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

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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].

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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

MSP, cells were fixed with 3% paraformaldehyde and washed with PBS. DAPI was added to the fixed cells for 5 min, after which they were examined by fluorescence microscopy (Nikon, Tokyo, Japan). The number of cells with apoptotic bodies was counted in 5 randomly chosen fields at 200× magnification and the percent apoptosis was calculated.

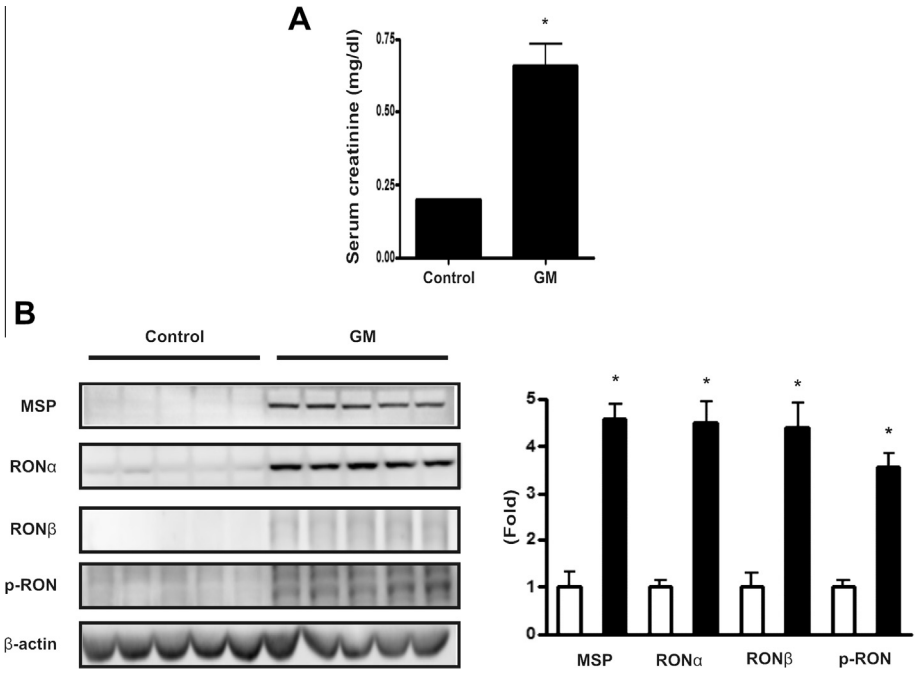

Fig. 1. Renal function and expression of the MSP/RON system. (A) Plasma creatinine increased in GM-treated rats vs. controls. (B) Expression of MSP, RONα, β, and phospho-RON increased in the cortex/OSOM of GM-treated rats. Each column represents mean ± SEM ($n = 5$). $^{*}P < 0.05$ vs. controls.

