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Mechanism of TNF-α-Induced Migration and Hepatocyte Growth Factor Production in Human Mesenchymal Stem Cells

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ABSTRACT

Accumulating evidence suggests that mesenchymal stem cells (MSCs) may decrease destructive inflammation and reduce tissue loss. Tumor necrosis factor-α (TNF-α) plays a central role in induction of proinflammatory signaling and paradoxically activates intracellular anti-inflammatory survival pathways. In this study, we investigated whether TNF-α could induce a chemotactic effect on human MSCs and stimulate their production of anti-inflammatory factors in vitro, as well as determined mechanisms that mediated this effect. Migration assays demonstrated that TNF-α had a chemotactic effect on MSCs. TNF-α increased both hepatocyte growth factor (HGF) mRNA expression in MSCs and HGF secretion in conditioned medium. These effects were dependent on the p38 MAPK and PI3K/Akt, but not JNK and ERK signaling pathways. Furthermore, these effects were inhibited by a specific neutralizing antibody to TNF receptor II, but not TNF receptor I. We conclude that TNF-α can enhance human MSCs migration and stimulate their production of HGF. These effects are mediated via a specific TNF receptor and signaling pathways. J. Cell. Biochem. 111: 469–475, 2010.

KEY WORDS: MESENCHYMAL STEM CELLS; HEPATOCYTE GROWTH FACTOR; TNF-α; CELL MIGRATION; ANTI-INFLAMMATORY CYTOKINE

Accumulating evidence suggests that mesenchymal stem cells (MSCs) may decrease destructive inflammation and reduce tissue loss after cardiac, kidney, and liver injury [Orlic et al., 2001; Fang et al., 2004; Lange et al., 2005; Oyagi et al., 2006]. Transdifferentiation and paracrine action are two functions that were supposed to contribute to the reparative effects of MSCs. Recently, there is a growing recognition that MSCs may exert organ-protective effects mainly through a paracrine mechanism, rather than transdifferentiation [di Bonzo et al., 2008; Psaltis et al., 2008; van Poll et al., 2008]. Although the particular growth factors contributing to these reparative effects remain to be defined, anti-inflammatory factors including hepatocyte growth factor (HGF) may be one of the main contributors [Rehman et al., 2004; van Poll et al., 2008].

Tumor necrosis factor-α (TNF-α) is induced in response to various injuries and plays a central role in induction of proinflammatory signaling [Schwabe and Brenner, 2006]. Paradoxically, TNF-α not only induces proapoptotic signals but also activates intracellular survival pathways [Abshagen et al., 2007]. TNF-α exerts its biological effects via activation of two distinct cell surface receptors, TNF receptor I (TNFRI) and TNF receptor II (TNFRII) [MacEwan, 2002]. Both TNF receptors are expressed in human MSCs [Croitoru-Lamoury et al., 2007], whereas it has not been well established which TNF receptor mainly mediated the known biological effects of TNF-α in human MSCs.

Based on these findings, we hypothesize that in a wound milieu, the locally produced TNF-α will have a chemotactic effect on MSCs promoting their migration to the site, while also inducing the production of the anti-inflammatory factor HGF and exert their reparative function. In this study, we investigated the chemotactic effect of TNF-α on human MSCs, and HGF production in human MSCs by stimulation of TNF-α.

MATERIALS AND METHODS

ISOLATION, CULTURE, AND CHARACTERIZATION OF HUMAN MSC

The use of human samples for scientific research has been approved by the Human and Animal Ethics Committee of Zhejiang University.

No competing financial interests exist.

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Bone marrow aspirates were obtained from donors with written informed consent. MSCs were isolated and proliferated as described previously [Ponte et al., 2007]. Briefly, aspirates were resuspended, centrifuged, and plated at a density of 5×10^5 cells/cm^2 in Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS; JRH Bioscience, Lenexa, KS), 2 mmol/L L-glutamine, 100 U/ml penicillin, and 50 mg/ml streptomycin. After 24, 48, and 72 h, nonadherent cells were removed by changing the culture medium. Adherent MSCs were harvested and plated into new flasks each time when 90% confluency was achieved. MSCs between passages 5 and 8 were used for all experiments.

