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Differential activation of CD95-mediated apoptosis related proteins in proximal and distal tubules during rat renal development

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ABSTRACT

The CD95-mediated apoptotic pathway is the best characterized of the death receptor-mediated apoptotic pathways. The present study characterized localization and expression of proteins involved in CD95-mediated apoptosis during rat renal development. Kidneys were obtained from embryonic (E) 18 and 20-day-old fetuses and postnatal (P) 1-, 3-, 5-, 7-, 14-, and 21-day-old pups. Immunohistochemical characterization revealed that CD95, FasL and cleaved caspase-3 were strongly expressed in proximal tubules and weakly expressed in distal tubules, but that expression of caspase-8 in distal tubules was stronger than that in proximal tubules. Results from terminal deoxynucleotidyl transferase dUTP nick end labeling assays showed that levels of apoptosis in proximal tubules slowly increased after E18, while those of distal tubules slowly decreased after P5. Western blotting demonstrated that expression of CD95, FasL and FADD was very weak during embryonic development, but rapidly increased at P14. Expression of cleaved caspase-3 was maintained at high levels after P1, while caspase-8 expression gradually reached a peak at P7. Results from this study reveal that the CD95-mediated apoptotic pathway is a key driver of apoptosis in proximal tubules during late postnatal kidney development in rats and suggest that apoptosis in distal tubules is mediated by a different apoptotic pathway.

1. Introduction

Tissue homeostasis reflects a balance of cellular proliferation, death, and differentiation (Vukusic Pusic et al., 2013; Lu et al., 2014; Yamaguchi and Miura, 2015). Apoptosis, or programmed cell death, plays a variety of crucial roles in multicellular organisms, including helping to maintain the balance between cell proliferation and death, regulating proper development, and protecting against disease (Musial and Zwolinska, 2011; Melo et al., 2015). Inappropriate regulation of apoptosis is associated with a wide variety of pathological disorders, such as cancer, lymphocyte depletion, and degenerative diseases (Kim et al., 2013; Wang et al., 2014). Because of the critical nature of apoptosis, it is tightly regulated at multiple levels, each of which is mediated by families of related molecules (Hovater and Sanders, 2012). Apoptotic pathways can be divided into the caspase-independent and caspase-dependent pathways, and the death receptor-mediated pathway is one of the best characterized of the caspase-dependent pathways (Li et al., 2014; Wang et al., 2001).

The death receptor apoptotic pathway is initiated by activation of the CD95 death receptor through binding to FasL expressed by an adjacent cell (Sanchez-Nino et al., 2010). CD95, the Fas death receptor, is a key physiological regulator of cell death that activates a signal transduction pathway that results in apoptosis of CD95-bearing cells. Binding of CD95 to FasL leads to the assembly of the multi-component death-inducing signaling complex (DISC) at the cell membrane. The DISC, in turn, binds specialized domains present in several intracellular proteins, collectively referred to as adaptor molecules, such as the Fas-associated death domain (FADD) protein (Gonzalvez et al., 2012; Majkut et al., 2014). Upon binding DISC, FADD serves as a bridge between the death receptors and the initiator caspase pro-caspase-8. Recruitment of pro-caspase-8 to the complex induces cleavage-mediated activation of caspase-8 and initiates a downstream caspase cascade involving the effector caspase-3 that leads to apoptosis (Monian and Jiang, 2016).

CD95 and related downstream signaling proteins are thought to be the primary regulators of lymphoid cell apoptosis (Nagata, 1996; Karev, 2014) and have also been implicated in the induction

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of apoptosis in many other tissues and organs, as well as several renal diseases including chronic renal failure and ischemia reperfusion injury (Leh et al., 2011; Kaushal, 2012; Wang et al., 2014). However, little is known about the mechanisms that promote tubular apoptosis or the importance of apoptotic signaling during renal development. Furthermore, the evidence available regarding regulation of apoptosis in the kidney is somewhat controversial (Bedir et al., 2015; Bonegio et al., 2011; Fainberg et al., 2013; Huang et al., 2013; van de Wetering et al., 2011). For example, Kim et al. (2000) reported that FasL is constitutively expressed in all nephron segments, but that Fas is expressed in all tubules, except for glomeruli (Kim et al., 2000). However, our previous study used immunohistochemistry and three-dimensional computer tracking to reveal that the apoptotic regulators of Bcl-2 and Bax were strongly expressed in proximal tubules, but only weakly in distal tubules (Song et al., 2012).

Thus, to better characterize differential expression of apoptosis-related proteins in proximal and distal tubules and to further clarify the roles of them these proteins in rats kidney development, in the present study, we have used immunohistochemical analysis, Western blotting, and TUNEL assays to create a detailed map of expression of protein involved in CD95-mediated apoptosis during rat renal development.

