RhoA/Phosphatidylinositol 3-Kinase/Protein Kinase B/Mitogen-Activated Protein Kinase Signaling after Growth Arrest–Specific Protein 6/Mer Receptor Tyrosine Kinase Engagement Promotes Epithelial Cell Growth and Wound Repair via Upregulation of Hepatocyte Growth Factor in Macrophages

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ABSTRACT

Growth arrest–specific protein 6 (Gas6)/Mer receptor tyrosine kinase (Mer) signaling modulates cytokine secretion and helps to regulate the immune response and apoptotic cell clearance. Signaling pathways that activate an epithelial growth program in macrophages are still poorly defined. We report that Gas6/Mer/RhoA signaling can induce the production of epithelial growth factor hepatic growth factor (HGF) in macrophages, which ultimately promotes epithelial cell proliferation and wound repair. The RhoA/protein kinase B (Akt)/mitogen-activated protein (MAP) kinases, including p38 MAP kinase, extracellular signal-regulated protein kinase, and Jun NH2-terminal kinase axis in RAW 264.7 cells, was identified as Gas6/Mer downstream signaling pathway for the upregulation of HGF mRNA and protein. Conditioned medium from RAW 264.7 cells that had been exposed to Gas6 or apoptotic cells enhanced epithelial cell proliferation of the epithelial cell line LA-4 and wound closure. Cotreatment with an HGF receptor-blocking antibody or c-Met antagonist downregulated this enhancement. Inhibition of Mer with small interfering RNA (siRNA) or the RhoA/Rho kinase pathway by RhoA siRNA or Rho kinase pharmacologic inhibitor suppressed Gas6-induced HGF mRNA and protein expression in macrophages and blocked epithelial cell proliferation and wound closure induced by the conditioned medium. Our data provide evidence that macrophages can be reprogrammed by Gas6 to promote epithelial proliferation and wound repair via HGF, which is induced by the Mer/RhoA/Akt/MAP kinase pathway. Thus, defects in Gas6/Mer/RhoA signaling in macrophages may delay tissue repair after injury to the alveolar epithelium.

Introduction

Growth arrest–specific protein 6 (Gas6) is a member of the vitamin K–dependent protein family. Gas6 comprises an N-terminal γ-carboxylglutamic acid domain followed by four epidermal growth factor–like domains and a large C-terminal region homologous to the sex hormone binding globulin (Manfioletti et al., 1993; Saller et al., 2006). Gas6 is expressed and secreted by a variety of cell types, including fibroblasts, endothelial cells (Manfioletti et al., 1993), smooth muscle (Nakano et al., 1995), bone marrow cells (Avanzi et al., 1997), and leukocytes (Muñoz et al., 2004; Ekman et al., 2010; Loges et al., 2010). Gas6 is a common ligand of the Tyro3/Axl/Mer receptor tyrosine kinase (Mer), or TAM, receptor subfamily (Godowski et al., 1995; Stitt et al., 1995). These receptors share significant domain similarity, all containing two extracellular N-terminal immunoglobulin-like domains as well as two fibronectin III-like domains followed by a tyrosine kinase domain that lies at the C-terminal cytoplasmic end of the receptors (Fischer et al., 1991; Lemke and Rothlin, 2008). On binding a TAM receptor, Gas6 induces signaling (Nagata et al., 1996; Gould et al., 2005) to mediate cell survival (Avanzi et al., 1997; Nakano et al., 1997), proliferation (Goruppi et al., 1996; Li et al., 1996), phagocytosis

ABBREVIATIONS: Akt, protein kinase B; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal–regulated protein kinase; FBS, fetal bovine serum; Gas6, growth arrest–specific protein 6; HGF, hepatocyte growth factor; JNK, Jun NH2-terminal kinase; LY 294002, 2-(4-morpholino)-8-phenyl-4H-1-benzopyran-4-one hydrochloride; MAP, mitogen-activated protein; Mer, Mer receptor tyrosine kinase; M-MLV, Moloney murine leukemia virus; PD 98059, 2-(2-amino-3-methoxyphenyl)-4-(2-[4-(methylsulphonyl)phenyl]-1H-imidazol-4-yl)pyridine hydrochloride; PHA-665752, (2H-imidazol-4-yl)[(2,6-dichlorophenyl)methyl]sulfonfonyl-1,2-dihydro-2-oxo-3H-indol-3-ylidenemethyl]-2,4-dimethyl-1H-pyrrol-3-yl(carbonyl]-2-{1-pyrrolidinylmethyl}pyrroliidine; PIAK, phosphatidylinositol 3-kinase; SB 203580, 4-[5-(fluorophenyl)-2-[4-(methylsulphonyl)phenyl]-1H-imidazol-4-yl]pyridine hydrochloride; siRNA, small interfering RNA; TAM, Tyro3/Axl/Mer; TGF–β, transforming growth factor β; VEGF, vascular endothelial growth factor; Y-27632, trans-4-{[1F]-1-aminoethyl}-N-4-pyridinylcyclohexanecarboxamide dihydrochloride.
(Mark et al., 1996; Lemke and Rothlin, 2008), differentiation, cell adhesion (McCloskey et al., 1997; Nakano et al., 1997; Fridell et al., 1998), cell migration (Nakano et al., 1995; Allen et al., 2002), platelet function, and thrombus stabilization (Angelillo-Scherrer et al., 2005; Gould et al., 2005). Recent studies have also demonstrated that Gas6 signaling modulates cytokine secretion to regulate the immune response (Alciato et al., 2010).

