The TAM-family receptor Mer mediates production of HGF through the RhoA-dependent pathway in response to apoptotic cells

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ABSTRACT The TAM receptor protein tyrosine kinases Tyro3, Axl, and Mer play important roles in macrophage function. We investigated the roles of the TAM receptors in mediating the induction of hepatocyte growth factor (HGF) during the interaction of macrophages with apoptotic cells. Mer-specific neutralizing antibody, small interfering RNA (siRNA), and a recombinant Mer protein (Mer/Fc) inhibited HGF mRNA and protein expression, as well as activation of RhoA, Akt, and specific mitogen-activated protein (MAP) kinases in response to apoptotic cells. Inhibition of Axl or Tyro3 with specific antibodies, siRNA, or Fc-fusion proteins did not prevent apoptotic cell-induced HGF mRNA and protein expression and did not inhibit activation of the postreceptor signaling molecules RhoA and certain MAP kinases in response to apoptotic cells. Inhibition of Axl or Tyro3 with specific antibodies, siRNA, or Fc-fusion proteins did not inhibit the activation of Akt and p38 MAP kinase in response to apoptotic cells. In addition, none of the TAM receptors mediated the effects of apoptotic cells on transforming growth factor-β or epidermal growth factor mRNA expression. However, they were involved in the induction of vascular endothelial growth factor mRNA expression. Our data provide evidence that when macrophages interact with apoptotic cells, only Mer of the TAM-family receptors is responsible for mediating transcriptional HGF production through a RhoA-dependent pathway.

INTRODUCTION Apoptotic cell clearance is essential in maintaining immune homeostasis. The interaction of apoptotic cells and phagocytes suppresses the production of inflammatory mediators, creating an anti-inflammatory milieu around the site of apoptosis (McDonald et al., 1999; Huynh et al., 2002). This process is mediated through the actions of transforming growth factor-β (TGF-β), prostanooids, peroxidase proliferator–activated receptor-γ, liver-X receptors, and interleukin (IL)-10 (Fadok et al., 1998; McDonald et al., 1999; Henson et al., 2001; Freire-de-Lima et al., 2006; Majai et al., 2007; A-Gonzalez et al., 2009; Mukundan et al., 2009). Furthermore, the interactions of apoptotic cells with professional or nonprofessional phagocytic cells play important roles in the regeneration and repair process of damaged tissues by inducing growth factors such as vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF; Morimoto et al., 2001; Golpon et al., 2004). We previously demonstrated that RhoA is a key signaling molecule that up-regulates HGF transcription in response to apoptotic cells through the phosphatidylinositol 3-kinase (PI3K)/Akt/mitogen-activated protein (MAP) kinase signaling pathway (Park et al., 2011). However, the relationship of a specific macrophage recognition receptor that interacts with apoptotic cells and triggers the activation of this signaling pathway has not been defined.
FIGURE 1: Specific neutralizing anti-Mer antibody inhibits apoptotic cell–induced HGF expression and activation of downstream intracellular signaling molecules. (A, B) Phosphorylation of Mer in RAW 264.7 cells in response to apoptotic cells or Gas6. RAW 264.7 cells were stimulated with apoptotic (ApoJ; A) or 400 ng/ml Gas6 (B) for the time indicated. Immunoblots of total cell lysates were analyzed for phospho-Mer/Mer. Relative values for phosphorylated Mer vs. unphosphorylated Mer are indicated below the gel. (C) RAW 264.7 cells were pretreated with 10 or 20 μg/ml anti-Mer antibody or IgG for 1 h and stimulated with apoptotic cells for 2 h. HGF mRNA levels were analyzed by relative quantitative RT-PCR and normalized to β-actin mRNA levels. (D–I) RAW 264.7 cells were pretreated with 20 μg/ml anti-Mer antibody or IgG for 1 h and stimulated with HGF protein levels in the conditioned medium were measured by ELISA. (E) The levels of p38 MAPK, phospho-MAP kinase, p38 MAP kinase, phospho-ERK/ERK, or phospho-JNK/JNK. Relative values for phospho-Mer vs. unphosphorylated kinase are indicated below the gel. Values represent means ± SE of three separate experiments. * p < 0.05.

Mer (Mer), a receptor tyrosine kinase in the Tyro3/Axl/Mer (TAM) family, is crucial for apoptotic cell clearance by macrophages in vivo and in vitro (Lu and Lemke, 2001; Lemke and Rothlin, 2008; Scott et al., 2001; Cohen et al., 2002). Growth arrest–specific protein 6 (Gas6) is a common ligand of the TAM receptor subfamily (Godowska et al., 1995; Stitt et al., 1995). Gas6 binds to phosphatidyserine expressed on the inverted plasma membrane of apoptotic cells (Mark et al., 1996; Lemke and Rothlin, 2008). Macrophage recognition of a Gas6–phosphatidylserine complex facilitates binding and clearance of apoptotic cells. Mer<sup>−/−</sup> mice have macrophages deficient in the clearance of apoptotic thymocytes and demonstrate spontaneous autoantibody production and lupus-like autoimmunity (Scott et al., 2001; Cohen et al., 2002). Furthermore, mice lacking all three TAM receptors are defective in apoptotic cell clearance and develop a severe lymphoproliferative disorder accompanied by broad-spectrum autoimmunity greater than that seen in mice lacking Mer alone (Lu and Lemke, 2001). Recent studies, however, suggest that Mer appears to be critical for the engulfment and efficient clearance of apoptotic cells in macrophages and organs, including the thymus and retina, although all three receptors contribute to these events (Scott et al., 2001; Seitz et al., 2007).

