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Macrophage-stimulating protein attenuates gentamicin-induced inflammation and apoptosis in human renal proximal tubular epithelial cells

Ko Eun Lee [a], Eun Young Kim [b], Chang Seong Kim [a], Joon Seok Choi [a], Eun Hui Bae [a], Seong Kwon Ma [a], Kyung Keun Kim [c], Jong Un Lee [b], Soo Wan Kim [a,*]

[a] Department of Internal Medicine, Chonnam National University Medical School, Gwangju 501-757, Republic of Korea
[b] Department of Physiology, Chonnam National University Medical School, Gwangju 501-757, Republic of Korea
[c] Department of Pharmacology, Chonnam National University Medical School, Gwangju 501-757, Republic of Korea

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ABSTRACT

The present study aimed to investigate whether macrophage-stimulating protein (MSP) treatment attenuates renal apoptosis and inflammation in gentamicin (GM)-induced tubule injury and its underlying molecular mechanisms. To examine changes in MSP and its receptor, recepteur d'origine nantais (RON) in GM-induced nephropathy, rats were injected with GM for 7 days. Human renal proximal tubular epithelial (HK-2) cells were incubated with GM for 24 h in the presence of different concentrations of MSP and cell viability was measured by MTT assay. Apoptosis was determined by flow cytometry of cells stained with fluorescein isothiocyanate-conjugated annexin V protein and propidium iodide. Expression of Bcl-2, Bax, caspase-3, cyclooxygenase (COX)-2, inducible nitric oxide synthase (iNOS), nuclear factor-kappa B (NF-κB), IκB-α, and mitogen-activated protein kinases (MAPKs) was analyzed by semiquantitative immunoblotting. MSP and RON expression was significantly greater in GM-treated rats, than in untreated controls. GM-treatment reduced HK-2 cell viability, an effect that was counteracted by MSP. Flow cytometry and DAPI staining revealed GM-induced apoptosis was prevented by MSP. GM reduced expression of anti-apoptotic protein Bcl-2 and induced expression of Bax and cleaved caspase 3; these effects and GM-induced expression of COX-2 and iNOS were also attenuated by MSP. GM caused MSP-reversible induction of phospho-ERK, phospho-JNK, and phospho-p38. GM induced NF-κB activation and degradation of IκB-α; the increase in nuclear NF-κB was blocked by inhibitors of ERK, JNK, p-38, or MSP pretreatment. These findings suggest that MSP attenuates GM-induced inflammation and apoptosis by inhibition of the MAPKs/NF-κB signaling pathways.

1. Introduction

Gentamicin (GM), one of the aminoglycoside antibiotics, is widely used to treat gram-negative bacterial infections; however, clinical use is limited by its potential nephrotoxicity. GM-induced nephropathy is characterized by proximal tubular apoptosis or necrosis and provokes acute kidney injury [1]. GM-induced renal toxicity is caused by formation of reactive oxygen species, transforming growth factor-β1 secretion [2,3]. Furthermore, GM preferentially accumulates in the renal proximal convoluted tubules localized with endosomal and lysosomal vacuoles, which may induce tubulointerstitial inflammation and apoptosis [2,4].

A number of growth factors that influence cell division and differentiation participate in kidney injury and tissue repair. Epidermal growth factor and hepatocyte growth factor regulate tubular regeneration and repair [5–7]. Macrophage-stimulating protein (MSP) is a plasminogen-related growth factor and a member of the serine protease family [8]. MSP is mainly synthesized in hepatocytes and secreted into the circulation; it is also produced in renal epithelial cells [9]. Due to its mitogenic potential, MSP may play an important role in tubular regeneration and repair. Several studies have indicated that MSP has multiple biological activities, such as inhibition of nitric oxide production in LPS-stimulated mouse macrophages and antiapoptotic activity in cisplatin-induced and renal ischemic reperfusion injury [10–12].

The MSP receptor, recepteur d'origine nantais (RON), is a transmembrane tyrosine kinase and a member of the c-met proto-oncogene family. RON is expressed in a variety of tissues during development, but is expressed mainly on epithelial cells [13]. MSP binding causes RON dimerization and phosphorylation [14].

* Corresponding author. Address: Department of Internal Medicine, Chonnam National University Medical School, 42 Jebongro, Gwangju 501-757, Republic of Korea. Fax: +82 62 2258578.
E-mail addresses: skimw@chonnam.ac.kr, kdksw@hanmail.net (S.W. Kim).

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Recently, it has been suggested that MSP and RON attenuate the inflammatory response. RON-deficient mice exhibited more severe acute lung injury in response to LPS [15]. MSP and RON are upregulated during the remodeling that occurs during skin excisional wound repair [16]. RON is also expressed in glomerular mesangial and tubular epithelial cells, and the MSP/RON system may influence inflammatory processes in the kidney [9]. On the other hand, MSP induces superoxide anion production, leading to respiratory burst in human macrophages [17]. MSP neutralization is also protective against glomerular injury in anti-Thy 1 glomerulonephritis by inhibiting monocyte recruitment and reducing inflammation [18]. Thus, future investigations must define the physiological role of MSP/RON and its molecular mechanisms in kidney diseases.

The purpose of this study was to investigate changes in the MSP/RON system in GM-induced nephropathy and to determine whether MSP treatment attenuates apoptosis and inflammation in GM-induced tubular injury.

