours (data not shown) and more marked at 72 hours. At 72 hours, blood urea increased from a median of 41.8 mmol/L in cisplatin-treated DR3<sup>+/+</sup> mice (n = 4) to a median of 52.2 mmol/L in cisplatin-treated DR3<sup>−/−</sup> mice (n = 4). P = 0.024 for these two values being different by chance. Consistent with these data, kidneys from saline-treated control groups showed normal histological features, with no morphological evidence of apoptotic cell death, such as nuclear fragmentation (Figure 1, A and B). In contrast, TEC damage and death were manifested in cisplatin-treated DR3<sup>−/−</sup> mice (in >25% of tubules), which further increased in DR3<sup>−/−</sup> mice to >50% (Figure 1, C and D).

TNF may mediate apoptotic cell death in cisplatin-induced renal injury. To test whether TNF was implicated in our model, mice were treated with cisplatin and GM6001, a TNF-α converting enzyme inhibitor that prevents TNF maturation and release. GM6001 reduced cisplatin-induced tubular damage in both DR3<sup>+/+</sup> (which were indistinguishable from DR3<sup>−/−</sup> mice) to <10% and DR3<sup>−/−</sup> (to approximately 25%) (Figure 1, E and F). To demonstrate the efficacy of GM6001 in blocking TNF production, we examined the expression of TNF. Parallel sections to those shown in Figure 1 were stained for TNF expression. There was no detectable staining in saline-treated controls from either DR3<sup>+/+</sup> (Figure 2A) or DR3<sup>−/−</sup> mice (data not shown). In contrast, sections from both DR3<sup>+/+</sup> and DR3<sup>−/−</sup> showed a substantial 22-fold increase in TNF expression, with no statistically significant differences in expression between the two genotypes (Figure 2, B and C, quantified in Figure 2E). Consistent with the results showing reduced kidney damage in mice treated with cisplatin and GM6001 (Figure 1), there was an 80% decrease in the number of TNF-positive tubules in DR3<sup>−/−</sup> (data not shown) and DR3<sup>−/−</sup> (Figure 2D, quantified for both genotypes in Figure 2E) mice, thus supporting the idea that the major part of cisplatin-induced TEC damage is mediated by TNF.

To quantify the amount of death, and to determine whether death was mediated by apoptosis, we counted the number of positive tubules per field (10 fields at ×40 magnification) expressing active (cleaved) caspase-3<sub>Asp175</sub> and determining the proportion of individual TECs displaying nuclear DNA fragmentation using the TUNEL assay (Figure 2, F–O). At 72 hours, the number of tubules containing active caspase-3 increased significantly by 2.4-fold in cisplatin-treated DR3<sup>−/−</sup> mice (n = 4) compared with these data, kidneys from saline-treated control groups showed normal histological features, with no morphological evidence of apoptotic cell death, such as nuclear fragmentation (Figure 1, A and B). In contrast, TEC damage and death were manifested in cisplatin-treated DR3<sup>−/−</sup> mice (in >25% of tubules), which further increased in DR3<sup>−/−</sup> mice to >50% (Figure 1, C and D).

TNF may mediate apoptotic cell death in cisplatin-induced renal injury. To test whether TNF was implicated in our model, mice were treated with cisplatin and GM6001, a TNF-α converting enzyme inhibitor that prevents TNF maturation and release. GM6001 reduced cisplatin-induced tubular damage in both DR3<sup>+/+</sup> (which were indistinguishable from DR3<sup>−/−</sup> mice) to <10% and DR3<sup>−/−</sup> (to approximately 25%) (Figure 1, E and F). To demonstrate the efficacy of GM6001 in blocking TNF production, we examined the expression of TNF. Parallel sections to those shown in Figure 1 were stained for TNF expression. There was no detectable staining in saline-treated controls from either DR3<sup>+/+</sup> (Figure 2A) or DR3<sup>−/−</sup> mice (data not shown). In contrast, sections from both DR3<sup>+/+</sup> and DR3<sup>−/−</sup> showed a substantial 22-fold increase in TNF expression, with no statistically significant differences in expression between the two genotypes (Figure 2, B and C, quantified in Figure 2E). Consistent with the results showing reduced kidney damage in mice treated with cisplatin and GM6001 (Figure 1), there was an 80% decrease in the number of TNF-positive tubules in DR3<sup>−/−</sup> (data not shown) and DR3<sup>−/−</sup> (Figure 2D, quantified for both genotypes in Figure 2E) mice, thus supporting the idea that the major part of cisplatin-induced TEC damage is mediated by TNF.