Gas6 and TAM receptors also play a role in the uptake of apoptotic cells by macrophages. Gas6 binds to phosphatidylserine expressed on the inverted plasma membrane of apoptotic cells (Lemke and Rothlin, 2008). Macrophage recognition of a Gas6-phosphatidylserine complex facilitates binding and clearance of apoptotic cells. Although macrophages express all three receptors, Mer plays a unique and nonredundant role in apoptotic clearance. In fact, apoptotic cell clearance in the thymus and retina of Axl- and Tyro3-deficient mice is still observed because Mer alone is sufficient for this process (Seitz et al., 2007). Consistent with this, macrophages of Mer knockdown mice cannot clear apoptotic thymocytes and therefore exhibit spontaneous autoantibody production and lupus-like autoimmunity (Cohen et al., 2002; Scott et al., 2001).

The microenvironment at the sites of inflammation and tissue injury is characterized by increased Gas6 production and dying cells derived from the surrounding tissue or immune cells undergoing activation-induced apoptosis (Ekman et al., 2010; Moon et al., 2010; Brecht et al., 2011). Apoptotic cell clearance has been reported to initiate a macrophage phenotype switch (Korns et al., 2011; Ortega-Gómez et al., 2013). Consequently, macrophages turn off production of proinflammatory cytokines and lipid mediators and launch an anti-inflammatory transcriptional program characterized by release of transforming growth factor-β (TGF-β), interleukin-10, and prostaglandin E2 (Fadok et al., 1998). Furthermore, our previous studies indicate that apoptotic cells and Gas6 are capable of triggering hepatocyte growth factor (HGF) production by macrophages (Park et al., 2011, 2012). Moreover, apoptotic cells bind the Mer receptor to induce HGF mRNA and protein expression in macrophages via a RhoA-dependent pathway (Park et al., 2012). However, the downstream signaling molecules involved in Gas6-mediated regulation of HGF production remain unknown. In this study, the RhoA/protein kinase B (Akt)/mitogen-activated protein (MAP) kinases axis in RAW 264.7 cells was identified as Gas6/Mer downstream signaling pathway for the upregulation of HGF mRNA and protein. We also performed in vitro coculture assays to determine whether and how macrophages programmed by

![Fig. 1. Activation of RhoA is required for Gas6-induced HGF mRNA and protein expression in a macrophage cell line. RAW 264.7 cells were preincubated with a specific Rho inhibitor, C3 transferase (C3T; 0.3–1 μg/ml), for 20 hours (A and E) or a Rho kinase inhibitor, Y-27632 (Y; 10–50 μM), for 20 hours (B and E), and then stimulated with 400 ng/ml Gas6 for 2 hours to detect HGF mRNA expression and for 24 hours to detect secreted HGF. (C and D) RAW 264.7 cells were transfected with RhoA siRNA or control vehicle (siRNA-green fluorescent protein) for 24 hours and then stimulated with Gas6 for 2 hours. (C) RhoA expression in RAW 264.7 cells was analyzed by Western blot using antibodies specific to RhoA. (A, B, and D) HGF mRNA levels were analyzed using semiquantitative reverse-transcription polymerase chain reaction. (E) HGF levels in the conditioned medium were measured using ELISA. Values represent the mean ± S.E.M of three independent experiments. *P < 0.05.](image-url)
Gas6 or apoptotic cells could promote proliferation and wound closure of murine LA-4 alveolar type II-like epithelial cells.

Materials and Methods

Reagents. Recombinant proteins of mouse Gas6, Mer/Fc, and Met/Fc were purchased from R&D Systems (Minneapolis, MN), LY-294002 [2-(4-morpholinyl)-8-phenyl-4H-1-benzo[4-5]pyran-4-one hydrochloride], wortmannin (Sigma-Aldrich, St. Louis, MO), PHA-665752 [(2R)-1-[[5-[[2,6-dichlorophenyl]methyl]sulfonyl]-1,2-dihydro-2-oxo-3H-indol-3-ylidene][methyl]-2,4-dimethyl-1H-pyrrol-3-yl]carboxyl][2-(1-pyrolidinylmethyl)pyrrolidine] (Tocris Bio Science, Ellisville, MO), SB 203580 [4-[5-(4-fluorophenyl)-2-[4-(methylsulfonyl)phenyl]-1H-imidazol-4-yl]pyridine hydrochloride] and PD 98059 [2-(2-amino-3-methoxyphenyl)-4H-1-benzo[4-5]pyran-4-one] (Biomol, Plymouth Meeting, PA), and Jun NH2-terminal kinase (JNK) inhibitor II (Calbiochem, San Diego, CA) were used as supplied. The gene Moloney murine leukemia virus (M-MLV)–specific relative reverse-transcription polymerase chain reaction kit and M-MLV transcriptase were purchased from Invitrogen/Life Technologies (Carlsbad, CA) and EnzymObios (Seoul, Korea), respectively. The G-LISA RhoA activation was obtained from Cytoskeleton, Inc. (Denver, CO).