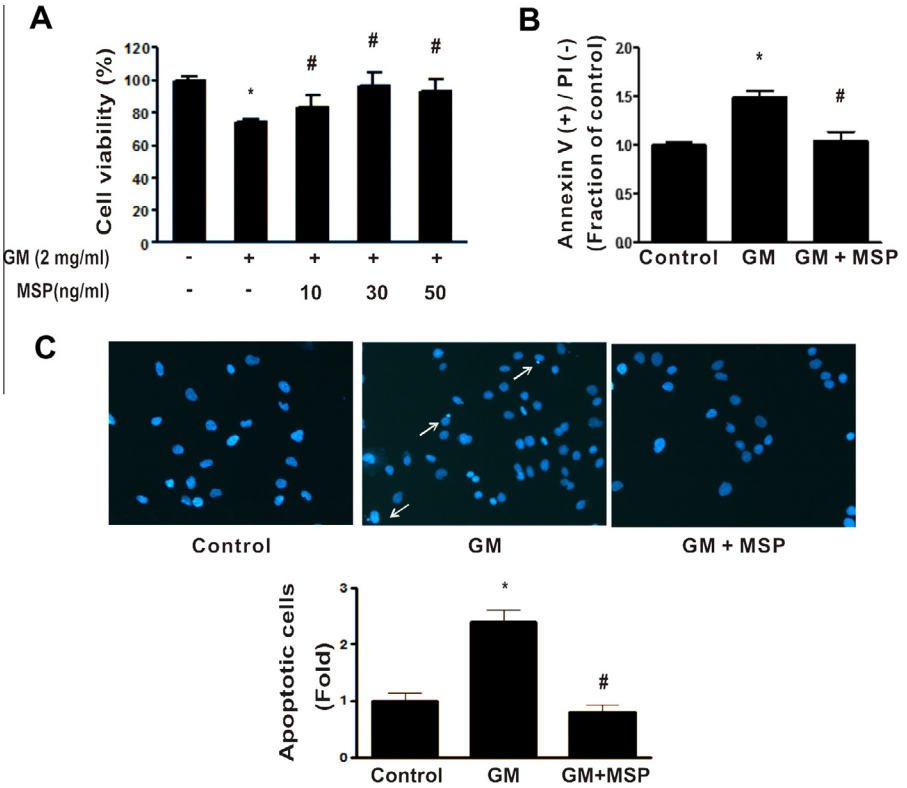

Fig. 2. Effect of MSP on cell viability and apoptosis in GM-treated HK-2 cells. (A) GM treatment caused an MSP-reversible reduction in cell viability. (B) HK-2 cells treated with 2 mg/ml GM for 24 h exhibited a progressive and MSP-reversible increase in annexin-V(+)/PI(−) staining. (C) Chromatin condensation and apoptotic bodies were stained bright blue (arrow) in GM-treated HK-2 cells; this effect was counteracted by MSP. Nuclear morphologies were examined by fluorescence microscopy. Results are presented as means ± SEM of 3 individual experiments. $^{*}P < 0.05$ vs. controls; $^{\#}P < 0.05$ vs. GM. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.9. Statistical analysis

Results are expressed as means ± SEM of 3 independent experiments. Multiple comparisons between groups were made by one-way ANOVA and post hoc Tukey's HSD test. Differences with values of $P < 0.05$ were considered significant.

3. Results

3.1. Expression of MSP, RONα, β, and phospho-RON in GM-induced nephropathy

Plasma creatinine increased in GM-treated rats compared with controls (Fig. 1A). Expression of MSP, RONα, β, and phospho-RON in the renal cortex/OSOM was significantly increased in GM-treated rats compared with controls (Fig. 1B). These results indicate the MSP/RON system is activated in rat kidney by GM treatment.

3.2. Effect of MSP on HK-2 cell viability and morphological apoptotic changes

GM treatment reduced HK-2 cell viability. The GM-induced reduction in cell viability was reversed by MSP pretreatment (10, 30, and 50 ng/ml) (Fig. 2A). Annexin-V and PI staining were used to detect apoptotic changes in GM-treated HK-2 cells. Double-stained cells were analyzed by flow cytometry; the results revealed that HK-2 cells treated with GM for 24 h exhibited a progressive increase in annexin-V(+)/PI(−) staining (apoptotic cells), which was prevented by MSP (Fig. 2B). Fig. 2C shows the apoptotic nuclei after