Before performing specific experiments, cultured cells were analyzed by flow cytometry and showed the typical MSC markers CD73, CD90, and CD105 (>95% positive cells), whereas they were negative for hematopoietic cell markers CD34 and CD45 [Bernardo et al., 2007b]. These cells gave rise to adipocytes, osteoblasts, and chondrocytes when placed in adequate differentiating conditions to show their multipotentiality [Bernardo et al., 2007a; in 't Anker et al., 2003].

TRANSWELL MIGRATION ASSAY

The assay for chemotaxis was performed in Corning Costar transwell chambers (Corning, Concorezzo, Italy). Briefly, 200 μl of medium containing human MSCs (5×10^4 cells) was added on the upper side of a porous polycarbonate membrane (pore size: 8 μm). Five hundred microliters of medium containing, or not containing, 50 ng/ml human recombinant TNF-α (Invitrogen, Carlsbad, CA) was added to the lower compartment. The plates were incubated at 37°C in 5% CO_2 for 24 h. In inhibition experiments, cells were preincubated with specific neutralizing antibodies (R&D Systems, Minneapolis, MN) for TNFRI (MAB625) or TNFRII (MAB726) at a final concentration of 10 μg/ml for 1 h prior to exposure to TNF-α. At the end of the incubation, the cells at the upper side of the filter were mechanically removed. Cells that had migrated to the lower side of the filter were fixed with 11% glutaraldehyde for 30 min and stained with hematoxylin and eosin. Numbers of the migrated cells were determined under microscope.

EFFECT OF TNF-α ON HGF EXPRESSION

To determine whether TNF-α stimulation can induce HGF expression in human MSCs, cells in low serum (2% FCS) medium were treated with 1–50 ng/ml TNF-α for 6 h. Then cells were harvested and quantification of HGF mRNA levels was performed by real-time PCR. Total RNA was extracted from MSCs using the Trizol method according to the manufacturer's instructions (Invitrogen). A total of 1 μg of RNA was transcribed into cDNA using 200 U of Super-Script II reverse transcriptase (Invitrogen) and 150 ng of random primers (Invitrogen). Real-time PCRs were performed using the SYBR green Master Mix according to the manufacturer's instructions (Applera, Foster, CA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference gene. Forward and reverse primers used for specific amplification of GAPDH and HGF were: GAAGGTGAAGGTCGGAGTC, GAAGATGGTGATGGGATTTC, and GGTACGCTACGAAGTCTG, TTGCCTGATTCTGTATGA, respectively. HGF mRNA expression was compared between different samples after normalization relative to GAPDH.

SIGNALING PATHWAYS MEDIATING TNF-α-INDUCED HGF EXPRESSION

To determine which signaling pathways were required for MSCs to express HGF in response to TNF-α, we utilized specific pharmacological inhibitors of kinases in the p38 mitogen-activated protein kinase (p38 MAPK), extracellular signal-regulated kinase (ERK), phosphoinositide 3-kinase (PI3K)/Akt and the c-Jun NH_2-terminal kinase (JNK) pathway. Cells were divided into groups as follows: untreated control, TNF-α (50 ng/ml), inhibitor, and TNF-α + inhibitor. In inhibitor experiments, cells were pre-treated with specific pharmacological inhibitors SB203580 (inhibitor of p38 MAPK, 10 μM), PD98059 (inhibitor of ERK kinase MEK-1, 30 μM), SP600125 (inhibitor of JNK, 10 μM), or LY294002 (inhibitor of PI3K, 10 μM) prior to treatment with TNF-α. Phosphorylation of relevant downstream substrates, namely HSP27, Erk-1/2, c-JUN, and Akt were measured. Production of HGF was also measured at the same time. All signaling inhibitors were purchased from Calbiochem (Nottingham, UK). All phospho-specific and total protein expression antibodies for these kinases were purchased from Cell Signaling Technology (Danvers, MA).