Binding of apoptotic cells or Gas6 to Mer elicits a number of postreceptor signals that are proposed to result in engulfment signals involving Src-mediated activation of focal adhesion kinase and increased formation of p130Cas/CrkII/Dock180 complex to activate Rac1 (Singh et al., 2007; Tibrewal et al., 2008). Furthermore, the Mer and PI3K/Akt pathways play key roles in the down-modulation of lipopolysaccharide- or zymosan A–induced expression of several proinflammatory cytokines, including tumor necrosis factor–α, IL-6, and IL-1β, in macrophages upon stimulation with Gas6- or phosphatidyserine-containing vesicles released from neutrophils (Aliotta et al., 2010; Eken et al., 2010). However, it is not known whether TAM receptor signaling is involved in the downstream production of HGF in response to apoptotic cells.

In the present study, we investigated the relative contribution of the three TAM receptors in mediating the production of HGF induced by the interaction of apoptotic cells with macrophages, which triggers the postreceptor signaling pathway.

RESULTS
Mer is involved in the apoptotic cell–induced signaling pathway that induces HGF production

Initial studies were performed to investigate the role of Mer in the induction of HGF and the postreceptor signaling pathway in macrophages in response to apoptotic cells.

Mer activation was examined in RAW 264.7 macrophages in response to apoptotic cells, viable cells, or Gas6 by Western blot analysis using an anti–phospho-Mer antibody. Phosphorylation of Mer peaked 5 min after exposure to apoptotic cells or Gas6, then gradually declined, and returned to resting levels at 120 and 30 min, respectively (Figure 1, A and B). However, exposure of macrophages to viable cells did not induce phosphorylation of Mer within the same time (Supplemental Figure S1A). The anti-Mer neutralizing antibody was used to specifically block the Mer activation by directing against the Mer extracellular domains. As expected,
antibody (Figure 1D) when compared with levels of HGF protein in the conditioned medium of RAW 264.7 cells pretreated with isotype IgG. The anti-Mer antibody also suppressed HGF protein expression in response to apoptotic cells (Supplemental Figure S2A).

Previously we demonstrated that apoptotic cells up-regulated transcription of HGF through the RhoA/Rho kinase/PI3K/Akt/MAP kinases, including p38 MAPK, extracellular signal-regulated protein kinase (ERK), and c-Jun NH2-terminal kinase (JNK) pathway (Park et al., 2011). Expression of these postreceptor signaling molecules peaked at 15 min after apoptotic cell treatment. Thus RhoA activity and phosphorylation of MAP kinases, including p38 MAPK, ERK1/2, and JNK1, were examined at this time point. RhoA activity, as well as the phosphorylation of these MAP kinases, was significantly decreased when apoptotic cell–induced macrophages were pretreated with the anti-Mer antibody (Figure 1, E–I). However, isotype IgG pretreatment did not affect apoptotic cell–induced HGF expression or phosphorylation of these signaling molecules.

To further examine the contribution of Mer signaling in apoptotic cell–induced HGF expression by RAW 264.7 cells, experiments were performed using Mer-specific small interfering RNA (siRNA). RAW 264.7 cells were transfected with Mer siRNA or negative-control siRNA and cultured for 48 h. The negative-control siRNA did not alter Mer protein levels in cells with or without apoptotic cell stimulation. After 48 h, Mer protein levels decreased ∼80% in cells transfected with Mer siRNA, but Axl and Tyro3 protein levels were not changed (Figure 2A). HGF expression and activation of the postreceptor signaling molecules were then assessed in Mer-specific siRNA–transfected RAW 264.7 cells. Mer-specific siRNA inhibited the apoptotic cell–induced expression of HGF mRNA and protein in the culture medium (Figure 2, B and C) and the cell lysates (Supplemental Figure S2B). Apoptotic cell–induced RhoA activity and phosphorylation of Akt and several MAP kinases were also inhibited by Mer-specific siRNA (Figure 2, D–H).

In previous experiments, it was shown that soluble Mer acts as a competitive inhibitor of the Gas6-mediated stimulation of membrane-bound Mer (Sather et al., 2007). Thus a chimeric recombinant protein consisting of the extracellular domain of murine Mer fused to the Fc domain of human immunoglobulin (Mer/Fc) was used to inhibit Mer activity in this study. Pretreatment of cells with 200 nM Mer/Fc resulted in a significant reduction of apoptotic cell–induced HGF mRNA expression and protein expression in the culture medium (Figure 3, A and B) and cell lysates (Supplemental Figure S2C). Activation of the signaling molecules RhoA, Akt, and MAP kinases in response to apoptotic cells was also significantly inhibited with anti-Mer antibody significantly inhibited phosphorylation of Mer at 5 and 15 min after exposure to apoptotic cells (Supplemental Figure S1D).