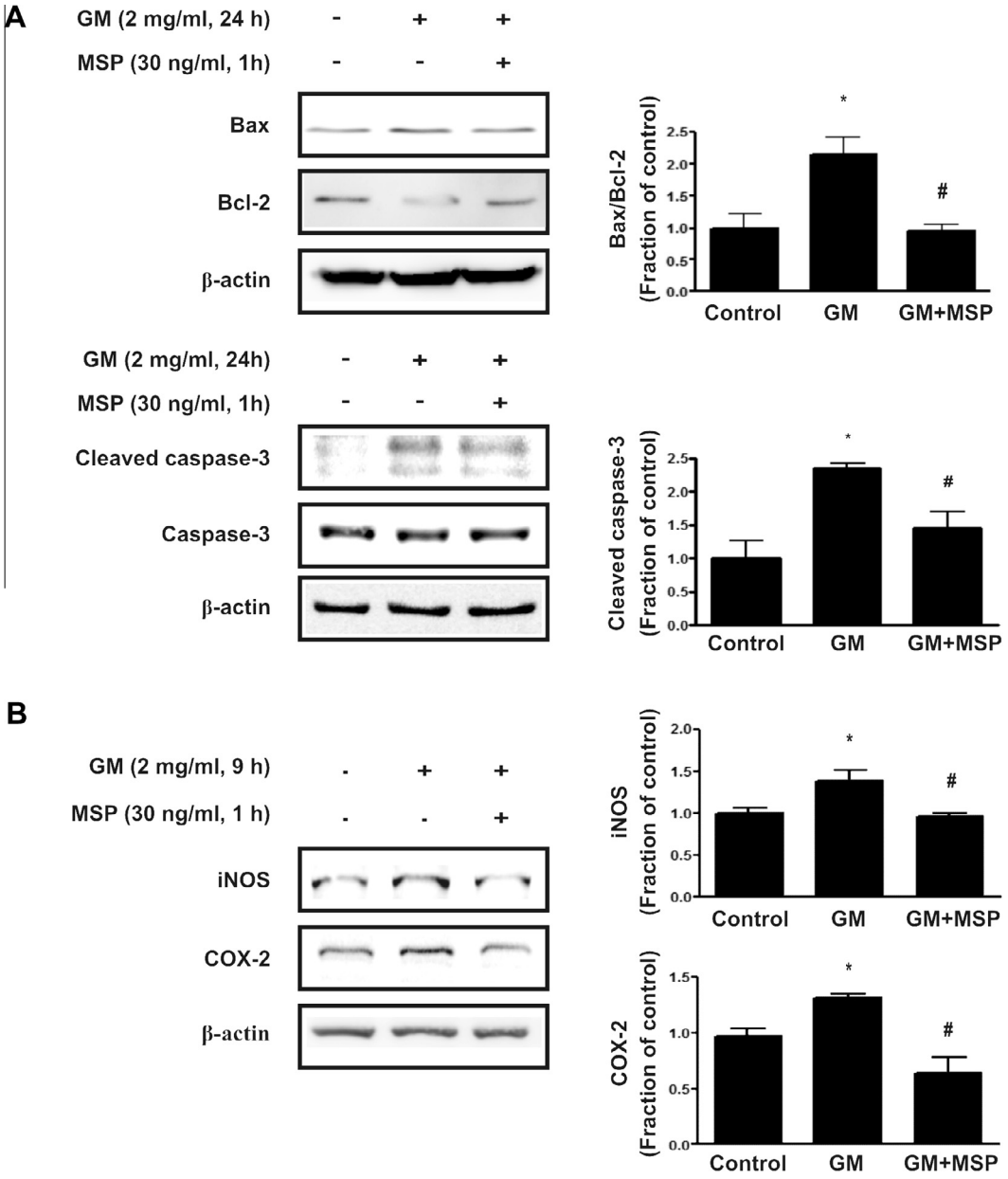

Fig. 3. Effect of MSP on apoptosis-related and inflammatory proteins in HK-2 cells incubated with GM. (A) Semiquantitative immunoblotting indicated that the level of pro-apoptotic marker Bax increased in GM-treated HK-2 cells, whereas that of anti-apoptotic protein Bcl-2 decreased. Increased cleaved caspase-3 expression was noted in GM-treated cells. Pretreatment with MSP attenuated all of these changes. (B) Expression of iNOS and COX-2 was increased by 2 mg/ml GM treatment; this effect was attenuated by MSP pretreatment (30 ng/ml). Results are presented as means ± SEM of 3 individual experiments. $^{*}P < 0.05$ vs. controls; $^{\#}P < 0.05$ vs. GM.

DAPI staining. We observed bright blue apoptotic nuclei, identified by chromatin condensation and apoptotic bodies in GM-treated HK-2 cells. MSP pretreatment significantly reduced these effects.

3.3. Effects of MSP on apoptosis-related and inflammatory proteins in HK-2 cells

Expression of Bax, Bcl-2, and cleaved caspase-3 was measured by immunoblotting (Fig. 3A). Incubation with GM increased Bax and cleaved caspase-3 activity and reduced Bcl-2 expression. MSP pretreatment attenuated these changes. Inducible NOS and cyclooxygenase-2 (COX-2) are synthesized during inflammation in renal disease, and induced by numerous physiologic stimuli [21]. Expression of iNOS and COX-2 was increased in GM-treated HK-2 cells and this effect was attenuated by pretreatment with MSP (Fig. 3B).