To quantify the amount of death, and to determine whether death was mediated by apoptosis, we counted the number of positive tubules per field (10 fields at ×40 magnification) expressing active (cleaved) caspase-3<sub>Asp175</sub> and determined the proportion of individual TECs displaying nuclear DNA fragmentation using the TUNEL assay (Figure 2, F–O). At 72 hours, the number of tubules containing active caspase-3 increased significantly by 2.4-fold in cisplatin-treated DR3<sup>−/−</sup> mice, which further increased to 5.8-fold in kidneys from cisplatin-treated DR3<sup>−/−</sup> mice (Figure 2, F–J). Quantitatively similar ratios were obtained when the apoptotic index (proportion of TUNEL-positive TECs per tubule nuclei) was quantified (Figure 20; DR3<sup>+/+</sup>, 2.3-fold; DR3<sup>−/−</sup>, 5.1-fold), indicating that TEC death occurred largely by apoptosis. Furthermore, treatment with GM6001 also significantly reduced by 40% to 50% the amount of caspase-3 activation and TUNEL labeling (Figure 2, E and J) in DR3<sup>−/−</sup> and DR3<sup>+/+</sup> mice.

To further demonstrate that cisplatin-induced death was apoptotic, we treated Bax-knockout, DR3<sup>−/−</sup> mice with cisplatin. Bax is the key regulator of mitochondrial permeabilization-mediated apoptosis induced by TNF.
Little TEC death (<5%; see Supplemental Figure S1 at http://ajp.ampathol.org) was detected in cisplatin-treated Bax-deficient mouse kidneys at 72 hours, confirming that TEC death induced by the chosen concentration of cisplatin is executed by the intrinsic pathway of apoptosis, as previously suggested in a different mouse strain.33 There was, moreover, little evidence of necrosis using this concentration of cisplatin.

**Figure 1.** Representative light micrographs of H&E staining of saline- or cisplatin-treated DR3+/+ (A, C, and E) or DR3−/− (B, D, and F) kidneys collected at 72 hours. Both saline-treated control groups showed normal histological characteristics, with no obvious pathological changes (A and B). In contrast, cisplatin-treated DR3+/+ mice showed moderate tubular cell damage in the cortex (25% to 50%), which was enhanced to severe in cisplatin-treated DR3−/− mice (50% to 100%; C and D). DR3+/+ and DR3−/− mice treated with cisplatin and GM6001 showed reduced tubular cell damage, mild in the case of DR3+/+ (<25%) and moderate in the case of DR3−/− (25% to 50%; E and F). Insets: Arrows, Fragmented nuclei in some TECs. Original magnification, X400. Scale bar = 20 μm. Glom, glomerulus; tu, TECs.

Cisplatin Treatment Reduces the Phosphorylation of NF-κBser276 in DR3−/− Kidneys

We previously showed that NF-κB is a downstream effector of TNFR1 and DR3,17 whose activation counteracts cell death induced by TNF or TL1A in kidney organ cultures.17 Activated NF-κB is stabilized by phosphorylation and is then targeted to the nucleus, where it promotes the
Figure 2. The absence of DR3 exacerbates apoptosis of TECs in cisplatin-treated mice and reduces NF-κB activation without increasing TNF production. DR3+/+ (A, B, F, G, K, L, P, and Q) or DR3−/− (C, D, H, I, M, N, R, and S) mice were injected with 20 mg/mL cisplatin or saline i.p., and sections of kidneys were collected at 72 hours. Arrows in L, M, and N denote TUNEL-positive nuclei in TECs. Sections were probed with anti-TNF (A–D) or anti-active (cleaved) caspase-3Asp175 antibody (F–I), examined for TUNEL staining (K–N), or probed with anti-phospho-NF-κBp65Ser276 antibody (P–S). Representative images are shown from three different experiments that showed similar findings. Immunostaining was quantified for TNF (E), cleaved caspase-3Asp175 (J), TUNEL (O), or nuclear phospho-NF-κBp65Ser276 (T). Data are given as the mean ± SD (n = 3 mice treated with saline (Sal), cisplatin (Cis), or cisplatin and GM6001 (Cis+Gm)). Immunostaining was developed by diaminobenzidine, and sections were counterstained with hematoxylin. Data were quantified as the number of positive tubules per high-power field of view in 10 fields per section per mouse. TUNEL staining is presented as the apoptotic index [proportion of positive (green) TEC nuclei of total TEC nuclei stained with Hoechst (blue)]. There was increased apoptosis in DR3−/− compared with DR3+/+ kidneys, and the opposite trend was noted for phosphorylated NF-κB. There was also a reduction in the amount of TNF in Cis + Gm-treated samples (one-way analysis of variance and Bonferroni post hoc test). 