To determine whether Mer plays a role in apoptotic cell–induced HGF production, we exposed RAW 264.7 macrophages to apoptotic Jurkat cells in the absence or presence of the specific Mer-neutralizing antibody or isotype immunoglobulin G (IgG). The anti-Mer antibody significantly inhibited apoptotic cell–induced expression of HGF mRNA, with complete inhibition at 20 μg/ml (Figure 1C). Enzyme-linked immunosorbent assay (ELISA) was used to demonstrate that apoptotic cell–induced HGF secretion was significantly decreased by pretreatment with 20 μg/ml anti-Mer antibody (Figure 1D) when compared with levels of HGF protein in the conditioned medium of RAW 264.7 cells pretreated with isotype IgG. The anti-Mer antibody also suppressed HGF protein expression in response to apoptotic cells (Supplemental Figure S2A).

FIGURE 2: Mer-specific siRNA inhibits apoptotic cell–induced HGF expression and activation of intracellular signaling molecules. (A) Mer, Axl, or Tyro3 expression in RAW 264.7 cells transfected with Mer siRNA or control vehicle (siRNA-GFP) for 48 h was analyzed by Western blot with anti-Mer, anti-Axl, or anti-Tyro3 antibody. RAW 264.7 cells were transfected with Mer siRNA or control vehicle for 48 h and then stimulated with apoptotic Jurkat cells for 2 h. (B) mRNA levels of HGF were analyzed by relative quantitative RT-PCR and normalized to β-actin mRNA levels. (C) HGF levels in the conditioned medium were measured by ELISA. (D) The levels of RhoA activity were quantified. (E–H) Immunoblots of total cell lysates were analyzed for phospho-Akt/Akt, phospho-p38 MAPK/p38 MAP kinase, phospho-ERK/ERK, or phospho-JNK/JNK. Relative values for phosphorylated kinase vs. unphosphorylated kinase are indicated below the gel. Values represent means ± SE of three separate experiments. *p < 0.05.
contribution of Axl and Tyro3 in mediating the apoptotic cell–in
are also involved in apoptotic cell engulfment by recognition of a
exposure to apoptotic cells (Supplemental Figure S1, E and F). In
phosphorylation of the relevant TAM receptors at 5 and 15 min after
confirmed that anti-Axl or Tyro3 antibody significantly inhibited
264.7 cells to viable cells (Supplemental Figure S1, B and C). It was
phorylation of Axl and Tyro3 did not occur after exposure of RAW
sively declined thereafter (Figure 4, C and D). As expected, phos -
Tyro3 in response to Gas6 stimulation peaked at 5 min and progres-
levels at 120 min (Figure 4, A and B). Phosphorylation of Axl and
peaked at 5 and 15–30 min, respectively, and returned to resting
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contrast to the inhibitory effects of anti-Mer antibody, specific anti-Axl or anti-Tyro3 antibody did not influence apoptotic cell–induced HGF mRNA (Figure 4E) or protein expression in the culture medium (Figure 4F) and the cell lysates (Supplemental Figure S2D). RhoA activity was also unaffected (Figure 4G).

To confirm that Axl and Tyro3 do not play a role in apoptotic cell–induced HGF expression, we transfected RAW 264.7 cells with Axl- or Tyro3-specific siRNA or a negative control and cultured for 48 h. Axl and Tyro3 protein levels were specifically decreased by ~82 and 72%, respectively in cells transfected with Axl- or Tyro3-specific siRNA (Figure 5, A and B). On exposure to apoptotic cells, the siRNA silencing of Axl or Tyro3 mRNA expression did not inhibit HGF mRNA (Figure 5, C and D) or protein expression (Figure 5E and Supplemental Figure S2E).

Increased activation of the intracellular signaling molecules RhoA (Figure 6A) and MAP kinases, including ERK and JNK (Figure 6, F–I), after exposure to apoptotic cells was not inhibited by siRNA silencing of Axl or Tyro3. However, activation of Akt (Figure 6, B and C) and p38 MAP kinase (Figure 6, D and E) was significantly inhibited by these siRNA products. On the basis of neutralizing antibodies and genetic approaches, our data indicate that only Mer among these three TAM receptors is required for HGF induction by macrophages in response to apoptotic cells. In addition, Mer triggers the downstream signaling pathways involving RhoA, Akt, and MAP kinases, including p38 MAP kinase, ERK, and JNK. Previous studies showed that these postreceptor molecules are required for HGF expression in response to apoptotic cells (Park et al., 2011). Thus these molecules seem to be essential to the Mer signaling pathway that leads to HGF mRNA and protein expression. However, activation of Akt and p38 MAP kinase does not appear to be essential for HGF induction in response to apoptotic cells.