3.4. Effects of MSP on activation of MAPKs and NF-κB in HK-2 cells

In HK-2 cells, expression of phospho-ERK, phospho-JNK, and phospho-p38 MAPKs increased after incubation with GM; this was attenuated by 1 h MSP pretreatment. In contrast, the expression of total ERK, JNK, and p38 was unaffected (Fig. 4A). Nuclear factor-kappa B (NF-κB) is released from inhibitory subunit IκBα and translocates to the nucleus, where NF-κB promotes transcriptional activation of target genes. Fig. 4B shows the changes of NF-κB in nuclear extracts and IκBα in the cytoplasm of HK-2 cells incubated with GM in the presence or absence of MSP. Expression of NF-κB increased in cells treated with GM for 1 h compared with untreated controls, whereas MSP pretreatment prevented this increase. In addition, GM exposure for 1 h caused MSP-reversible induction of cytosolic IκB-α expression (Fig. 4B). SB203580 (a specific chemical inhibitor of p38), PD98059 (ERK inhibitor), and SP600125 (JNK inhibitor) also attenuated GM-induced expression of NF-κB. These findings suggest that p38, JNK, and ERK MAPKs are involved in GM-induced NF-κB nuclear translocation.

4. Discussion

We investigated renal expression of the MSP/RON system in GM-induced kidney injury in rats. Markedly enhanced levels of MSP, RON, and the active phospho-RON, were observed in the kidneys of GM-treated rats. These findings suggest activation of the MSP/RON system occurs when there is kidney damage, and is consistent with a previous study that demonstrated plasma levels of MSP were elevated in patients with acute kidney injury for tissue regeneration [11].

Recent studies have indicated the MSP/RON system may play a proliferative role on several cell types stimulated by stress [22]. To investigate the possible protective effect of MSP against GM-induced programmed cell death, we performed an in vitro study using HK-2 cells. The annexin-V/PI staining method is widely used in flow cytometry to detect apoptosis and necrosis. Apoptotic changes induce binding of annexin-V to phosphatidylserine in the outer leaflet of the plasma membrane. Propidium iodide (PI), a fluorescent DNA intercalator permeates the necrotic cell membrane [23]. In this study, GM-treated HK-2 cells exhibited an increase in annexin-V(+)/PI(−) staining, but MSP pretreatment reduced this effect, indicating that MSP attenuates apoptosis.

Apoptosis is regulated by proteins of the Bcl-2 family that control mitochondrial permeabilization in response to stress or damage [24]. GM can cause apoptosis by triggering the mitochondrial pathway through release of cytochrome c and activation of caspase-3 in renal proximal tubular cells [25]. In this study, GM treatment increased expression of apoptotic molecules such as cleaved caspase-3 and the ratio of Bax/Bcl-2, which was attenuated by MSP, suggesting MSP can inhibit GM-induced apoptosis in HK-2 cells through suppression of cleaved caspase-3 and Bax/Bcl-2.

It is well known that inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX-2), derived prostaglandins, are involved in inflammatory response as a biological mediator [21]. Inducible NOS promotes nitric oxide (NO) production, and excessive production of NO plays a pathogenic role in acute and chronic inflammatory diseases [26]. Acute kidney injury caused by GM can also promote an inflammation [27]. In the present study, HK-2 cells