Cell Lines, Culture, and Stimulation. Murine RAW 264.7 macrophages (American Type Culture Collection, Manassas, VA) were plated at 10⁶ cells/ml and incubated overnight in Dulbecco’s modified Eagle’s medium (Mediatech, Inc., Washington, DC) supplemented with 10% fetal bovine serum (FBS), 2 mML-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C and 5% carbon dioxide (CO₂). Before stimulation, the medium was replaced with serum-free X-vivo 10. The human leukemia T-cell line Jurkat was obtained from American Type Culture Collection and cultured in RPMI 1640 (Mediatech, Inc.)

Fig. 2. Activation of the PI3K/Akt pathway and MAP kinases is required for Gas6-induced HGF mRNA and protein expression. (A–D) RAW 264.7 cells were pretreated with the PI3K inhibitor wortmannin or LY 294002 (LY) at the indicated concentrations for 1 hour and then stimulated with 400 ng/ml Gas6 for 15 minutes to detect phosphorylation of Akt (A and B), for 2 hours to detect HGF mRNA expression (C) and for 24 hours to detect secreted HGF (D). (E and F) RAW 264.7 cells were preincubated with inhibitors of p38 MAPK, ERK, or JNK (10 μM SB 203580 [SB], 30 μM PD 98059 [PD], or 30 μM JNK inhibitor II, respectively) for 1 hour, and then stimulated with Gas6 for 2 hours to detect HGF mRNA expression (E) and for 24 hours to detect secreted HGF (F). (A and B) Phospho-Akt/Akt was detected in total cell lysates using immunoblotting. Relative values for phosphorylated kinase versus unphosphorylated kinase are indicated below the gel. (C and E) HGF mRNA levels were analyzed using semiquantitative reverse-transcription polymerase chain reaction and normalized to β-actin mRNA levels. (D and F) Secreted HGF levels were measured using ELISA. Values represent the mean ± S.E.M. of three independent experiments. *P < 0.05.
containing 10% FBS. Murine LA-4 alveolar type II–like epithelial cells were purchased from American Type Culture Collection and grown in F12K medium (Lonza, Basel, Switzerland) containing 15% heat-inactivated FBS at 37°C in 5% CO₂.

**Induction of Cell Death.** Jurkat T cells were induced to undergo apoptosis by exposure to ultraviolet irradiation at 254 nm for 10 minutes and then cultured in RPMI 1640 for 2.5 hours at 37°C and 5% CO₂ before being added to macrophages. Irradiation caused approximately 70–80% of cells to undergo apoptosis as confirmed by evaluation of nuclear morphology using light microscopy (Fadok et al., 2001; Hoffmann et al., 2001). Lysed (necrotic) Jurkat T cells were obtained by multiple freeze-thaw cycles (Fadok et al., 2001). Apoptosis and necrosis were confirmed by Annexin V/propidium iodide (BD Biosciences, San Jose, CA) staining followed by flow cytometric analysis on a fluorescence-activated cell sorter (BD Biosciences) (Golpon et al., 2004).

**Coincubation of Macrophages with Gas6 or Apoptotic, Viable, or Necrotic Jurkat T Cells.** RAW 264.7 macrophages (10⁶ cells/ml) were incubated with Gas6 or Jurkat T cells (apoptotic, viable, or necrotic; 3 × 10⁶ cells/ml) for 20 hours in serum-free media.

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**Fig. 3.** PI3K/Akt pathway and MAP kinases are downstream of RhoA/Rho kinase. RAW 264.7 cells were pretreated with 1 µg/ml C3 transferase (C3T) for 20 hours (A, C, E, and G) or 10–50 mM Y-27632 (Y) for 2 hours (B, D, F, and H) and then stimulated with Gas6 for 15 minutes. (I–L) RAW 264.7 cells were transfected with RhoA siRNA or control vehicle (siRNA-green fluorescent protein) for 24 hours and then stimulated with Gas6 for 15 minutes. Phospho-Akt/Akt, phospho-p38 MAPK/p38 MAP kinase, phospho-ERK (P-ERK)/ERK1, or phospho-JNK1/JNK1 was detected in total cell lysates using immunoblotting. Relative values for phosphorylated kinase versus unphosphorylated kinase are indicated below the gel. (K) Lines indicate discontinuities in lanes 2 and 5. Values represent means ± S.E.M. of three separate experiments. *P < 0.05.
The conditioned medium was harvested by centrifugation and filtration and then used for enzyme-linked immunosorbent assay (ELISA) or proliferation and wound repair assays.

**Transient Transfections.** RAW 264.7 cells were transiently transfected with 75 nM control small interfering RNA (siRNA) or Mer-, Axl-, Tyro3-, or RhoA-specific siRNA (Bioneer, Seoul, Korea) using 5 μl of siRNA transfection reagent (Genlantis, San Diego, CA) according to the manufacturer’s protocol. The cells were then incubated in serum-free medium for 24 hours before further experimentation.