Similarly, soluble Axl or Tyro3 fusion proteins did not influence the apoptotic cell–induced HGF mRNA (Figure 7, A and B) and protein expression in the cell culture medium (Figure 7C) and the cell lysates (Supplemental Figure S2F). These data suggest that the Gas6/phosphatidylserine complexes formed on apoptotic cell surfaces are recognized by the Mer receptor rather than Axl or Tyro3, which mediates the induction of HGF expression. It is likely that there was no significant inhibition of RhoA activity (Figure 7D) and phosphorylation of ERK and JNK (Supplemental Figure S3, A and B) in response to apoptotic cells by Axl/Fc or Tyro3/Fc. However, these fusion proteins significantly inhibited phosphorylation of Akt and p38 MAP (Figure 7, E and F).

Mer is involved in the apoptotic cell–induced expression of HGF in murine peritoneal macrophages
The involvement of Mer in apoptotic cell–induced HGF mRNA expression was also examined in murine peritoneal macrophages.

200 nM Mer/Fc (Figure 3, C–G). However, the Met/Fc control pro-
tin did not yield a similar effect on HGF induction and the intracel-
lar signaling pathway (Figure 3, A–G).

Axl and Tyro3 do not contribute to the apoptotic cell–induced expression of HGF
Gas 6 is a common ligand for Mer, Axl, and Tyro3 (Godowskia et al., 1995; Stitt et al., 1995; Nagata et al., 1996). These TAM receptors are also involved in apoptotic cell engulfment by recognition of a Gas6–phosphatidylserine complex (Lemke and Lu, 2003). Thus the contribution of Axl and Tyro3 in mediating the apoptotic cell–induced HGF expression was also examined using receptor-specific neutralizing antibodies. We first examined the time course of Axl and Tyro3 activation after treatment with apoptotic cells or Gas6. Phosphorylation of Axl and Tyro3 in response to apoptotic cells peaked at 5 and 15–30 min, respectively, and returned to resting levels at 120 min (Figure 4, A and B). Phosphorylation of Axl and Tyro3 in response to Gas6 stimulation peaked at 5 min and progressively declined thereafter (Figure 4, C and D). As expected, phosphorylation of Axl and Tyro3 did not occur after exposure of RAW 264.7 cells to viable cells (Supplemental Figure S1, B and C). It was confirmed that anti-Axl or Tyro3 antibody significantly inhibited phosphorylation of the relevant TAM receptors at 5 and 15 min after exposure to apoptotic cells (Supplemental Figure S1, E and F). In

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FIGURE 3: Mer/Fc inhibits apoptotic cell–induced HGF expression and activation of intracellular signaling molecules. RAW 264.7 cells were pretreated with 200 nM Mer/Fc or 200 nM Met/Fc for 1 h and then stimulated with apoptotic cells for 15 min (C–G), 2 h (A), or 24 h (B). (A) HGF mRNA levels were analyzed by relative quantitative RT-PCR and normalized to β-actin mRNA levels. (B) HGF levels in the conditioned medium were measured by ELISA. (C) The levels of RhoA activity were quantified. (D–G) Immunoblots of total cell lysates were analyzed for phospho-Akt/Akt, phospho-p38 MAP kinase, phospho-ERK/ERK, or phospho-JNK/JNK. Relative values for phosphorylated kinase vs. unphosphorylated kinase are indicated below the gel. Values represent means ± SE of three separate experiments. *p < 0.05.
Mer receptor for the induction of HGF expression through the RhoA-dependent pathway in murine peritoneal macrophages.

**Mer is involved in Gas6-induced expression of HGF**

As in experiments with apoptotic cells, whether only Mer of the TAM receptors is required for HGF mRNA and protein expression upon exposure to Gas6 was examined. After 48 h, decreased Mer, Axl, and Tyro3 protein levels (∼80%) in RAW 264.7 cells transfected with specific siRNA of individual TAM receptors were confirmed (Supplementary Figure S4, A–C). Mer-specific siRNA inhibited the Gas6-induced expression of HGF mRNA and protein in the culture medium (Figure 9, A and B) and the cell lysates (Supplementary Figure S4D). Gas6-induced RhoA activity (Figure 9C) and phosphorylation of Akt and several MAP kinases (Supplementary Figure S5, A–D) were also inhibited by Mer-specific siRNA. However, the siRNA of Axl or Tyro3 did not inhibit HGF mRNA and protein expression in the culture medium (Figure 9, D–F) and the cell lysates (Supplementary Figure S4E). Increased activation of the intracellular signaling molecules RhoA (Figure 9G) and MAP kinases, including ERK and JNK (Supplemental Figure S6, E–H), after exposure to Gas6 was not inhibited by siRNA silencing of Axl or Tyro3. However, activation of Akt (Supplementary Figure S6, A and B) and p38 MAP kinase (Supplemental Figure S6, C and D) was significantly inhibited by these siRNA products. These results indicate that only Mer mediates Gas6-induced HGF expression through activation of the postreceptor signaling molecules, including RhoA, ERK, and JNK, as for apoptotic cell stimulation.