Three sets of procedures were performed in this step. Firstly, after MSCs attained 70% confluency, cells were pre-treated with inhibitor in low serum medium for 2 h before treatment with 50 ng/ml TNF-α for 1 h. Then cells were harvested and protein was extracted. Activation of specific signal transduction pathways were determined by Western blotting with phospho-specific and total protein expression antibodies. Secondly, after MSCs attained 70% confluency, cells were pre-treated with inhibitor in low serum medium for 2 h before treatment with 50 ng/ml TNF-α for 6 h. Then, the cells were harvested and RNA was extracted. HGF mRNA expression was determined by real-time PCR as described above. Thirdly, after MSCs attained 70% confluency, cells were plated in 12-well plates (Corning) at 1×10^5 cells/well. Cells were pre-treated with inhibitor in low serum medium for 4 h before treatment with TNF-α for 24 h. The conditioned medium was then collected for analysis of HGF secretion with ELISA. ELISA was performed using a commercially available kit (Invitrogen) according to the manufacturer's instructions.

EFFECT OF TNF RECEPTOR-NEUTRALIZING ANTIBODIES ON TNF-α-INDUCED SIGNALING AND HGF EXPRESSION

To determine which TNF-α receptor subtypes mediated the effects of TNF-α on signaling and HGF expression in MSCs, we utilized specific neutralizing antibodies to TNFRI and TNFII. Cells were divided into groups as follows: untreated control, TNF-α (50 ng/ml), TNF-α + TNFRI-neutralizing antibody, and TNF-α + TNFRII-neutralizing antibody. Phosphorylation of kinases in relevant signaling pathways, namely p38 MAPK, Erk-1/2, JNK, and Akt were measured. Production of HGF was also measured at the same time. All phospho-specific and total protein expression antibodies for these kinases were purchased from Cell Signaling Technology.

Three sets of procedures were performed in this step. Firstly, after MSCs attained 70% confluency, cells were pre-treated with

neutralizing antibodies in low serum medium for 1 h prior to exposure of TNF-α for 1 h. Then the cells were harvested and protein was extracted. Activation of specific signaling pathways was determined by Western blotting with phospho-specific and total protein antibodies as described above. Secondly, after MSCs attained 70% confluency, cells were pre-treated with TNF receptor-neutralizing antibodies in low serum medium for 2 h before treatment with 50 ng/ml TNF-α for 6 h. Then, the cells were harvested and RNA was extracted. HGF mRNA expression was determined by real-time PCR as described above. Thirdly, after MSCs attained 70% confluency, MSCs were plated in 12-well plates (Corning) at 1×10^5 cells/well. Cells were pre-treated with neutralizing antibodies in low serum medium for 1 h prior to exposure to TNF-α for 24 h. Then, the conditioned medium was collected for analysis of HGF secretion with ELISA as described above.

STATISTICAL ANALYSIS

Results are expressed as mean ± SD and analyzed using SPSS. Differences between normalized data were analyzed using paired ratio *t*-tests. Dose–response data were compared as ratios using repeated measures one-way analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

RESULTS

TNF-α INDUCES A CHEMOTACTIC EFFECT ON HUMAN MSCS

Addition of TNF-α to the lower compartment of a transwell stimulated migration of MSCs through polycarbonate filters towards TNF-α (Fig. 1A,E). To discriminate between the chemotactic and the chemokinetic effect of TNF-α on MSC migration, studies were