**Immunoblot Analysis.** RAW 264.7 macrophages (10^6 cells/ml) were plated and incubated in serum-free medium overnight. The stimulated cells were lysed in 0.5% Triton X-100–containing lysis buffer and then resolved on a 10% SDS-PAGE gel before transfer onto nitrocellulose. Membranes were blocked at room temperature with Tris-buffered saline containing 3% bovine serum albumin and then incubated at room temperature with various anti-primary antibodies and probed with mouse anti-mouse horseradish peroxidase–conjugated secondary antibody. Bands were visualized using enhanced chemiluminescence.

**Semi-quantitative Reverse-Transcription Polymerase Chain Reaction.** Total RNA was isolated from cultured cells using TRIzol reagent (Life Technologies, Grand Island, NY). The concentration and purity of the RNA samples were evaluated by spectrophotometry. RT was conducted for 60 minutes at 42°C with 2 μg of total RNA using M-MLV reverse transcriptase. cDNA was denatured for 5 minutes at 95°C and amplified using a GeneAmp PCR System 2400 (PerkinElmer, Waltham, MA). The PCR primer sequences used were mouse-specific HGF (sense 5'-GCT GAC TGC ATT CAT GTG AG-3' and anti-sense 5'-GCT GAC TGC ATT CAT GTG AG-3'). cDNA was denatured for 5 minutes at 95°C and amplified using a GeneAmp PCR System 2400 during 33 cycles of 95°C for 30 seconds, 54°C for 30 seconds, and 72°C for 30 seconds, followed by a 10-minute final extension at 72°C. Samples were visualized on 1–2% agarose gels stained with ethidium bromide. The relative amounts of HGF compared with β-actin were determined by densitometry.

**ELISA Measurement of HGF Protein.** Culture supernatants were collected and HGF concentration was measured using ELISA according to the manufacturer’s instructions (R&D Systems).

**Cell Proliferation Assay.** Murine LA-4 alveolar type II–like epithelial cells were plated into 96-well culture plates (10^4 cells/well) and cultured overnight in 200 μl RPMI 1640 containing 10% FBS. Cells were synchronized in RPMI 1640 containing 0.5% heat-inactivated FBS for 6 hours and then treated for 18 hours with conditioned medium from RAW 264.7 cells (Golpon et al., 2004). In some experiments, an antibody against the HGF receptor, VEGF (neutralizing), was added to the conditioned medium. After incubation, the cells were washed three times with PBS before determining cell proliferation of LA-4 cells using the CyQuant Cell Proliferation Assay Kit (Molecular Probes/Life Technologies, Carlsbad, CA) according to the manufacturer’s protocol. Fluorescence was measured using the Synergy H1 microplate reader (BioTek, Winooski, VT) with excitation at 480 nm and emission detection at 520 nm.

**In Vitro Epithelial Wound Repair Assay.** LA-4 cells (3 x 10^5 cells/well) grown in six-well plates were serum-starved for 18 hours before being scratched in a defined area using a small pipette tip (10 μl). Detached cells were removed before incubation in serum-free medium alone or conditioned medium for 24 hours (Golpon et al., 2004). Inactivated FBS for 6 hours and then treated for 18 hours with conditioned medium from RAW 264.7 cells (Golpon et al., 2004). In some experiments, an antibody against the HGF receptor, VEGF (neutralizing), TGF-β (neutralizing), isotype IgG, or the c-Met antagonist PHA-665752 was added to the conditioned medium. After incubation, the cells were washed three times with PBS before determining cell proliferation of LA-4 cells using the CyQuant Cell Proliferation Assay Kit (Molecular Probes/Life Technologies, Carlsbad, CA) according to the manufacturer’s protocol. Fluorescence was measured using the Synergy H1 microplate reader (BioTek, Winooski, VT) with excitation at 480 nm and emission detection at 520 nm.

**Results**

**Activation of RhoA/Rho Kinase, Akt, and MAP Kinases Is Required for Gas6-Induced HGF mRNA and Protein Expression.** Our previous study demonstrated that Gas6/mer signaling induces activation of RhoA, Akt, and three MAP kinases (Park et al., 2012). In the present study, to
identify Gas6/Mer downstream signaling pathway leading to induction of HGF production, we first investigated whether these molecules are required for the HGF mRNA and protein expression in response to Gas6. To examine the involvement of RhoA/Rho kinase pathway in HGF expression, RAW 264.7 cells were pretreated overnight with 0.3–1.0 μg/ml of the RhoA-specific inhibitor C3 transferase, which inactivates Rho proteins through ADP-ribosylation, or 10–50 μM Y-27632, an inhibitor of the RhoA downstream molecule Rho kinase, for 2 hours in the presence of Gas6. Both inhibitors reduced Gas6-induced HGF mRNA expression in a concentration-dependent manner (Fig. 1, A and B).

