Kidney International, Vol. 67 (2005), pp. 1397–1409

IL-2–mediated apoptosis of kidney tubular epithelial cells is regulated by the caspase-8 inhibitor c-FLIP

CAIGAN DU, QIUNONG GUAN, ZIQIN YIN, ROBERT ZHONG, and ANTHONY M. JEVNIKAR

Department of Medicine, The University of Western Ontario, London, Canada; Department of Microbiology and Immunology, The University of Western Ontario, London, Canada; Department of Surgery, The University of Western Ontario, London, Canada; The Lawson Health Research Institute, London, Ontario, Canada; The Robarts Research Institute, London, Ontario, Canada; Multi-Organ Transplant Program, London Health Science Center, London, Ontario, Canada

IL-2–mediated apoptosis of kidney tubular epithelial cells is regulated by the caspase-8 inhibitor c-FLIP.

Background. Tubular epithelial cells (TECs) are essential in the maintenance of kidney function. Apoptosis of TECs occur during acute and chronic renal allograft rejection as well as other forms of renal injury, including autoimmune nephritis. The regulation of TEC apoptosis by proinflammatory cytokines associated with renal inflammation [e.g., interleukin (IL)-2 and interferon-gamma (IFN-γ)] has not been extensively investigated.

Methods. Apoptosis in murine TECs was determined by FACS with annexin-V or ligation-mediated-polymerase chain reaction (LM-PCR) and mRNA levels by reverse transcription (RT)-PCR or Northern blot. Protein expression was observed using Western blot.

Results. IL-2R (CD25) was expressed by murine TECs and up-regulated by IL-2. Both IL-2 and IFN-γ induced TEC apoptosis and activated caspase-8. Apoptosis with IL-2 was concentration-dependent and blocked by z-IETD-fmk, a specific caspase-8 inhibitor. Apoptosis with IFN-γ was associated with increased surface expression of Fas, while IL-2 had no effect on Fas. IL-2 did not induce apoptosis in Fas-deficient TECs (M3.1-*lpr*) suggesting IL-2 regulation of caspase-8 activity requires Fas. Consistent with this, IL-2 but not IFN-γ was found to decrease mRNA and protein expression of c-FLIP, an endogenous caspase-8 inhibitor in murine TECs. Overexpression of c-FLIP in TECs (CS3.7-FLIP) blocked apoptosis and caspase-8 activation with both IFN-γ and IL-2. c-FLIP expression was found in kidney cortex, primary and cloned TECs, suggesting c-FLIP is likely a key regulator of caspase-8–mediated apoptosis in vivo.

Conclusion. This is the first report of c-FLIP regulation by IL-2 in renal TECs. Augmentation of c-FLIP in TECs may enhance an endogenous mechanism by which TECs normally resist injury to caspase-8–mediated apoptosis and thus may be a useful and novel strategy to prevent tubular injury in transplant rejection and autoimmune nephritis.

Key words: Renal tubular epithelium, apoptosis, cytokine, caspase, c-FLIP, transplantation, nephritis.

Tubular epithelial cells (TECs) comprise the majority of renal parenchymal cells. Their susceptibility to injury impacts on renal function as tubular injury can be a primary cause for nephron loss [1]. Kidney cell death occurs by apoptosis or programmed cell death and necrosis (for review, see [2–4]), which have considerable overlap in etiologies and pathways [4]. Apoptosis is initiated by a variety of stimuli, including growth factor withdrawal, physical factors, and death receptor signals. The process is efficient and usually induces minimal inflammation. Apoptosis is frequently observed in renal injury, including ischemia [5], cisplatinum toxicity [6], proteinuria [6], obstruction [7], systemic sclerosis [8], calcineurin inhibitor toxicity [9], and allograft rejection (for review, see [2]). The short half-life of apoptotic cells in kidneys, however, can make the process inconspicuous [2]. While apoptosis is required for kidney remodeling and repair, excessive cell death can lead to loss of function or in the case of transplants, premature graft failure.

There is a growing subfamily of death receptors which belong to the tumor necrosis factor (TNF)/nerve growth factor receptor superfamily. CD95 (APO-1/Fas) is perhaps the best characterized in renal injury. Fas is highly expressed on many parenchymal cells, including tubular epithelium, and is up-regulated by cytokines such as interferon-gamma (IFN-γ). Other members of this family (TNF-R1, DR3, TRAIL-R1, TRAIL-R2, and DR6) [10] could also have important roles in renal injury as they share intracellular signaling pathways. Death receptors of this family are characterized by extracellular domain homology and a cytoplasmic death domain. Both receptor-related and receptor-independent apoptosis are mediated by members of the caspase family, a series of highly regulated intracellular cysteine proteases [11] which can be blocked by variably specific synthetic peptides such as YVAD, DEVD, IETD, and zVAD [12]. Binding to Fas promotes receptor oligomerization (trimerization) and cytoplasmic recruitment of several adaptor proteins and procaspases to a receptor complex,

death-inducing signaling complex (DISC) [13]. The Fas receptor DISC includes a Fas-associated death domain (FADD), an adaptor protein that contains a death domain that allows interaction with the death domain in death receptors, and a "death effector domain" (DED) that allows it to interact with DED domain-containing procaspase-8. In the DISC, a high concentration of procaspase-8 leads to self processing of the mature enzyme (caspase-8) which then cleaves downstream caspases [3, 6, 7]. On the molecular level, the apoptotic death program can be divided into three parts: initiation, execution, and termination. In most cases the execution phase is characterized by membrane inversion and exposure of phosphatidylserine, blebbing (zeiosis), dissipation of the mitochondrial transmembrane potential gradient, release of proteins such as cytochrome c and apoptosis inducing factor (AIF), fragmentation of the nucleus, chromatin condensation, and DNA degradation. In the termination phase, "apoptotic bodies" are engulfed by phagocytes [10]. The process is efficient and usually induces minimal inflammation, which would be important for efficient kidney repair and remodeling.