**Effects of Mer, Axl, and Tyro3 on apoptotic cell–induced mRNA expression of other growth factors**

We also examined whether the activation of Mer, Axl, and Tyro3 mediate the effects of the apoptotic cell induction of other key growth factors, including TGF-β, epidermal growth factor (EGF), and VEGF. Anti-Mer antibody or Mer/Fc did not suppress apoptotic cell–induced TGF-β1 mRNA expression in RAW 264.7 cells (Figure 10A) or peritoneal macrophages (Figure 10C). Similarly, blocking Axl or Tyro3 with receptor-specific antibodies or Fc-fusion proteins did not inhibit apoptotic cell–induced TGF-β1 mRNA expression in RAW 264.7 cells (Figure 10B) or peritoneal macrophages (Figure 10D). Apoptotic cell–induced expression of EGF mRNA in RAW 264.7 cells was not inhibited by any of the TAM receptor–specific siRNAs (Figure 10, E and F). However, the apoptotic cell–induced expression of VEGF mRNA induction was significantly inhibited (Figure 10, G and H). These data suggest that the three TAM

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**FIGURE 4:** Effects of Axl- and Tyro3-neutralizing antibodies on apoptotic cell–induced HGF expression. (A–D) Phosphorylation of Axl and Tyro3 after exposure to apoptotic cells or Gas6. RAW 264.7 cells were stimulated with apoptotic Jurkat T-cells (A, B) or Gas6 (C, D) for the time indicated. (A, C) Immunoblots of total cell lysates were analyzed for phospho-Axl/Axl. (B, D) Total cell lysates were immunoprecipitated with anti-Tyro3 antibody, and immunoprecipitates were analyzed by immunoblotting with anti-phosphotyrosine or anti-Tyro3 antibody. (E–G) RAW 264.7 cells were pretreated with 20 μg/ml anti-Axl or Tyro3 antibody or 20 μg/ml IgG for 1 h and then stimulated with apoptotic cells for 15 min (G), 2 h (E), or 24 h (F). (E) HGF mRNA levels were analyzed by relative quantitative RT-PCR and normalized to β-actin mRNA levels. (F) HGF protein levels in the conditioned medium were measured by ELISA. (G) The levels of RhoA activity were quantified. Values represent means ± SE of three separate experiments. *p < 0.05.
Axl or Tyro3-specific siRNA does not inhibit apoptotic cell–induced HGF expression. (A, B) Mer, Axl, and Tyro3 protein expression in RAW 264.7 cells transfected with Axl-siRNA, Tyro3-siRNA, or control vehicle (siRNA-GFP) for 48 h was analyzed by Western blot with anti-Mer, anti-Axl, or anti-Tyro3 antibodies. RAW 264.7 cells were transfected with Axl siRNA, Tyro3 siRNA, or control vehicle for 48 h and then stimulated with apoptotic Jurkat cells for 2 h (C, D) or 24 h (E). (C, D) HGF mRNA levels were analyzed by relative quantitative RT-PCR and normalized to β-actin mRNA levels. (E) HGF protein levels in the conditioned medium were measured by ELISA. Values represent means ± SE of three separate experiments. *p < 0.05.

FIGURE 5: Axl- or Tyro3-specific siRNA does not inhibit apoptotic cell–induced HGF expression. In addition to Mer, other TAM receptors—Axl and Tyro3—are also involved in clearance of apoptotic cells in dendritic phagocytes (Nagata et al., 1996; Lu and Lemke, 2001; Seitz et al., 2007). Of importance, Mer activation was also required for apoptotic cell–induced HGF mRNA expression, as well as the RhoA/PI3K/Akt/MAP kinases signaling pathway in murine peritoneal macrophages. In contrast to Mer, Axl and Tyro3 were not involved in the apoptotic cell induction of HGF mRNA and protein expression in RAW 264.7 or murine peritoneal macrophages, as inhibition of Axl and Tyro3 with receptor-specific neutralizing antibodies, siRNA, or Fc-fusion proteins had no effect on apoptotic cell–induced HGF mRNA and protein expression or RhoA activity. In addition, siRNA specific for Axl or Tyro3 did not affect apoptotic cell–induced phosphorylation of MAP kinases, including ERK and JNK, but they did significantly suppress the apoptotic cell–induced phosphorylation of Akt and p38 MAP kinase. These data suggest that Axl and Tyro3 are not involved in mediating the effect of apoptotic cells on HGF induction through the RhoA-dependent pathway, including ERK and JNK. However, Akt and p38 MAP kinase might not be essential molecules leading to HGF induction. These TAM receptors have been shown to use PI3K/Akt-dependent pathways for other roles in macrophages, such as ingestion of apoptotic cells (Leverrier and Ridley, 2001; Tibrewal et al., 2008), antiapoptotic effects (Linger et al., 2008; Zheng et al., 2009), and inhibition of NF-κB activation (Sen et al., 2007). However, the role that p38 MAP kinase plays in PI3K/Akt-dependent pathways for these cellular functions has not been determined.