Fig. 4. Interaction between PI3K/Akt pathway and MAP kinase pathways in RAW 264.7 cells stimulated with Gas6. RAW 264.7 cells were pretreated with PI3K inhibitors (wortmannin [W] or LY 294002 [LY]) at the indicated concentrations, or with inhibitors of p38 MAPK, ERK, or JNK (10 μM SB 203580 [SB], 30 μM PD 98059 [PD], or 30 μM JNK inhibitor II, respectively) for 1 hour. RAW cells were then stimulated with 400 ng/ml Gas6 for 15 minutes to detect phosphorylation of MAP kinases (A–F) or Akt (G). Phospho-Akt/Akt, phospho-p38 MAPK/p38 MAP kinase, phospho-ERK (P-ERK)/ERK, or phospho-JNK (P-JNK)/JNK were detected in total cell lysates using immunoblotting. Relative values for phosphorylated kinase versus unphosphorylated kinase are indicated below the gel. (A and B) Lines indicate discontinuities in a blot. Values represent means ± S.E.M of three separate experiments. *P < 0.05.
To further confirm the role of RhoA in Gas6-induced HGF gene expression, we examined the level of HGF mRNA in RAW 264.7 cells transfected with 75 nM RhoA-specific siRNA or pharmacologic inhibitors. Whereas the negative control siRNA did not alter RhoA protein levels in cells regardless of Gas6 exposure, RhoA protein levels were completely diminished at 24 hours after transfection with RhoA siRNA (Fig. 1C). The absence of RhoA led to decreased Gas6-induced HGF mRNA without affecting the level of β-actin, which was assessed as a control (Fig. 1D). Moreover, pharmacologic inhibition of RhoA/Rho kinase with 1 μg/ml C3 transferase or 50 μM Y-27632 suppressed HGF protein levels in the culture medium (Fig. 1E).

In addition, we examined the involvement of phosphatidylinositol 3-kinase (PI3K)/Akt pathway and MAP kinase pathway in Gas6-induced HGF mRNA and protein expression in RAW 264.7 cells. Pretreatment with the PI3K inhibitor wortmannin (30–100 nM) (Fig. 2A) or LY 294002 (30–100 μM) for 1 hour before Gas6 stimulation reduced Akt phosphorylation in a concentration-dependent manner (Fig. 2B), as well as HGF mRNA expression (Fig. 2C) and protein production (Fig. 2D). RAW 264.7 cells were also pretreated with SB

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**Fig. 5.** RhoA-dependent signaling after Gas6/Mer engagement is required for HGF mRNA induction in murine peritoneal macrophages. (A–F) Peritoneal macrophages were transfected with Mer siRNA or control vehicle for 48 hours. (G–L) Peritoneal macrophages were pretreated with C3 transferase (C3T; 1 μg/ml) for 20 hours, a Rho kinase inhibitor Y-27632 (Y) (50 μM), PI3K inhibitor (100 nM wortmannin), or inhibitors of p38 MAPK, ERK, or JNK (10 μM SB 203580 [SB], 30 μM PD 98059 [PD], or 30 μM JNK inhibitor II, respectively) for 1 hour. Cells were then stimulated with 400 ng/ml Gas6 for 15 minutes to detect phosphorylation of Akt or MAP kinases and for 2 hours detect HGF mRNA expression. (A, G, and H) mRNA levels of HGF were analyzed by relative quantitative reverse-transcription polymerase chain reaction and normalized to β-actin mRNA levels. (B) The levels of RhoA activity were quantified. (C–F and I–L) Phospho-Akt/Akt, phospho-p38 MAPK/p38 MAP kinase, phospho-ERK (P-ERK)/ERK, or phospho-JNK (P-JNK)/JNK were detected in total cell lysates using immunoblotting. Relative values for phosphorylated kinase versus unphosphorylated kinase are indicated below the gel. Values represent means ± S.E. of three separate experiments. *P < 0.05.
203580, a specific p38 MAP kinase inhibitor, PD 98059, a specific mitogen-activated kinase kinase 1 inhibitor, or the JNK inhibitor II (30 μM) for 1 hour before exposure to Gas6; 10 μM SB 203580, 30 μM PD 98059, or 30 μM JNK inhibitor II, at which concentrations were shown previously to be effective and without cell toxicity (Xiao et al., 1998, 2002), reduced the levels of apoptotic cell–induced HGF mRNA expression (Fig. 2E) and secreted HGF protein (Fig. 2F). These data suggest that activation of the PI3K/Akt pathway and these MAP kinases is required for Gas6-induced HGF mRNA and protein expression in macrophages.

Gas6 Stimulates RhoA/Rho Kinase/PI3K/Akt/MAP Kinases Axis in RAW 264.7 Cells. We examined first whether the RhoA/Rho kinase pathway acts as an upstream regulator of PI3K/Akt and MAP kinases. Pharmacologic inhibitors of RhoA/Rho kinase, C3 transferase and Y-27632 (Fig. 3, A–H), and RhoA siRNA (Fig. 3, I–L) inhibited Gas6-induced phosphorylation of Akt and MAP kinases. We then examined whether the PI3K/Akt pathway acts upstream of the MAP kinases in response to apoptotic cells. The PI3K inhibitor wortmannin (100 nM) or LY 294002 (100 μM), the PI3K inhibitor wortmannin (100 nM) or LY 294002 (100 μM), the MAP kinase inhibitors SB 203580 (10 μM), PD 98059 (30 μM), or JNK inhibitor II (30 μM) suppressed significantly HGF mRNA expression (Fig. 5, G and H) and phosphorylation of Akt and MAP kinases, including p38 MAP kinase, ERK1/2, and JNK1 (Fig. 5, I–L). These findings indicate that RhoA/PI3K/Akt/MAP kinase signaling is required for HGF mRNA induction in primary cells of peritoneal macrophages on exposure to Gas6 as for that seen in RAW 264.7 cell line.