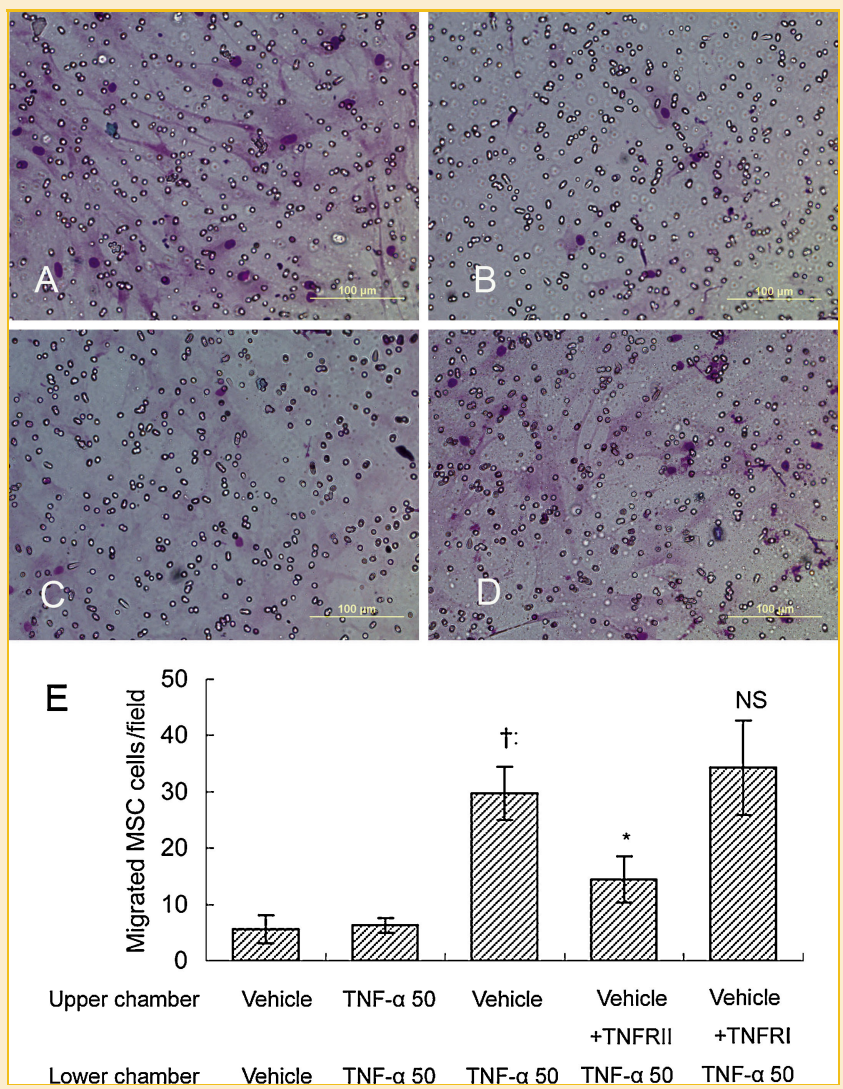

Fig. 1. Effect of TNF-α and TNF receptor-neutralizing antibodies on MSC migration. A–D: Representative micrographs of MSC migration. Plenty of MSCs migrated to the lower side of the membrane when challenged with 50 ng/ml TNF-α (A); only few MSCs migrated to the lower side of the membrane when TNF-α presented on both sides of the chamber (B); MSC migration was significantly inhibited when preincubated with TNFRII-neutralizing antibody (C) but not when preincubated with TNFRI-neutralizing antibody (D). Scale bar: 100 μm. E: Statistics of migrated cell number. Values are expressed as the mean ± SD of migrated cell number every 100× magnification field (n = 3). ANOVA with Newmann–Keuls' multicomparison test was performed. †$P < 0.05$ for effect of TNF-α versus control. *$P < 0.05$ for effect of TNFRII-neutralizing antibody versus TNF-α alone. NS: not significant for effect of TNFRI-neutralizing antibody versus TNF-α alone.

performed in the presence of TNF-α on both sides of the chamber. The migratory effect of TNF-α was abolished when 50 ng/ml TNF-α was added to both sides of the chamber (Fig. 1B,E). This result suggests chemotaxis rather than chemokinesis of TNF-α on MSC migration. Pre-incubation of MSCs with a neutralizing monoclonal antibody to TNFRII significantly inhibited TNF-α-induced MSC migration (Fig. 1C,E). Preincubation of MSCs with TNFRI antibody did not inhibit MSC migration (Fig. 1D,E).

TNF-α INDUCES HGF EXPRESSION IN HUMAN MSCS IN A DOSE-DEPENDENT MANNER

In untreated MSCs, a basal level of HGF expression was determined. After stimulation with various concentrations of TNF-α, steady increases in HGF expression was observed in a concentration-dependent manner (Fig. 2). The concentration of TNF-α for effective stimulation of HGF production was determined as 50 ng/ml. Thus, all subsequent experiments were performed with 50 ng/ml TNF-α.

