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Pivotal Role of Apoptosis Signal-Regulating Kinase 1 in Monoclonal Free Light Chain–Mediated Apoptosis

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Renal failure, a major complication associated with multiple myeloma, is usually related to deposition of monoclonal immunoglobulin free light chains (FLCs) and directly contributes to morbidity and mortality in this disease. The present study focused on the cytotoxic effects of monoclonal FLCs. Human proximal tubular epithelial cells (HK-2) were examined after incubation with two human monoclonal FLCs (termed κ2 and λ3). Incubation of HK-2 cells for 24 and 48 hours with either FLCs at 1 mg/mL promoted activation of caspase-9 and caspase-3 and increased the rate of apoptosis. Because prior studies demonstrated that FLCs generated intracellular oxidative stress, our studies focused on the redox-sensitive mitogen-activated protein kinase kinase kinase known as apoptosis signal-regulating kinase 1 (ASK1). A time-dependent increase in phosphorylation of ASK1 at T845, indicating activation of this enzyme, was observed. Small interfering RNA designed to reduce ASK1 expression in HK-2 cells successfully decreased ASK1, which was confirmed by Western blot analysis. Incubation of ASK1-depleted HK-2 cells with the two FLCs prevented the increase in apoptosis while pretreating HK-2 cell with nontargeting small interfering RNA did not prevent FLCs-mediated apoptosis. The combined data demonstrate that monoclonal FLCs activated the intrinsic apoptotic pathway in renal epithelial cells by activation of ASK1. ***(Am J Pathol 2012, 180:41–47; DOI: 10.1016/j.ajpath.2011.09.017)***

A major function of proximal tubular epithelium is reabsorption of proteins that are present in glomerular ultrafiltrate. This process integrally involves the heteromeric receptor composed of megalin and cubilin.[1–4] As low-molecular-weight proteins, immunoglobulin free light chains (FLCs) are filtered relatively freely and are presented to the proximal tubule. Unlike other low-molecular-weight proteins, however, monoclonal FLCs have high nephrotoxic potential.[5–8] Batuman's laboratory in particular has demonstrated that monoclonal FLCs are directly cytotoxic, promoting apoptosis of proximal tubular cells. Apoptosis required endocytosis of the FLCs and subsequent activation of mitogen-activated protein (MAP) kinase pathways.[9–12]

A novel human protein kinase, apoptosis signal-regulating kinase 1 (ASK1, alias MAP3K5, MEKK5, and MAPKKK5) was cloned in 1996 and was found to function as a MAP kinase kinase kinase (MAP3K).[13] This ubiquitously expressed MAP3K functions as an upstream activator of the c-Jun N-terminal kinase and p38 MAP kinase pathways.[14,15] Overexpression of ASK1 promotes apoptosis specifically by inducing Bax translocation and cytochrome *c* release from mitochondria and activation of caspase-9 and caspase-3.[16] ASK1 is inhibited by association with reduced cytoplasmic thioredoxin-1 and mitochondrial thioredoxin-2.[17,18] Reactive oxygen species, particularly hydrogen peroxide, oxidize thioredoxin, releasing ASK1 and permitting phosphorylation at T845 and activation of this kinase, which results in apoptosis.[19–22] ASK1 is also involved in promoting release of inflammatory molecules in ischemic events that include acute kidney injury.[23,24] Intriguingly, protein kinase B (Akt) phosphorylates ASK1 at S83, which mitigates ASK1-mediated apoptosis.[25] Thus, ASK1 is a highly regulated key element in stress-induced apoptosis.

The redox state of the cell modulates signal transduction activity and is a critical determinant of cell survival.[26] Recently, endocytosis of monoclonal FLCs has been

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shown to generate intracellular oxidative stress sufficient to activate c-Src, the 60-kDa product of *c-src*, also known as $pp60^{c\text{-}src}$, and the NF-κB pathway.[27–29] We tested the hypothesis that monoclonal FLCs promotes apoptosis of renal epithelial cells through activation of ASK1.