Macrophages Exposed to Gas6 or Apoptotic Cells Enhance LA-4 Epithelial Cell Proliferation and Wound Closure. The normal response to injury to the epithelial surface involves a complex repair process that includes epithelial migration, proliferation, and differentiation (Aarbiou et al., 2004). To assess whether factors secreted by macrophages in response to Gas6 or apoptotic cells are indeed biologically effective, cell supernatants were incubated for 18 hours with murine LA-4 alveolar type II–like epithelial cells, and cell proliferation was measured. The conditioned medium derived from RAW 264.7 cells exposed to 200 or 400 ng/ml Gas6 had similar or comparable stimulatory effects on the proliferation of LA-4 cells (Fig. 6A). Likewise, epithelial cell proliferation was enhanced by incubation with conditioned medium derived from macrophages exposed to apoptotic cells. This effect was not observed with medium derived from naive epithelial cells or with conditioned media derived from coculture with control, viable, or necrotic Jurkat cells. Culture supernatant derived from apoptotic Jurkat cells alone also did not induce proliferation.

To investigate whether exposure of macrophages to Gas6 or apoptotic cells affects epithelial wound closure, mechanically wounded LA-4 cell monolayers were incubated for 24 hours with supernatants from RAW 264.7 cells that had been exposed
to Gas6 and apoptotic, viable, or necrotic Jurkat cells. Conditioned medium from RAW 264.7 cells that had been exposed to Gas6 or apoptotic cells promoted wound closure, whereas medium from RAW 264.7 cells cocultured with viable or necrotic Jurkat cells did not (Fig. 6, B and C). Supernatant from cultured apoptotic Jurkat cells alone also did not promote wound closure (Fig. 6B).

**Direct Exposure of LA-4 Cells to Gas6 or Apoptotic Cells Does Not Enhance Epithelial Cell Growth and Repair.** Previous data demonstrated that Gas6 functions as a growth factor in cultured human lens epithelial cells (Valverde et al., 2004) and cardiac fibroblasts (Stenhoff et al., 2004). Thus, we examined whether Gas6 directly regulates LA-4 cell proliferation. Direct exposure of LA-4 cells to 400 ng/ml Gas6 did not significantly affect cell proliferation and wound closure after epithelial injury (Fig. 7, A and B). Similarly, incubation of LA-4 cells with apoptotic cells did not increase in epithelial growth. Moreover, cotreatment of LA-4 cells with a Gas6 inhibitor, Mer/Fc, did not affect epithelial proliferation and wound closure after epithelial injury induced by conditioned medium isolated from macrophages exposed to Gas6 or apoptotic cells (Fig. 7, C and D). These data indicate that epithelial cell growth and wound closure are induced by bioactive factors secreted by macrophages that have switched functional program upon Gas6 or apoptotic cell stimulation.

**HGF Secretion from Macrophages Causes Epithelial Cell Proliferation and Wound Repair.** To identify the secreted factor involved in epithelial cell growth, conditioned medium was added to LA-4 cells in the presence of a blocking antibody against the HGF receptor or neutralizing antibodies against either TGF-β or VEGF. Only samples treated with anti-HGF receptor antibodies inhibited LA-4 cell proliferation induced by conditioned medium derived from RAW 264.7 cells stimulated with Gas6 or apoptotic cells (Fig. 8A). A c-Met antagonist, PHA-665752, produced a similar inhibitory effect (Fig. 8D). In contrast to these findings, the TGF-β1 and VEGF neutralizing antibodies did not inhibit cell proliferation, indicating that these growth factors are not involved (Fig. 8, B and C).