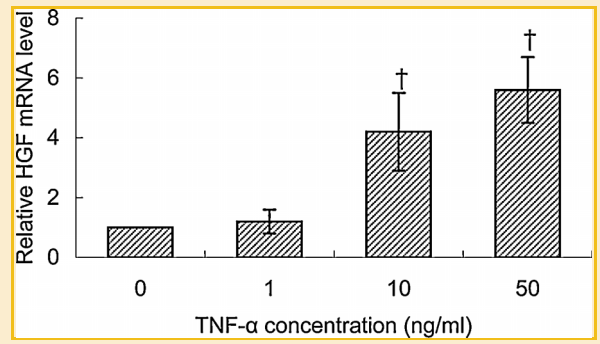

Fig. 2. Effect of TNF-α on HGF mRNA expression in MSCs. HGF mRNA expression was normalized to GAPDH and data are expressed as relative to untreated cells (n = 5). Effective stimulation of HGF production was observed in response to 50 ng/ml TNF-α. †$P < 0.05$ for effect of TNF-α versus control.

p38 MAPK AND PI3K, NOT JNK AND ERK SIGNALING PATHWAYS MEDIATE TNF-α-INDUCED HGF EXPRESSION IN HUMAN MSCS

SB203580, PD98059, SP600125, and LY294002 significantly inhibited phosphorylation of HSP27, Erk-1/2, c-JUN, and Akt, respectively (Fig. 3A). After MSCs were pre-treated with inhibitors of p38 MAPK and PI3K, TNF-α-induced HGF mRNA expression was reduced significantly (Fig. 3B). HGF secretion by MSCs in the conditioned medium was reduced correspondingly (Fig. 3C). Pre-incubation of MSCs with inhibitors of ERK kianse MEK-1 and JNK did not reduce the TNF-α-induced HGF mRNA expression and protein secretion. Therefore, TNF-α-induced expression of HGF in human MSCs was mediated specifically by the p38 MAPK and PI3K pathways, but not by the ERK and JNK pathways.

TNF RECEPTOR II MEDIATE TNF-α-INDUCED HGF EXPRESSION IN HUMAN MSCS

A TNFRII-neutralizing antibody significantly inhibited TNF-α-induced phosphorylation of p38 MAPK, Erk-1/2, JNK, and Akt, whereas a TNFRI-neutralizing antibody had no significant effect on any of these pathways (Fig. 4A). Pre-incubation of MSCs with TNFRII-neutralizing antibody resulted in a significant reduction of expression of HGF mRNA in MSCs (Fig. 4B) and HGF secretion in conditioned medium (Fig. 4C). In contrast, the reduction of HGF mRNA expression and protein secretion did not occur when the