**U**: Immunoblot demonstrating increased levels of cisplatin-induced active caspase-3Asp175 in kidneys from DR3−/− compared with DR3+/+ mice (saline treated ++) and cisplatin treated (++). 

**E**: ***P < 0.0001 for Cis versus Sal; J**: ***P < 0.0001 for Cis versus Sal; O**: ***P < 0.0001 for Cis versus Sal; T**: ***P < 0.0001 for Cis+GmKO versus Cis+GmWT; **P < 0.001 for CisKO versus Cis+GmKO; and *P < 0.001 for Cis+GmKO versus Cis+GmWT. Original magnification, ×235. Scale bar = 20 μm. Glom, glomerulus; hpf, high-power field; KO, knockout; ns, not significant; WT, wild type.
expression of anti-apoptotic genes. To test whether the absence of DR3 altered the activation of NF-κB in response to cisplatin, we stained sections for the presence of nuclear phospho-NF-κBp65Ser276. The number of tubules per field, each showing >90% of cells with nuclear phospho-NF-κBp65Ser276, increased by eightfold in cisplatin-treated DR3+/+ kidneys, consistent with the idea that TNF and DR3 signaling was activated in the DR3+/+ mice (Figure 2, P–T). However, there was only a 3.2-fold increase in nuclear phospho-NF-κBp65Ser276 in cisplatin-treated DR3−/− kidneys, 60% less than in the DR3+/+ mice, indicating that NF-κB activation is mediated, in part, by DR3 signaling. Cotreatment with cisplatin and GM6001 caused a reduction in phospho-NF-κBp65Ser276 of 60% in DR3−/− animals, consistent with the idea that the signal is derived from TNF. However, the relative reduction in phospho-NF-κBp65Ser276 staining was much less in the DR3−/− TECs, in keeping with the idea that DR3 signaling is also responsible for a proportion of NF-κB activation. Thus, the reduced intensity of the NF-κBp65Ser276 signal is inversely correlated with the enhanced activation of caspase-3 and TUNEL staining observed in DR3−/− compared with DR3+/+ mice.

Immunoblot analysis of extracts from kidneys isolated from saline- or cisplatin-treated DR3+/+ and DR3−/− mice further confirmed increased levels of active caspase-3ΔAsp175 in cisplatin-treated DR3−/− compared with DR3+/+ mice (Figure 2U).

**Figure 3.** Cisplatin activates expression of DR3 and TL1A in TECs. Frozen kidney sections from mice injected with saline (A) or 20 mg/mL cisplatin i.p. (B, DR3+/+, C, DR3−/−) for 72 hours were costained for cytokeratin (red) and DR3 (green). Nuclei were stained with Hoechst (blue). Arrows indicate TECs, in which there is strong colocalization. D–F: Frozen kidney sections were stained for TL1A (green); nuclei are in blue (Hoechst). Original magnification, ×65. Scale bar = 100 μm. There is a similarity in induction of TL1A in DR3+/+ (E) and DR3−/− (F) kidneys compared with control (D). Images are representative of three different experiments with similar findings.