To address whether released HGF leads to enhanced epithelial wound repair, conditioned medium was added to mechanically wounded LA-4 cell monolayers, along with anti-HGF receptor antibody or PHA-665752. Both agents significantly inhibited wound repair induced by conditioned medium harvested from RAW 264.7 cells stimulated with Gas6 or apoptotic cells (Fig. 8, E and F) or apoptotic cells (Fig. 8, G and H). To confirm that released HGF (from basal 60 pg/ml increasing to 150 pg/ml) contributes to epithelial cell proliferation and wound closure in a paracrine manner, we investigated the effects of exogenous HGF at 60, 150, and 1000 pg/ml in epithelial cells. At the basal concentration, HGF enhanced little, but 150 and 1000 pg/ml HGF enhanced significantly, LA-4 cell proliferation in a concentration-dependent manner, compared with those of conditioned medium alone or the basal concentration of HGF (Fig. 8I). Similarly, direct exposure of LA-4 cells to HGF enhanced (150 and 1000 pg/ml) enhanced significantly wound repair compared with those of control conditioned medium with or without the basal concentration of HGF (Fig. 8, J and K). Taken together, these data indicate that both cell proliferation and wound closure in LA-4 cells were evoked by Gas6- or apoptotic cell–induced HGF activity.
Gas6 or Apoptotic Cell–Induced Mer/RhoA Signaling Provokes Macrophage-Dependent Epithelial Cell Proliferation and Wound Repair. To determine the functional relevance of Mer signaling in a macrophage-dependent epithelial proliferation, RAW 264.7 cells were transfected with Mer-, Axl-, or Tyro3-specific or negative-control siRNA and then cultured for 24 hours before exposure to Gas6 or apoptotic cells for 20 hours. After 24–48 hours post-transfection, Mer, Axl, or Tyro3 protein levels were decreased approximately 80% in cells transfected with the specific siRNA (Park et al., 2012). As expected, the negative-control siRNA did not alter Mer or Axl protein levels. The conditioned medium derived from macrophages incubated with Gas6 or apoptotic cells in the presence of Mer siRNA reduced the enhancement of LA-4 epithelial cell proliferation by 72 or 100%, respectively (Fig. 9, A and B), as well as caused complete inhibition of wound closure (Fig. 9, C–E). Axl- or Tyro3-specific siRNA produced little to no inhibitory effects on enhanced epithelial proliferation and wound closure induced by the conditioned medium.

Transfection of RAW 264.7 cells with RhoA siRNA before exposure to Gas6 or apoptotic cells resulted in a reduction in enhanced epithelial cell proliferation (Fig. 9, A and B) and wound closure by the conditioned media (Fig. 9, C–E) compared with cells incubated with conditioned medium derived from negative control siRNA-transfected macrophages (Fig. 9, A and B). Furthermore, treatment with 10–50 μM Y-27632 suppressed this cell proliferation (Fig. 10A) and wound closure by the Gas6 or apoptotic cell conditioned media (Fig. 10, B and C). These results suggest that epithelial cell proliferation and wound closure were evoked by macrophage production of HGF, which was induced by Gas6 or an apoptotic cell/Mer/RhoA/Rho kinase–dependent pathway.

Discussion

Macrophages have been shown to play key roles in orchestrating the biologic events involved in inflammation and restoration of tissue integrity in vivo and in vitro (Pull et al., 2005; Qualls et al., 2006; Seno et al., 2009; D’Angelo et al., 2013). These beneficial effects are due mainly to the release of trophic factors macrophages into the environment, particularly on parenchymal cells. Previously, we demonstrated that, like exposure to apoptotic cells, treatment of macrophages with Gas6 leads to HGF transcription induced through Mer receptor activation (Park et al., 2012). HGF is a paracrine growth, motility, and morphogenic factor (Yi et al., 1998). Thus, we hypothesize that Gas6 could reprogram macrophages into tissue repair–promoting cells for maintaining tissue homeostasis in injured epithelium through HGF induction. Previously, we reported activation of all three TAM-family receptors (Mer, Axl, and Tyro3) in RAW 264.7 cells in response to apoptotic cells or Gas6. However, only Mer was found to be responsible for mediating HGF gene expression and production (Park et al., 2012). In this study, we elucidated a signaling mechanism...
downstream of the Mer receptor that is involved in HGF expression by macrophages stimulated by Gas6. Studies have demonstrated that RhoA/Rho kinase–dependent signaling pathways, including PI3K/Akt, p38 MAP kinase, ERK, and JNK, mediate Gas6-induced HGF mRNA and protein expression. We found that p38 MAP kinase and ERK act downstream of PI3K/Akt, which also participates in cross-talk with JNK during Gas6 stimulation. Notably, these data suggest that Gas6 and apoptotic cells use the same signaling pathways for HGF expression.