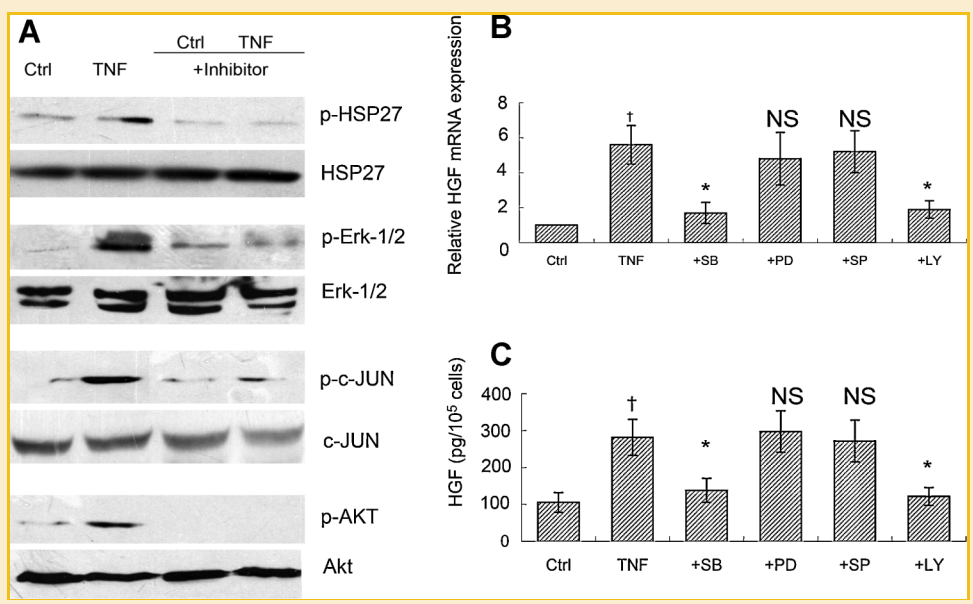

Fig. 3. Effect of pharmacological inhibitors on TNF-α-induced signaling pathways and HGF expression in MSCs. A: Activation of specific signal transduction pathways was determined by Western blotting. Blots are representative of n = 5. B: HGF mRNA expression was determined by real-time PCR. Data are expressed as relative to untreated sample (n = 6). C: HGF secretion in conditioned medium was determined by ELISA. Data are expressed as mean ± SD of HGF concentration normalized to cultured cell numbers (n = 6). †$P < 0.05$ for effect of TNF-α versus control. *$P < 0.05$ for effect of inhibitors versus TNF-α alone. NS: not significant for effect of inhibitors versus TNF-α alone.

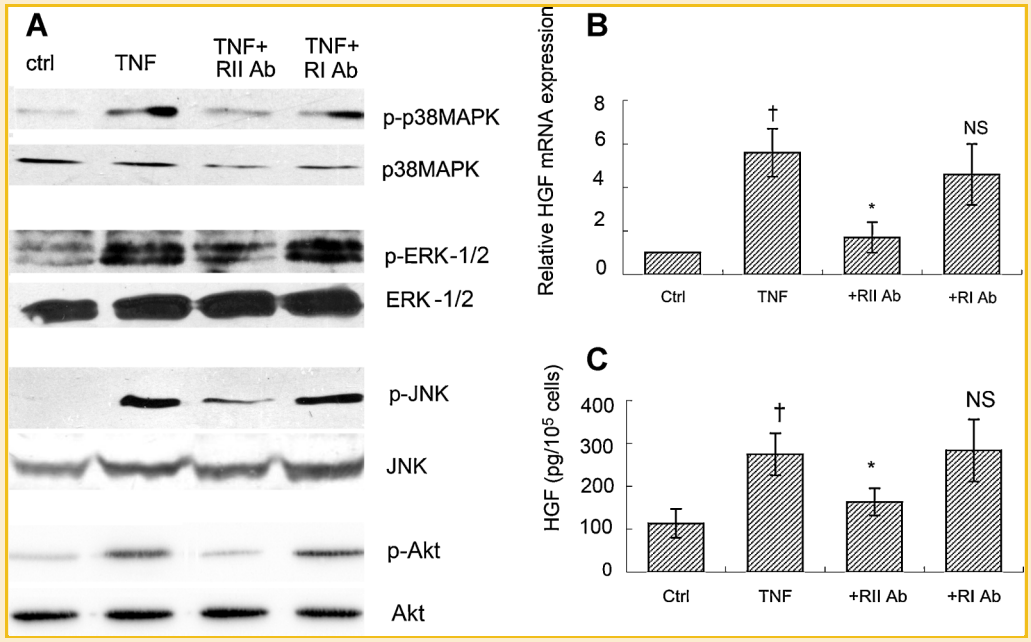

Fig. 4. Effect of TNF receptor-neutralizing antibodies on TNF-α-induced signaling and HGF expression in MSCs. A: Activation of specific signal transduction pathways was determined by Western blotting. Blots are representative of n = 5. B: HGF mRNA expression was determined by real-time PCR. Data are expressed as relative to untreated cells (n = 5). C: HGF secretion in conditioned medium was determined by ELISA. Data are expressed as mean ± SD of HGF concentration normalized to cultured cell numbers (n = 5). †$P < 0.05$ for effect of TNF-α versus control. *$P < 0.05$ for effect of TNFRII-neutralizing antibody versus TNF-α alone. NS: not significant for effect of TNFRI-neutralizing antibody versus TNF-α alone.

MSCs were pre-incubated with TNFRI-neutralizing antibody. Therefore, TNF-α-induced HGF expression in human MSCs was mediated specifically by TNFRII, but not by TNFRI.

DISCUSSION

Our results reveal several major findings on the effect of TNF-α on migration and HGF production in human MSCs. Firstly, TNF-α has a chemotactic effect on MSC migration. Secondly, TNF-α increases both mRNA expression and protein secretion of HGF in human MSCs in vitro. Thirdly, these effects are mediated by p38 MAPK and PI3K/Akt, but not JNK and ERK signaling pathways. Finally, these effects are mediated specifically via TNFRII but not TNFRI.