Recent studies demonstrated that expression of all three TAM receptors in macrophages and platelets appear to be required for efficient heterodimerization subsequent to Mer tyrosine phosphorylation, indicating interaction among these receptors (Angelillo-Scherrer et al., 2005; Seitz et al., 2007). Nonetheless, our report is the first to demonstrate that only Mer among the TAM receptors plays a key role in mediating effects of apoptotic cells on HGF induction. Previous reports also provide evidence that Gas6-induced Mer activation is responsible for the reduction of inflammatory cytokine expression in cells only expressing Mer but not Axl or Tyro3 (Alciato et al., 2010). Moreover, Mer−/− mice display similar innate immunity alterations as TAM−/− mice, and Axl and Tyro3 single mutants do not significantly alter monocyte function (Lu and Lemke, 2001; Lemke and Lu, 2003). These data support the significant importance of Mer in immune cells, in particular, macrophages.
However, there are no data to support the involvement of RhoA in these additional Mer functions. This study advances our knowledge of the molecular basis of the postreceptor signaling cascade of Mer, namely the RhoA-dependent pathway involving the downstream molecules ERK and JNK, leading to apoptotic cell–induced HGF expression.

We previously showed that RhoA activity rapidly and substantially increased by 5 min, continued to increase lightly for 15 min, and maintained two-thirds of peak activity up to 2 h after apoptotic cell exposure (Park et al., 2011). This observation is independent of phagocytic activity, and therefore it is not a consequence of the rate of uptake of the apoptotic cells. The uptake rate in macrophages correlates to the length of exposure to apoptotic Jurkat cells, up to 90 min (Erwig et al., 2006). In addition to RhoA activation, Mer activates Rac1 through the Crk-DOCK180-ELMO signaling pathway, inducing apoptotic cell engulfment (Wu et al., 2006). Thus we examined the role of individual TAM receptors in phagocytosis of apoptotic cells. The phagocytic index in J774 and murine peritoneal macrophages transfected with specific siRNA of Mer upon exposure to apoptotic cells was more significantly reduced than those with siRNA of Axl or Tyro3 (Supplemental Figure S7, A and B). In accordance with this observation, Seitz et al. (2007) reported the differential contribution of TAM family receptors for efferocytosis, using peritoneal macrophages from Mer$^{−/−}$, Axl$^{−/−}$, and Tyro3$^{−/−}$ mice. These data indicate that all three TAM members play a role, but Mer is the most important for apoptotic cell clearance by macrophages. Molecular mechanisms involved in these differential contributions for efferocytosis need to be further studied.

Mer is emerging as an important cell surface receptor that bridges innate immune responses and regulation of autoimmune disease. However, affinities or activities of the Gas6–phosphatidylserine complex for Mer, Axl, or Tyro3 and the levels of expression of these receptors on phagocytic cells may dictate the differential contributions of this receptor family (Seitz et al., 2007). Indeed, we found that only Mer/Fc, but not Axl/Fc and Tyro3/Fc, among Gas6 inhibitors suppressed HGF expression at the gene and protein levels. Moreover, only Mer of the three TAM receptors is involved in gene and protein expression of HGF and activation of the downstream molecules RhoA, ERK, and JNK upon exposure to a Mer ligand—Gas6—as in apoptotic cell stimulation. Although Axl/Fc and Tyro3/Fc clearly have some effect on signaling through Akt and p38, these data indicate that affinity of the Gas6–phosphatidylserine (on the outer leaflet of an apoptotic cell membrane) for Mer is crucial for receptor function.

Recent studies also demonstrated an essential role for Mer in the regulation of the PI3K/Akt and NF-xB pathways in apoptotic cell– or Gas6–induced inhibition of proinflammatory cytokine production (Sen et al., 2007, Alciato et al., 2010; Eken et al., 2010). Mer is also involved in the regulation of Akt and ERK for the promotion of macrophage survival after exposure to oxidative stress (Anwar et al., 2009).
Mer mediates HGF production

For each of the three TAM receptors in order from greatest to least is Axl, Tyro3, and Mer (Nagata et al., 1996).

Of interest, the TAM receptors were not involved in apoptotic cell induction of TGF-β and EGF mRNA but did contribute to the induction of VEGF mRNA expression. These data suggest that the apoptotic cell–induced signaling mediated by Mer to accomplish expression of HGF is different from other TAM receptors.

In conclusion, among the TAM receptors, only Mer mediates the apoptotic cell– or Gas6-induced expression of HGF. In addition, we expanded the knowledge base on postreceptor signaling in response to phosphatidylserine on surface apoptotic cells, namely the Gas6–Mer/RhoA/ERK and JNK signaling pathway. Strategies aimed at the activation of Mer signaling might be important for anti-inflammatory responses, as well as for tissue repair and epithelial regeneration that might limit the development of fibrosis after tissue injury through the induction of HGF.