Previously, it was reported that the conditioned medium from HC-11 epithelial cells exposed to apoptotic cells also...
enhanced epithelial wound repair (Mackay and Hall, 1998). However, the bioactive mediators responsible for this enhanced wound closure have yet to be elucidated. With respect to the study of wound repair, simple cell assays, such as proliferation and cell migration, are widely used in a laboratory despite limitations of the methods (Liang et al., 2007; Ansell et al., 2012). In particular, one of the major advantages of in vitro scratch wound assay is that it mimics, to some extent, the migration of cells in vivo. Another advantage is its particular suitability to study the regulation of cell migration by cell interaction with extracellular matrix and cell-cell interactions. However, the complex extracellular environment and range of cell types involved in wound repair are not considered in these simple cell assays. In vitro organotypic culture models have been developed. On the other hand, a number of ex vivo skin explant systems contain local cell types, including some resident inflammatory cells (Pistoor et al., 1996). However, these models remain particularly undeveloped (Ansell et al., 2012). Here, using these simple cell assays, we demonstrated that LA-4 epithelial cell proliferation and wound closure were enhanced by conditioned medium derived from macrophages exposed to Gas6. It is highly likely that aspects of the observed biologic properties of the conditioned medium were mediated by HGF since anti-HGF receptor antibodies and the c-Met antagonist completely inhibited epithelial cell proliferation and wound closure elicited by the conditioned medium. Moreover, the paracrine effects of HGF after exposure of macrophages to Gas6 on epithelial proliferation and wound closure were quite similar to those after exposure of macrophages to apoptotic cells. This hypothesis was confirmed by the experiments where exogenous HGF at the concentration of 150 pg/ml as released from macrophages for 20 hours in response to Gas6-enhanced epithelial cell proliferation, as well as wound closure. Other uncharacterized factors produced by macrophages after exposure to Gas6 or apoptotic cells likely also contribute to epithelial growth. VEGF and TGF-β are released from macrophages after incubation with apoptotic cells (Fadok et al., 1998; Golpon et al., 2004). VEGF was initially thought to be a specific endothelial cell mitogen (Brown et al., 2001). More recent studies showed that VEGF also stimulates proliferation of other cell types, including renal tubular epithelial cells and hepatocytes (Kanellis et al., 2000; Lecouter et al., 2003). Recent studies showed that exogenous VEGF upregulates acid-exposed alveolar lining epithelial cells but not resting cells (Ohwada et al., 2003). In contrast, TGF-β is a potent growth inhibitor for most cell types, including epithelial cell and endothelial cells (Moses et al., 1991). Data from our study using neutralizing antibodies against VEGF and TGF-β suggested that VEGF, like TGF-β, does not play a role in LA-4 epithelial cell proliferation and wound closure in the present experimental setting.

Next, we focused on the role of Gas6/Mer/RhoA signaling in epithelial cell proliferation and wound repair induced by conditioned medium. Incubation with a specific Mer siRNA, RhoA siRNA, or Rho kinase inhibitor (Y-27632) suppressed the proliferation and wound repair of epithelial cells elicited by Gas6-treated RAW 264.7 cells. Similar results were found when RAW 264.7 cells were stimulated with apoptotic cells in the same experimental model. Collectively, these data implicate that Gas6/Mer signaling or Gas6/phosphatidylserine complexes present on apoptotic cell surfaces are recognized by the Mer receptor, which induces HGF expression via a RhoA-dependent pathway to mediate epithelial cell proliferation and wound repair. Notably, data from our previous study using siRNA suggested that neither Axl nor Tyro3 affects RhoA activity in response to apoptotic cells or Gas6 (Park et al., 2012). Thus, our findings emphasize that RhoA is
a critical signaling molecule for macrophage-dependent epithelial growth activated by Mer receptor binding. In addition to RAW 264.7 cells, we confirmed the involvement of Mer in Gas6-induced RhoA activity and phosphorylation of Akt and several MAP kinases, as well as HGF mRNA expression, using Mer-specific siRNA. Moreover, RhoA/Rho kinase/PI3K/Akt/MAP kinase signaling after Gas6/Mer engagement leading to HGF mRNA expression was also confirmed in primary cells of murine peritoneal macrophages. Thus, although not explored directly herein, it seems reasonable to assume that released HGF from primary macrophage cells after exposure to Gas6 contributes to epithelial cell proliferation and wound closure.

In conclusion, data from our studies demonstrate a novel mechanism for Gas6-induced macrophage-dependent epithelial growth and wound repair. Thus, defects in Gas6/Mer/RhoA signaling or apoptotic cell recognition in macrophages may delay tissue repair after epithelial injury,
thereby contributing to the pathogenesis of chronic lung inflammatory diseases and autoimmunity.

**Authorship Contributions**

**Participated in research design:** Lee, H.-J. Park, Kang.

**Conducted experiments:** Lee, H.-J. Park.

**Contributed new reagents or analytic tools:** Woo, E. M. Park.

**Performed data analysis:** Lee, H.-J. Park, Kang.

**Wrote or contributed to the writing of the manuscript:** Kang.

**References**


Alciato F, Sainaghi PP, Sola D, Castello L, and Avanzi GC (2010) TNF-alpha, IL-6, LA-4 cell monolayers were incubated with the conditioned medium, and the distance between the opposite edges of the wound was measured at the indicated times. (C) Photographs depict a representative epithelial wound closure. Values represent the mean ± S.E.M. of three independent experiments. *P < 0.05; **P < 0.05 for Gas6 CM versus Y-27632 + Gas6 CM or Apo CM versus Y-27632 + Apo CM at a given time.

Fig. 10. Inhibition of Rho kinase blocks macrophage-dependent epithelial cell proliferation and wound repair. (A) RAW 264.7 cells were pretreated with a Rho kinase inhibitor Y-27632 (10–50 μM) for 2 hours before stimulation with Gas6 or apoptotic cells (Apo). After 20 hours, conditioned medium was collected and added to LA-4 cells. Cell proliferation was assessed 18 hours after the addition of conditioned medium (CM). (B) Mechanically wounded LA-4 cell monolayers were incubated with the conditioned medium, and the distance between the opposite edges of the wound was measured at the indicated times. (C) Photographs depict a representative epithelial wound closure. Values represent the mean ± S.E.M. of three independent experiments.

**+** P < 0.05; **+** P < 0.05 for Gas6 CM versus Y-27632 + Gas6 CM or Apo CM versus Y-27632 + Apo CM at a given time.


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