Bone marrow-derived MSCs have been shown to be capable of positive remodeling and regeneration of healthy, functional tissue. Recent studies suggest that MSCs exert their reparative function through paracrine, rather than transdifferentiation mechanisms [di Bonzo et al., 2008; Psaltis et al., 2008; van Poll et al., 2008]. Homing to the injured tissue might be the necessary step for MSCs to exert their paracrine function. Several studies have shown that MSCs are able to home to and persist long-term in a wide range of tissues including kidney, heart, and liver following systemic infusion into the bloodstream [Karp and Leng Teo, 2009; Li et al., 2009; Sordi, 2009]. The signals that guide MSCs to appropriate microenvironments are determined as some soluble factors including platelet-derived growth factor-AB, insulin-like growth factor 1, and stromal-derived factor-1 [Ponte et al., 2007]. Recently, TNF-α has been reported to exert chemotactic effect on MSCs [Fu et al., 2009], but the mechanism is yet to be clarified. In the present study, we clearly showed that TNF-α had a definitively chemotactic effect on MSCs migration. Neutralizing TNFRII resulted in inhibition of MSCs migrating to the site with higher concentrations of TNF-α, whereas neutralizing TNFRI did not. It could be proposed that expression of cell surface TNFRII and a high concentration TNF-α in an inflammatory microenvironment is involved in the localization of MSCs to injured tissue. However, the migration of MSC to a TNF-α gradient was not completely inhibited by TNFRII-neutralizing antibody even a high dose of neutralizing antibody was used (100 μg/ml, data not shown). This might suggest that other members of the TNF receptor superfamily, such as CD40 or Fas, may partly be responsible for TNF-α stimulated MSC migration.

TNF-α is induced in various injury conditions and is mainly responsible for localized responses, such as inflammation and apoptosis. Paradoxically, TNF-α not only induces proapoptotic signals but also activates intracellular survival pathways [Abshagen et al., 2007]. It has been well established that TNF-α induces MSCs to produce numerous cytokines including vascular endothelial growth factor, insulin-like growth factor 1, and HGF [Wang et al., 2006; Wang et al., 2008, 2009], which are considered as angiogenic and anti-inflammatory factors. HGF is an important mediator of MSC paracrine effects. It has been reported that MSCs mediate protective effects on ischemic myocardium through elevated HGF [Wairiuko et al., 2007]. Genetically modified MSCs to overexpress HGF have been shown to enhance MSC-induced protection in the ischemic liver [Yu et al., 2007]. In the present study, it was clearly shown that TNF-α-induced MSCs to produce HGF in a dose-dependent manner. This result suggests that the locally produced TNF-α may exert

important effects on implanted stem cell function. Furthermore, the related mechanism has been investigated in the present study.

HGF gene transcription can be induced via several different intracellular signaling pathways, including MAPK and PI3K/Akt signaling pathways. In human fibroblasts, HGF expression induced by maleic acid requires activation of ERK and JNK, but not p38 MAPK [Motoki et al., 2008], whereas inflammatory cytokine IL-1beta and IFN-gamma-induced HGF production in human fibroblasts requires activation of ERK and p38 MAPK but not of JNK [Takami et al., 2005]. In contrast, HGF production of human MSCs stimulated by LPS, or hypoxia depends on p38 MAPK activation but does not depend on increased ERK or JNK phosphorylation [Crisostomo et al., 2008]. In the present study, we demonstrated that TNF-α-induced HGF production in human MSCs were dependent on p38 MAPK and PI3K/Akt pathways, but were not dependent on ERK and JNK signaling pathways. Our results also demonstrated that neutralizing of TNFRII had a significant effect on inhibition of phosphorylation of p38 MAPK, ERK and JNK as well as Akt. Neutralizing TNFRII significantly inhibited TNF-α-induced HGF production while neutralizing TNFRI did not. It suggests that TNF-α-induced HGF production in MSCs is mediated specifically via TNFRII but not TNFRI.

Our study highlights the effect of TNF-α, as an important local factor, on cell migration and HGF production in MSCs. Furthermore, our study demonstrates that the TNF-α-induced HGF production in MSCs is mediated via a specific TNF receptor and signaling pathways. This study brings us one step closer to understanding the roles of MSC may play in wound healing and control of tissue inflammation.

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