MATERIALS AND METHODS

Reagents

Recombinant proteins of mouse Gas6 (986-GS-025), Mer/Fc (591-MR), Axl/Fc (854-AX), Tro3/Fc (759-DT), and Met/Fc (527-ME) were purchased from R&D Systems (Minneapolis, MN). The gene-specific relative reverse transcription (RT)-PCR kit was from Invitrogen (Carlsbad, CA). M-MLV reverse transcriptase was from Enzynomics (Seoul, Korea). The G-LISA RhoA activation assay and recombinant C3 transferase were obtained from Cytoskeleton (Denver, CO). For the detection of TAM-family receptors, the antibodies anti–phospho Mer (PMKT-140AP) from Fab Gennix (Frisco, TX), anti-Mer (sc-67281), anti-Axl (sc-1097), and anti-Tyro3 (sc-1095) from Santa Cruz Biotechnology (Santa Cruz, CA), and anti–phospho Axl (D12B2) from Cell Signaling Technology (Danvers, MA) were used as supplied. The anti–phospho-p38 MAP kinase (sc-17852-R), anti-p38 MAP kinase (sc-535-G), anti–phospho-ERK1/2 (sc-7383), anti-ERK1 (sc-93-G), anti–JNK1 (SC-6254), anti–JNK1 (sc-1648), anti–phospho-Akt (sc-8312), anti–Akt (sc-7945), anti–HGF α chain (SC-7949) were from Santa Cruz Biotechnology, and anti–phosphotyrosine 4G10 (#05-321) was from Upstate Biotechnology (Lake Placid, NY).

Antibody blockade of TAM receptors

The antibodies used to block the TAM receptors in the range of concentration 10–50 μg/ml were raised against the extracellular N-terminus domains of the respective TAM receptor family members (Toft et al., 2004; Sharif et al., 2006; Sen et al., 2007; Wallet et al., 2008; Yi et al., 2009; Zhu et al., 2010; Png et al., 2011; Lee et al., 2012). A goat polyclonal anti-mouse Mer antibody (AF591),
The ligand-binding sites for protein S and Gas6 on the TAM receptors are located within the second immunoglobulin-like domain in their respective N-terminus extracellular domains (Lemke and Lu, 2003).

A goat polyclonal anti-mouse Axl antibody (AF854), a goat polyclonal anti-mouse Tyro3 antibody (AF759), and a normal goat IgG antibody (AB-108-C) were purchased from R&D Systems. In direct ELISAs and Western blots, the antibody shows no cross-reactivity with other TAM receptor members (description from R&D Systems). The ligand-binding sites for protein S and Gas6 on the TAM receptors are located within the second immunoglobulin-like domain in their respective N-terminus extracellular domains (Lemke and Lu, 2003).

FIGURE 8: Inhibition of Mer, but not Axl or Tyro3, suppresses apoptotic cell-induced HGF mRNA expression and activation of intracellular signaling molecules in murine peritoneal macrophages. Peritoneal macrophages were pretreated with 20 μg/ml of individual receptor-specific antibody; 20 μg/ml IgG; 200 nM Fc-fusion proteins of Mer, Axl, and Tyro3; or 200 nM Met/Fc for 1 h and then stimulated with apoptotic cells for 15 min (F–J) and 2 h (A–E). (A–E) HGF mRNA levels were analyzed by relative quantitative RT-PCR and normalized to β-actin mRNA levels. (F) The levels of RhoA activity were quantified. (G–J) Immunoblots of total cell lysates were analyzed for phospho-Akt/Akt, phospho-p38 MAPK/p38 MAP kinase, phospho-ERK/ERK, or phospho-JNK/JNK. Relative values for phosphorylated kinase vs. unphosphorylated kinase are indicated below the gel. Values represent means ± SE of three separate experiments. *p < 0.05.
Resident peritoneal macrophages were isolated using 5 ml of ice-cold sterile Hank's balanced salt solution to lavage the peritoneum after killing mice with CO₂. Lavage fluid was centrifuged and resident peritoneal cells plated at 5 × 10⁶ cells/well and cultured in DMEM supplemented with 10% heat-inactivated FBS, 2 mM l-glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin in humidified 10% CO₂ at 37°C. The macrophages were stimulated with apoptotic Jurkat T-cells (3 × 10⁶ cells/ml) in serum-free medium. Suspended peritoneal macrophages were >95% viable, as determined by trypan blue dye exclusion.

Induction of apoptosis
Cell lines of human Jurkat T-cells were exposed to UV irradiation at 254 nm for 10 min and cultured in RPMI 1640 (Media Tech) for 2.5 h at 37°C and 5% CO₂ before addition to macrophages. After irradiation, these cells were ∼70–80% apoptotic by evaluation of nuclear morphology using light microscopy (Hoffman et al., 2001).