**DR3 and TL1A Expression Levels Are Induced by Cisplatin in Injured TECs**

DR3 and TL1A expression levels are enhanced in allograft-rejected human kidney transplant tissue. To investigate whether a similar event occurred in response to cisplatin, kidneys from saline- or cisplatin-treated DR3+/+ and DR3−/− mice were analyzed for co-expression of DR3 and cytokeratin immunocytochemically. As in human kidney, DR3 expression was rarely observed in saline-treated controls (Figure 3A) but was markedly up-regulated in cytokeratin-positive TECs in kidneys from cisplatin-treated DR3+/+ mice (Figure 3B). We confirmed that no DR3 expression was detected in cisplatin-treated DR3−/− kidneys (Figure 3C). Likewise, TL1A expression in TECs was also dramatically increased in TECs of cisplatin-treated mice and, interestingly, this enhanced expression occurred similarly irrespective of genotype (Fig-
Figure 4. Cisplatin-induced apoptosis is enhanced in DR3+/− organ cultures. Kidney organ cultures from DR3+/− or DR3−/− mice were either untreated or treated with 80 μmol/L cisplatin for 3 hours at 37°C and then immunostained for TNF, active caspase-3Asp175, TUNEL, or phospho-NF-κBser276. A rare signal for TNF is present in untreated DR3+/− or DR3−/− cultures, with a notable greater than sixfold increased signal detected in TECs of DR3−/− and DR3+/− mice (A–D). Similarly, staining for active caspase-3 (F–I) or TUNEL (K–N) is rare in TECs (arrows) in untreated cultures, with a sevenfold higher signal detected in cisplatin-treated DR3+/−, whose intensity is doubled to near 17-fold in DR3−/− mice. Original magnification, ×235. Scale bar = 20 μm. The opposite trend occurs with phospho-NF-κBser276 staining, in which there is more staining in DR3−/− compared with DR3+/− tubules (P–S). Quantitative analysis of immunostaining for TNF (E), active caspase-3Asp175 (J), TUNEL (O), and phospho-NF-κBser276 (T) of samples treated as previously noted, with 80 μmol/L cisplatin. Data are given as the mean ± SD (n = 3 animals each, one-way analysis of variance, Bonferroni post hoc test). ***P < 0.0001, †P < 0.0001 versus CisKO, and ‡P < 0.0001 versus UTKO. U: Immunoblot demonstrating increased levels of cisplatin-induced active caspase-3Asp175 in DR3−/− mice compared with that reduced in the presence of TL1A and exacerbated in the presence of TNF (one of three different experiments with similar findings). Cis, cisplatin; Glom, glomerulus; KO, knockout; ns, nonsignificant; UT, untreated; WT, wild type.
These data confirm the presence of a DR3 signaling pathway in DR3−/− TECs after treatment with cisplatin and support the idea that DR3 activation by TL1A occurs in response to renal injury.

Enhancement of Cisplatin-Induced Apoptosis in DR3−/− TECs Is Conserved in Organ Cultures

We previously showed that the increased apoptosis in DR3−/− kidneys was not because of the enhanced production of TNF, but this does not address whether the damage is systemic, possibly via an activation of an inflammatory immune response, or direct. We previously showed that death receptor signaling and apoptosis in kidney organ cultures is recapitulated within hours of addition of TNF; thus, we turned to organ cultures to investigate these questions. Kidney organ cultures from DR3−/− and DR3+/− mice were treated with 80 μmol/L cisplatin for 3 hours, and the number of TECs expressing TNF, active caspase-3Asp175, TUNEL, and phospho-NF-κBp65Ser276 was scored on immunostained slides. Cisplatin induced the same number of TNF-positive TECs per tubule in DR3−/− and DR3+/− kidneys (Figure 4, A–D; quantified in Figure 4E), confirming the idea that the higher responsiveness of DR3+/− cells to cisplatin is not because of the increased production of TNF. Likewise, cisplatin induced expression of active caspase-3 and TUNEL in cultures of both genotypes (Figure 4, F–I, quantified in Figure 4J; Figure 4, K–N, quantified in Figure 4O). However, despite the similar amounts of TNF, there was a further 2.5-fold increase in the number of TECs expressing active caspase-3Asp175 and TUNEL in the cisplatin-treated DR3−/− organ cultures, compared with the DR3+/+ tissue. Conversely, there was a significant reduction in the production of phospho-NF-κBp65Ser276 in DR3−/− TECs compared with DR3+/+ cells (Figure 4, P–S; quantified in Figure 4T). Images of fields of immunostained active caspase-3Asp175 and phospho-NF-κBp65Ser276 sections (60 to 80 tubules per field) were quantified in an unbiased manner using ImageJ analysis. Consistent with the counting data, average active caspase-3 intensity increased 3.1 ± 0.1-fold in cisplatin-treated DR3+/+ samples and doubled to 6 ± 0.2-fold in kidneys from DR3−/− mice. Conversely, the intensity of NF-κBp65Ser276 staining increased by 5.4 ± 0.2-fold in DR3+/+ kidneys but was reduced to 2.3 ± 0.1-fold in DR3−/− kidneys, consistent with counting data (see Supplemental Figure S2 at http://ajp.ampathol.org). Immunoblot analysis of extracts from the organ cultures further confirmed increased levels of active caspase-3Asp175 in cisplatin-treated DR3−/− compared with DR3+/+ organ cultures (Figure 4U).