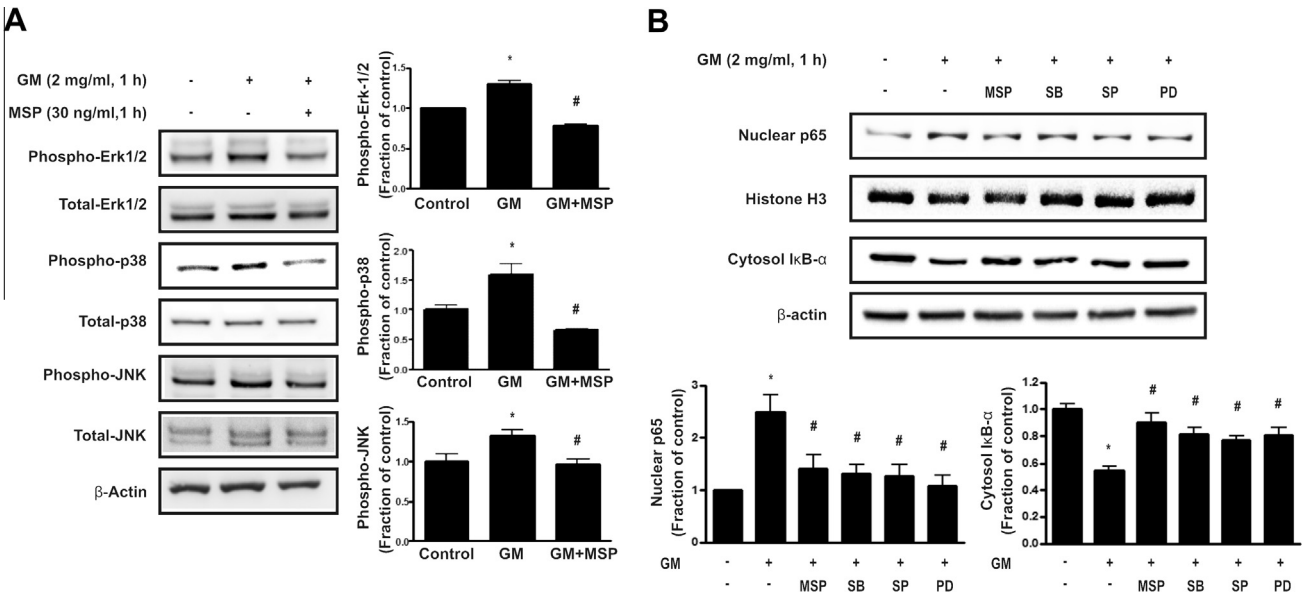

Fig. 4. Effect of MSP on activation of MAPKs and NF-κB in HK-2 cells. (A) Expression of p-ERK-1/2, p-p38 and p-JNK was increased by GM (2 mg/ml) treatment; this effect was attenuated by pretreatment with MSP (30 ng/ml). (B) Pretreatment with MSP (30 ng/ml) for 1 h before exposure to 2 mg/ml GM for 1 h suppressed the overexpression of nuclear NF-κB p65. Incubation with SB203580 (p38 inhibitor, 10 μM), SP600125 (JNK inhibitor, 10 μM), and PD98059 (ERK inhibitor, 10 μM) for 1 h before exposure to GM attenuated expression of the nuclear NF-κB p65 subunit. Total cytoplasmic IκBα expression decreased after GM incubation, and these changes were attenuated by MSP and by MAPK inhibitors. Results are presented as means ± SEM of 3 individual experiments $^{*}P < 0.05$ vs. controls; $^{\#}P < 0.05$ vs. GM.

treated with GM showed increased iNOS and COX-2 expression in comparison to untreated controls; this was attenuated by pretreatment with MSP. Out results are consistent with those of an earlier study in which MSP suppressed inflammatory cytokines in lipopolysaccharide-induced rheumatoid arthritis synovial fibroblasts [28]. These findings indicate the inflammatory process has a significant role in the pathogenesis of GM-induced nephrotoxicity, and MSP can attenuate the resultant renal damage by suppression of iNOS and COX-2 protein levels.

We examined the involvement of MAPK/NF-κB signaling molecules that have been implicated as stimulators of inflammation and apoptosis [29]. We demonstrated MSP pretreatment in GM-treated HK-2 cells decreased the phosphorylation of p38, Erk-1/2, JNK MAPKs, and NF-κB p65. Extracellular MAPK signals activate the IKK complex, which regulates the transcription factor NF-κB. NF-κB is released from IκB-α, then translocated to the nucleus, where it promotes transcription of a large number of proteins involved in inflammation, apoptosis and cell proliferation [30]. While the net effect of NF-κB is usually anti-apoptotic [31], NF-κB activation in the kidney can also lead to stimulation of apoptosis in renal cells [32]. This is exemplified by TRAIL-mediated NF-κB activation, which increases death receptor 5 expression, and amplifies the apoptotic response of TRAIL in epithelial cells [33]. In agreement with this, renal tubule cell apoptosis is associated with activation of MAPKs/NF-κB signaling pathways [32,34,35]. We observed that MSP inhibits GM-induced apoptosis by the modulation of Bax, Bcl-2, and caspase-3 in HK-2 cells. The possible mechanism involving these anti-apoptotic effects of MSP may relate to the interruption of the NF-κB and MAPK signaling pathways.

Our results indicate the MSP/RON system is activated in rat kidney damaged by GM and may have a beneficial role in the response to tissue injury. MSP could act as an anti-apoptotic agent in GM-treated HK-2 cells through attenuation of Bax/Bcl-2 and caspase-3 activity. Moreover, MSP showed anti-inflammatory effects by inhibiting iNOS and COX-2 via inactivation of the MAPK and NF-κB pathways. Although further studies would be required to elucidate the underlying molecular mechanisms, our study suggests that MSP may lead to the development of clinical intervention strategies for the treatment of renal disease.

Acknowledgments

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