ELISA measurement of HGF protein
RAW 264.7 cells (2 × 10⁶ cells/ml) were stimulated with apoptotic Jurkat T-cells (6 × 10⁶ cells/ml) for 24 h. Culture supernatants were collected, and HGF concentrations were measured by ELISA according to the manufacturer's instructions (R&D Systems).

Semiquantitative RT-PCR
Total RNA was isolated from cultured cells using reagent solution (iNtRon Biotechnology, Seoul, Korea). The concentrations and purities of the RNA samples were evaluated by spectrophotometry. Reverse transcription was conducted for 60 min at 42°C with 2 μg of total RNA using M-MLV reverse transcriptase (Enzynomics). Levels of HGF and TGF-β1 mRNA were determined using a semiquantitative RT-PCR kit (Invitrogen). The primer sequences used were as follows: mouse-specific HGF (sense, 5′-ATC CAC GAT GTT CAT GAG AG-3′; antisense, 5′-GCT GAC TGC ATT TCT CAT TC-3′), mouse-specific TGF-β1 (sense, 5′-AAT AGT TAT CCA GGA TGC CC-3′; antisense, 5′-ATG CTA CCA CCC TCG ACG CA-3′), mouse-specific EGF (sense, 5′-AGT TAT CCA GGA TGC CC-3′; antisense, 5′-GTT CAT ACC CAG GAA AGC AA-3′), mouse-specific VEGF (sense, 5′-GCG GGC TGC CTC GCA GTC-3′; antisense, 5′-TCA CCG CCT TGG CTT GTC AC-3′), and mouse-specific β-actin (sense, 5′-GAT GAC GAT ATC GCT GCG CTG-3′; antisense, 5′-GTA CGA CCA GAG GAA AGC AA-3′). The cDNA was denatured for 5 min at 95°C and amplified using a GeneAmp PCR System 2400 (PerkinElmer, Waltham, MA) during 33 cycles of 95°C for 30 s, 54°C for 30 s, and 72°C for 30 s, followed by a 10-min final extension at 72°C. Samples were visualized on 1–2% agarose gels stained with GelRed (Biotium, Hayward, CA). The relative amounts of HGF to β-actin were evaluated by densitometry.

Cell line culture and stimulation
Murine RAW 264.7 macrophages (American Type Culture Collection, Rockville, MD) were plated at 10⁶ cells/ml and incubated overnight in DMEM (Media Tech, Washington, DC) supplemented with 10% fetal bovine serum (FBS), 2 mM l-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C and 5% CO₂. Before stimulation, the medium was replaced with serum-free X-vivo 10. RAW 264.7 cells were pretreated with 10 or 20 μg/ml anti-Mer antibody or IgG for 1 h. The macrophages were stimulated with apoptotic cells (3 × 10⁶ cells/ml) or Gas6 (400 ng/ml) in serum-free medium.

Experimental animals and isolation and culture of primary cells
Pathogen-free male C57BL/6 mice (Orient Bio, Sungnam, Korea) weighing 20–22 g were used to isolate resident peritoneal macrophages. The Animal Care Committee of the Ewha Medical Research Institute (Seoul, Korea) approved the experimental protocol.
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Serum-free culture medium for 48 h before further experimentation.

**RhoA activity assay**

RhoA activity was measured in RAW 264.7 cell lysates using an ELISA-based RhoA activation assay Biochem Kit (G-LISA; Cytoskeleton) according to the manufacturer’s instructions. Briefly, cell lysates were added to a RhoA-GTP affinity plate that was coated with the Rhotekin binding domain of RhoA for 30 min. The active GTP-bound form of RhoA was measured using indirect immunodetection, followed by a colorimetric reaction at 490 nm on a microplate spectrophotometer.

**Immunoblotting analysis**

RAW 264.7 macrophages and murine peritoneal macrophages (10^6 cells/ml) were plated and incubated in serum-free medium overnight at 37°C in 5% CO2. The stimulated cells were lysed in 0.5% Trion X-100 lysis buffer and resolved on a 10% SDS–PAGE gel. Separated proteins were electrophoretically transferred onto nitrocellulose and blocked at room temperature with Tris-buffered saline containing 3% bovine serum albumin. Membranes were incubated at room temperature for 1 h with various anti-primary antibodies and probed with mouse anti-mouse HRP-conjugated secondary antibody. Membranes were developed using the enhanced chemiluminescence system.

**Immunoprecipitation of Tyro3**

Whole-cell lysates were prepared from RAW 264.7 cells, and Tyro3 was immunoprecipitated with Tyro3 antibody for 2 h at 4°C, followed by incubation with protein A–Sepharose for 1 h at 4°C. The pellet was then washed three times with ice-cold lysis buffer, dissolved in Laemmli sample buffer, and separated on 10% SDS–PAGE gels.

**Statistical analysis**

Data are expressed as mean ± SEM. Analysis of variance was applied for multiple comparisons, and Tukey’s post hoc test was applied where appropriate. Student’s t test was used for comparisons of two sample means. p < 0.05 was considered statistically significant.

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