Sections were also analyzed for expression of DR3 and TL1A. Figure 5 shows that expression of DR3 was increased by cisplatin treatment and that TL1A was induced to a similar extent in TECs of both genotypes. Also similar to the situation in vivo, there was a 7.5-fold increase in the amount of TUNEL staining in the cisplatin-treated DR3+/+ TECs, but this almost doubled to 14-fold...
in DR3−/− TECs, thus supporting the findings from our in vivo studies that cisplatin-induced death of TECs is a direct response and is mediated by apoptosis.

**TL1A Reduces, whereas TNF Increases, Cisplatin-Induced Apoptosis of Cultured Immortalized Proximal Tubular Cells (TKPTS)**

Although the data from the organ culture experiments suggest that systemic inflammation is not implicated in cisplatin-mediated renal injury and protection, we asked whether TL1A and TNF can directly affect cisplatin-induced toxicity to TECs. For this purpose, we used TKPTS cells, an immortalized mouse renal proximal tubular cell line. TKPTS cells were treated with 50 μmol/L cisplatin alone or cisplatin and 10 ng/mL TNF or 100 ng/mL TL1A (previously identified as saturating doses) for 24 hours and analyzed for the amount of caspase-3 cleavage by immunoblotting. As observed in the kidneys isolated from cisplatin-treated animals and in organ cultures, cisplatin induced a marked increase in caspase-3 in the cells. Moreover, apoptosis was further exacerbated by the addition of TNF but was attenuated by the addition of TL1A (Figure 6A). Neither ligand induced apoptosis on its own, as is commonly observed in isolated cells. Thus, when apoptosis is triggered by cisplatin, TNF and TL1A exert a direct cell autonomous effect on TECs, in which TNF activity is pro-apoptotic, whereas TL1A activity is anti-apoptotic.

**Discussion**

In this article, we investigated what role DR3 plays in renal injury, using cisplatin to induce controlled nephrotoxicity that results in apoptosis. Our principle findings are as follows. i) Cisplatin induces expression of DR3 and TL1A in TECs, as previously noted in human kidney after allograft rejection or ischemic injury. ii) The absence of DR3 enhanced apoptosis (eg, caspase-3 activation and TUNEL staining) in kidney TECs and increased blood urea levels in response to cisplatin, whereas the nuclear phospho-NF-κBp65Ser276 signal intensity was reduced in DR3−/− mice compared with cisplatin-treated DR3+/+ mice. A similar exacerbation of apoptotic cell death occurred in DR3−/− kidney organ cultures within 3 hours of cisplatin addition, demonstrating that the effect is direct rather than systemic. iii) DR3 acted by antagonizing the pro-apoptotic effects of TNF because cotreatment with cisplatin and GM6001, a TNF-α converting enzyme inhibitor that reduced the amount of mature TNF present in serum and kidney tissue of cisplatin-treated mice, and that induced TNF similarly in DR3−/+ and DR3−/− kidneys, reduced the amount of apoptotic death in TECs induced by cisplatin. iv) Finally, the pathways induced by TNF and TL1A act directly on TECs because TNF exacerbated, whereas the DR3 ligand TL1A inhibited, cisplatin-induced apoptosis in the proximal tubule cell line TKPTS. Neither ligand induced marked cell death in the absence of cisplatin, indicating that these receptors and pathways are particularly pertinent to renal injury.