Materials and Methods

Cells and Reagents

Human Immunoglobulin Monoclonal FLCs

Two unique monoclonal FLCs, one κ and the other λ, labeled κ2 and λ3, respectively, were purified using standard methods from the urine of patients who had multiple myeloma and light chain proteinuria.[7] These patients had clinical evidence of renal damage, although renal biopsy was not performed. The FLCs were endotoxin-free (Limulus Amebocyte Lysate, QCL-1000; Lonza, Walkersville, MD) and observed to generate H_2O_2 and promote intracellular oxidative stress in human proximal tubular epithelial cells (HK-2) cells in culture.[27]

HK-2 Cells

HK-2 cells, which have previously been characterized,[30] were obtained from the American Type Culture Collection (Manassas, VA). Monolayers of HK-2 cells were grown on six-well plates (Corning-Costar; Corning Incorporated Life Sciences, Lowell, MA) that were precoated with 5 μg/cm^2 type 1 collagen (rat tail collagen type 1; Invitrogen Corporation, Carlsbad, CA), and incubated at 37°C with 5% CO_2/95% air in keratinocyte serum-free medium (Gibco, Invitrogen Corporation, Carlsbad, CA) supplemented with recombinant human epidermal growth factor (5 ng/mL) and bovine pituitary extract (50 μg/mL). Medium was exchanged at 48-hour intervals, and cells were not used beyond 25 to 30 passages. In the present experiments, confluent cell monolayers were incubated at 37°C in medium containing a unique FLCs, 1 mg/mL, for 24 and 48 hours before study. This FLCs concentration was within the expected concentration range to which proximal tubular cells are exposed in patients with multiple myeloma.[31]

To suppress c-Src activity in HK-2 cells in some experiments, simultaneously with the addition of the FLCs, 4-amino-5-(4-chlorophenyl)−7-(*tert*-butyl)pyrazolo [3,4-*d*] pyramidine (PP2; EMD Biosciences, Gibbstown, NJ) was added to the medium in a final concentration of 10 μmol/L.[32]

Western Blot Analyses

After incubation, cells were lysed in radioimmunoprecipitation assay buffer containing a protease inhibitor cocktail (Complete; Roche, Indianapolis, IN) and clarified by centrifugation; lysates were then stored at −70°C until they were assayed. Total soluble proteins in lysates were determined with the BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL). Protein extracts (20 to 60 μg) were boiled for 3 minutes in Laemmli buffer and separated by 7% to 12% SDS-PAGE (BioRad Laboratories, Hercules, CA) before transfer onto polyvinylidene diflouride membranes. The membranes were blocked in 5% skim milk and incubated at 4°C overnight with one of the following primary antibodies: rabbit–anti-human polyclonal antibody to ASK1, phospho-ASK1 (T845), and phospho-ASK1 (S83); all were obtained from Cell Signaling Technology (Danvers, MA). Glyceraldehyde 3-phosphate dehydrogenase, determined using mouse anti-human glyceraldehyde 3-phosphate dehydrogenase (Abcam Inc., Cambridge, MA), served as a loading normalization control. Gels were developed in standard fashion using enhanced chemiluminescence (SuperSignal West Dura Chemiluminescent Substrate; Pierce Biotechnology), and densitometry was performed by Quantity One software (BioRad Laboratories).

Determination of Cytoplasmic Caspase-9 Activity and Concentration

Cytoplasmic caspase-9 activity was quantified with a fluorometric assay (Caspase-9 Activity Assay Kit; Calbiochem EMD Chemicals Inc., Darmstadt, Germany), following the protocol provided by the manufacturer. Briefly, caspase-9 activity was detected in cell lysates by using LEHD peptide substrate labeled with a fluorophore, 7-amino-4-trifluoromethyl coumarin. Cytoplasm was collected from pelleted cells using kit lysis buffer supplemented with protease inhibitors (Complete Protease Inhibitor tablets; Roche Diagnostics GmbH, Mannheim, Germany) and dithiothreitol (Sigma-Aldrich, St. Louis, MO). Lysates were added with substrate into a 96-well plate. The kit provided both a positive control, which consisted of frozen HL-60 cells previously cultured and treated by the vendor with 0.5 μg/mL actinomycin D for 19 hours to induce apoptosis, and a negative control that used the same cells also treated with a specific caspase-9 inhibitor. After incubation, caspase-9 activity was quantified with a fluorescent plate reader (Spectramax $M2^e$ Microplate Reader; Molecular Devices, Sunnyvale, CA) with an excitation of 400 nm and emission at 505 nm.

Cytoplasmic caspase-9 concentration was determined with an enzyme-linked immunosorbent assay (ELISA) (Human Caspase-9 ELISA; BioVendor Research and Diagnostic Products, Candler, NC), following the protocol provided by the manufacturer. Briefly, collected cells were pelleted and the pellets were resuspended in kit lysis buffer at a concentration of 5×10^6 cells/mL. The lysates were added to an antibody-coated 96-well plate and then incubated with the detection antibody at room temperature for 2 hours. Anti-rabbit horseradish peroxidase antibody was then added to all wells, followed by 3,3′,5,5′-tetramethylbenzidine substrate solution. Caspase-9 levels were quantified with a colorimetric plate reader (Molecular Devices Spectramax $M2^e$ reader) at 450 nm.