In previous work, we have demonstrated that the induction of TEC apoptosis by IFN-γ and TNF-α is dependent on Fas/FasL expression which allows a cell contact form of tubular self-injury or "fratricide" [14]. Given the serious consequences of indiscriminant binding to death receptors such as Fas, TECs must possess mechanisms of self-protection as they express both Fas and its ligand FasL. Furthermore, as Fas-mediated death utilizes caspase-8 activation, TECs must possess capacity to regulate potentially lethal caspase activity. c-FLIP is a major endogenous inhibitor of caspase-8, and has been shown to alter resistance of human renal carcinoma cells to chemotherapeutic-induced apoptosis [15]. In the present study, we demonstrate for the first time that both primary culture and cloned TECs express c-FLIP, suggesting c-FLIP is likely used by normal TECs for protection from apoptosis. We also show that interleukin (IL)-2 receptors expressed by TECs are up-regulated by IL-2 and we present the novel observation that IL-2 induces apoptosis in TECs through inhibition of endogenous c-FLIP likely by allowing Fas activation of caspase-8 rather than a direct effect on caspase-8 itself. These data suggest that augmentation of c-FLIP may enhance an endogenous mechanism used by TECs to resist injury to caspase-8–mediated apoptosis and thus may be a useful and novel strategy to prevent tubular injury during diverse forms of renal inflammation.

METHODS

Cells and reagents

Primary cultures of TECs were isolated and characterized from wild-type C3H-HeJ (H-2^k) using methods described previously [16]. Mice were handled in accordance with the Canadian Council on Animal Care Guidelines under protocols approved by the Animal Use Subcommittee at our institution. Cloned and immortalized TECs (H-2^k) were obtained using origin defective SV40 DNA as described from C3H-HeJ (CS3.7) mice and were used as well as primary cells in experiments as indicated in results. CS3.7 TECs are highly differentiated proximal tubular cells [17]. M3.1-*lpr* TECs were similarly derived using Fas-deficient MRL-*lpr* mice and cloned and immortalized as above. All TECs were grown in complete K1 culture medium [Dulbecco's modified Eagle's medium (DMEM):Hams F12] (50:50) (Invitrogen-Gibco, Carlsbad, CA, USA), supplemented with 5% bovine calf serum, hormone mix [5 μg/mL insulin, 1.25 ng/mL prostaglandin E_1 (PGE_1), 34 pg/mL triiodothyronine, 5 μg/mL transferrin, 1.73 ng/mL sodium selenite, and 18 ng/mL of hydrocortisone) and 25 ng/mL epidermal growth factor (EGF). Cells were grown and passaged on 100 mm plastic tissue culture plates (Sarstedt, Inc., Newton, NC, USA) and removed by brief trypsinization. Media were replaced in confluent monolayer cultures before experiments with K1 media without serum or growth factors to arrest cell division. TECs were cultured for up to 24 hours during apoptosis assays. Recombinant mouse IFN-γ and TNF-α, anti-Fas antibody (Jo2 clone, for induction of apoptosis and FACS analysis) and anti-CD25 (7D4 clone, murine high-affinity IL-2R) were purchased from BD PharMingen (Mississauga, Ontario, Canada). Recombinant mouse IL-2 was purchased from R&D Systems (Minneapolis, MN, USA). Rabbit anti-Fas (M20) and anti-FasL (C178) IgG for Western blot analyses were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-c-FLIP polyclonal antibody was obtained from Stressgen Biotech (Victoria, British Columbia, Canada). Specific primer sequences for reverse transcription-polymerase chain reaction (RT-PCR) were designed using MacVector 6.5 software (Accelrys, San Diego, CA, USA), for murine IL-2R and control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA: IL-2Rα chain (584 bp) sense 5′-CAG CCT GGT CTA CAA AGT AAG TGC C-3′, antisense 5′-TCT ATG AAG CCT TGG TTG CCC-3′; IL-2Rβ chain (506 bp) sense 5′-CGT AGG GTA AAG ACC TGC GAC TTC-3′, antisense 5′-GGG ATG TGG CAC TTG AGA ACT G-3′; IL-2Rγ chain (355 bp) sense 5′-TGA CTT CTA CAG CCC CTG AAC ACC-3′, antisense 5′-AGA TTT TCT GGA GCC CGT GG-3′; and GAPDH (430 bp product) sense 5′-ATC ACT GCC ACC CAG AAG ACT G-3′, antisense 5′-CCC TGT TGC TGT AGC CGT ATT C-3′.

Overexpression of c-FLIP in TECs

Full length murine cFLIP-$_L$ cDNA, was generously provided by Dr. J. Tschopp (University of Lausanne, Switzerland). It was subcloned into a modified herpes