Concomitant activation of caspase-3 and NF-κB is a typical hallmark of death receptor signals, especially those induced by TNFR superfamily members. NF-κB activation can counteract apoptosis induced by TNF and TL1A by inducing transcription of survival factors, including the caspase-8 inhibitor cFLICE inhibitory protein, A20, and mitogen-activated protein kinase–specific phosphatases, and microRNA-146 NF-κB–dependent induction of microRNA-146, an inhibitor targeted to signaling proteins of innate immune responses. The balance between the various signaling pathways downstream of their receptors (TNFR1/2 and DR3) determines the overall extent of cell death in cisplatin-treated kidneys, as in TNF signaling paradigms generally. Previously, we showed that NF-κB activation in response to TNF is anti-apoptotic in mouse kidney organ culture because drugs (eg, pyrrolidine dithiocarbamate or thalido-
mide) that blocked NF-κB activation, indicated by phospho-NF-κB Ser276 staining, exacerbated TNF-induced death. Although we use cisplatin to initiate renal damage in the present experiments, our data using GM6001 suggest that TNF is an important mediator of cisplatin-induced toxicity, in keeping with studies showing that cisplatin induces TNF mRNA and protein expression in TECs. In support, we show that mature TNF was highly up-regulated in TECs of both genotypes. TNF expression was markedly reduced when GM6001 was present during treatment with cisplatin, and cisplatin-induced apoptosis of TKTPS cells was exacerbated by adding TNF. The similarity in the amount of TNF produced in DR3−/− and DR3+/+ TECs excludes the possibility that the increased responsiveness of DR3+/+ tissue to cisplatin is the result of more production of TNF.

Against the backdrop of cisplatin and TNF signaling, DR3 appears to be anti-apoptotic. Thus, apoptosis induced by cisplatin was greatly enhanced in DR3−/− mice, and we noted a concomitant decrease in the number of cells displaying nuclear phospho-NF-κB Ser276. We also observed that the DR3 ligand TL1A reduced caspase-3 cleavage in cisplatin-treated TKTPS cells, consistent with our in vivo and organ culture data that DR3 signaling is protective. We previously showed that TL1A is capable of inducing mild caspase-3 Asp175 activation in mouse kidney organ cultures. Therefore, the question arises about why DR3 signaling is largely anti-apoptotic in the present study. In the previous study, we found that the extent of caspase-3 activity induced by TL1A was only approximately 25% of that induced by TNF, whereas the expression of phospho-NF-κB Ser276 was markedly enhanced. Our proposed explanation, shown schematically in Figure 6B, is as follows: on stimulation with cisplatin, the kidney produces high amounts of TNF that enhance caspase-3-dependent death while the anti-apoptotic signal mediated by NF-κB is relatively low. Up-regulated TL1A stimulates DR3, whose expression is also increased on TECs after injury. However, the predominant signal from DR3 is not activation of caspase-3 because this only occurs at approximately 25% the intensity of the signal activated by TNF. Rather, the added phosphorylation and activation of NF-κB are predominant, leading to protection against cisplatin/TNF-induced cell death. Thus, in the face of a strong apoptotic stimulus, such as that provided by cisplatin via TNF, the NF-κB moiety of DR3 signaling is more likely to have a strong anti-apoptotic impact and contribute to protection, whereas the impact of the weaker pro-apoptotic signaling component of DR3 is likely to be minor when this is added to the strong stimulus already produced by cisplatin/TNF. Thus, in this context, DR3 signaling is behaving essentially as a classic partial agonist (namely, weakly activating on its own) but antagonistic to a stronger signal that operates on the same pathway. To our knowledge, the present data describe a unique case in which one member of the TNFR superfamily (DR3) antagonizes the responses of another member of the same family (TNF) in the same target cells. There was a similarity between the large increase in DR3 expression that occurs on TECs in the cisplatin-treated wild-type animals and in allograft rejection in human transplanted kidney, suggesting that both injuries share common mechanisms. Our data suggest that DR3 agonists may be beneficial in attenuating cisplatin-induced nephrotoxicity by signaling within the kidney, in addition to the suggested use of agents that counteract immune cell responses for this purpose. Because renal injury can be fatal if left untreated, amelioration of the injury response via stimulation of DR3 externally is worthy of consideration.

Acknowledgments

We thank Prof. Elsa Bello-Reuss (Texas Tech University Health Sciences Center, Lubbock) for her generous gift of the TKTPS cells, the Tissue Bank (Department of Histopathology, University of Cambridge) for assistance with processing of tissue blocks, Keith Burling (University of Cambridge) for urea analysis, and the Animal House staff for their help with mouse husbandry.

References