2. Material and methods

2.1. Animals

Kidneys were isolated from 10 prenatal and 30 postnatal Sprague-Dawley rats maintained under standard pathogen-free conditions. Embryonic day 0 (E0) was designated as the first day on which the cervical mucus plug was observed in the pregnant rat, and the day of birth was designated as postnatal day 0 (P0). On days E18, E20 P1, P3, P5, P7, P14, and P21, kidneys were collected from 5 rats each from a separate litter. Animal experiments were performed in accordance with the code of Ethics of the World Medical Association (Declaration of Helsinki) and were approved by the Medical Ethics Committee of Jinzhou Medical University.

2.2. Antibodies

Rabbit polyclonal antibody against CD95, Rabbit polyclonal antibody against FasL and Rabbit polyclonal antibody against caspase-8 were purchased from Abcam. Rabbit monoclonal antibody against cleaved caspase-3 was purchased from Cell Signaling Technology. Goat polyclonal antibody against FADD and Monoclonal anti-β-actin antibody produced in mouse were purchased from Santa Cruz Biotechnology.

2.3. Preparation of renal tissues

Fetal kidneys were extracted several minutes after injection of pentobarbital sodium (50 mg/kg body weight) into the peritoneal cavities of the pregnant mice. Pup kidneys were removed several minutes after inhalation of diethyl ether. The left kidneys from each rat were fixed in 4% paraformaldehyde and stored at 4 °C for immunohistochemical analysis. The right renal cortices were dissected under a microscope, frozen in liquid nitrogen, and stored at −80 °C for protein extraction.

Kidneys used for immunohistochemical studies were dehydrated in an ascending ethanol series, cleared in xylene and embedded in paraffin wax. A microtome was used to generate consecutive serial sections (4-μm thickness), which were subsequently mounted on poly-L-lysine-coated slides.

2.4. Immunohistochemistry

Tissue sections prepared as described above were stained using the Envision Dual Link System-HRP. Briefly, deparaffinized kidney sections were heated in a microwave oven for antigen retrieval. The sections were then incubated with rabbit anti-CD95 IgG (1:50), rabbit anti-FasL IgG (1:100), rabbit anti-caspase-8 IgG (1 μg/ml), or rabbit anti-cleaved-caspase-3 IgG (1:200) overnight at 4 °C. Sections incubated in phosphate-buffered saline (PBS) instead of primary antibodies served as negative controls. The following day, sections were incubated with peroxidase-conjugated secondary anti-rabbit antibodies (1:200) for 30 min at 37 °C, followed by visualization with diaminobenzidine (DAB). The sections were counterstained with hematoxylin for less than 2 min, rinsed in running water, dehydrated in graded ethanol, soaked in xylene, and mounted with Eukitt. Images were captured using a light microscope equipped with a Nikon DS digital camera.

The numbers of cells positive for expression of caspase-8, CD95, FasL and cleaved caspae-3 in the proximal and distal tubules were quantified in 10 randomly selected high power fields by a blinded observer. The results from this analysis are expressed as a percentage of the total number of proximal or distal tubular cells counted. Subsequently, the results are versus E18 proximal or distal tubule samples.

2.5. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) analysis

DNA fragmentation in apoptotic cells was detected using TUNEL staining kit (*In situ* Cell Death Detection kit, POD, Roche). Paraffin-embedded sections were deparaffinized in xylene and rehydrated by incubation in a graded ethanol series, followed by immersion in PBS. Tissue sections were then permeabilized by incubation with proteinase K working solution for 30 min at 37 °C. Sections were then treated with the TUNEL reaction mixture for 30 min at 37 °C, followed by visualization with DAB. After three rins with PBS, sections were counterstained with hematoxylin for 2 min, dehydrated in a graded ethanol series, soaked in xylene, and mounted with Eukitt.

The number of TUNEL-positive cells and total cells in the proximal and distal tubules were counted in 10 random fields from in the cortical region. Cells considered TUNEL-positive included cells with darkly stained nuclei, lightly stained nuclei, and pyknotic nuclei with apoptotic bodies. The resulting data are expressed as percentages of total tubular cells counted, and then versus E18 proximal or distal tubule samples.

2.6. Western blot analysis

Kidney samples were minced into small pieces, homogenized in lysis buffer [150 mM NaCl, 50 mM Tris-HCL, 1% Nonidet P-40, 0.25% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mg/ml leupeptin, 1 mM Na_3VO_4, 1 mM NaF], and incubated overnight at 4 °C. The homogenate was then centrifuged at 12,000 rpm for 30 min, and the clarified supernatant was aliquoted and frozen at −80 °C. Protein extracts (50 μg) were resolved on SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (Millipore, CA). Nonspecific binding sites were blocked in Tris-buffered saline/0.1% Tween-20 (TBST) containing 5% bovine serum albumin for 1 h at room temperature. The membranes were then incubated overnight at 4 °C with the following primary antibodies: rabbit anti-CD95 (1:1000), rabbit anti-FasL (1 μg/ml), goat anti-FADD (1:500), rabbit anti-caspase-8 (0.5 μg/ml), rabbit anti-cleaved-caspase-3 (1:1000) and mouse anti-β-actin antibody (1:5000).