2. Materials and methods

2.1. Animal model

Animal experiments were performed in accordance with the Ethics Committee of Chonnam National University Medical School. All rats weighed 200–220 g at the start of the experiment. Gentamicin (GM, Choongwae Pharma Co., Seoul, Korea, 150 mg/(kg day), $n = 5$) was injected once a day intramuscularly (i.m.) over 7 days. Control rats ($n = 5$) received vehicle alone (i.e., sterile 0.9% saline, i.m.). Rats were maintained on a standard rodent diet and allowed free access to drinking water. In the control group, rats were offered the amount of food corresponding to the mean intake of food consumed by gentamicin-treated rats during the previous day (pair-feeding). Thus, food intake was matched between groups. On the day of the experiment, the rats were anesthetized with isoflurane. Blood samples were collected from the inferior vena cava and analyzed for creatinine. The right kidney was rapidly removed, dissected into cortex/outer stripe of outer medulla (OSOM), and processed for semiquantitative immunoblotting as described below. Plasma creatinine in rats was measured using the Jaffe method (Olympus 5431; Olympus Optical, Tokyo, Japan).

2.2. Cell culture and reagents

Human renal proximal tubular epithelial cells (HK-2; ATCC, Manassas, VA) were cultured and passaged every 3–4 days in 100-mm dishes containing combined Dulbecco's modified Eagle's medium-F12 (Sigma, St Louis, MO) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma–Aldrich). The cells were treated with GM in the presence or absence of MSP (R&D systems, Minneapolis, MN, USA).

2.3. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Cells were grown to confluence in DMEM/F-12 medium containing 10% FBS, harvested by trypsinization, and plated at 5×10^3 cells/well in a 96-well plate. To examine the effect of MSP, the cells were incubated for 24 h in the presence or absence of MSP (10, 30, and 50 ng/ml) for 1 h prior to exposure to GM. Cell viability was determined by MTT assay. After incubation, 50 μl of 5 mg/ml MTT (Sigma–Aldrich) were added to each well and incubated for 3 h at 37 °C. Supernatants were removed by aspiration and dimethyl sulfoxide was added to dissolve the precipitated dyes. Absorbance at 570 nm was measured on an ELISA reader (Biotek Inc., Winooski, VT, USA).

2.4. Protein extraction and semiquantitative immunoblotting

The dissected cortex/OSOM was homogenized in ice-cold isolation solution containing 0.3 M sucrose, 25 mM imidazole, 1 mM EDTA, 8.5 μM leupeptin, and 1 mM phenylmethylsulfonyl fluoride (pH 7.2). The homogenates were centrifuged, and the total protein concentration was measured (Pierce BCA protein assay kit, Pierce, Rockford, IL). All samples were adjusted with isolation solution to normalize the protein concentrations, solubilized at 65 °C for 15 min in SDS-containing sample buffer, and the stored at −20 °C.

HK-2 cells were harvested, washed with cold PBS and resuspended in lysis buffer (20 mM Tris–HCl, pH 7.4, 0.01 mM EDTA, 150 mM NaCl, 1 mM PMSF, 1 μg/ml leupeptin, 1 mM Na_3VO_4) and prepared for immunoblotting. The separated proteins were transferred onto nitrocellulose membranes using Bio-Rad Mini Protean II apparatus (Bio-Rad, Hercules, CA). The blots were blocked with 5% milk in TBST (20 mM Tris–HCl, 140 mM NaCl, 0.1% Tween 20, pH 8.0) for 1 h and incubated overnight at 4 °C with primary antibodies, followed by incubation with secondary anti-rabbitor anti-mouse horseradish peroxidase-conjugated antibodies. Labeling was visualized with an enhanced chemiluminescence system.

2.5. Primary antibodies

The anti-extracellular signal-regulated kinases (ERK), anti-phosphorylated ERK , anti-p-p38, anti-total p38, anti-c-Jun N-terminal kinase (JNK), anti-p-JNK, anti-Bcl-2, anti-Bax, anti-cleaved caspase 3, anti-IκBα, anti-NF-κB p65, and anti-histone H3 (Cell Signaling Technology, Danvers, MA), anti-RONβ and phospho-RONβ (Santa Cruz Biotechnology, Santa Cruz, CA), anti-COX2, anti-iNOS, anti-RONα (BD Transduction Laboratories, Lexington, KY), anti-MSP (R&D Systems), and β-actin (Sigma–Aldrich) antibodies were incubated with the blots.

2.6. Nuclear extract preparation

For nuclear extracts, cells were lysed using NE-PER nuclear extraction reagent (NER; Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's protocol. Briefly, HK-2 cells incubated with GM or GM+MSP were harvested and centrifuged at 14,000*g* for 5 min. After removing the supernatant, 100 μl of ice-cold cytoplasmic extraction reagent (CER) I was added to the dried cell pellet. After incubation on ice for 10 min, ice-cold CER II was added to the tube. The tube was centrifuged at 16,000*g* for 5 min, and the pellet fraction was suspended in 50 μl ice-cold NER. After centrifugation at 16,000*g* for 15 min, the supernatant (nuclear extract) fraction was transferred to a new tube [19].

2.7. Annexin V/propidium iodide staining assay

HK-2 apoptosis assessed by using an apoptosis detection kit (Koma Biotech, Seoul, Korea). After exposure to GM for 24 h in the presence or absence of MSP, HK-2 cells were harvested and washed with pre-cooled PBS and re-suspended in a binding buffer containing fluorescein isothiocyanate (FITC)-conjugated annexin-V protein and PI. Annexin-V binding and PI staining were determined by a FACSCalibur™ flow cytometry (Becton Dickinson, San Jones, CA, USA). Apoptotic cells were defined as PI-negative and Annexin V-FITC positive [20].

2.8. DAPI staining assay

Apoptotic nuclei were detected using the DNA-specific fluorescent dye 4′-6-diamidino-2-phenylindole (DAPI) (Invitrogen, Seoul, Korea). After exposure to GM for 24 h in the presence or absence of