Identification of Mannose Receptor as Receptor for Hepatocyte Growth Factor β-Chain

NOVEL LIGAND-RECEPTOR PATHWAY FOR ENHANCING MACROPHAGE PHAGOCYTOSIS*5

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Background: Proteolytic cleavage of hepatocyte growth factor (HGF) yields a HGF-β fragment, but its function remained unknown.

Results: Mannose receptor (MR) was identified as an HGF-β-binding receptor, and HGF-β enhanced phagocytosis by macrophages.

Conclusion: HGF-β is the first MR ligand to enhance apoptotic cell phagocytosis.

Significance: Growth factor proteolysis and its fragment recognition by scavenger receptors provide new insights in cell clearance possibly during inflammation.

Hepatocyte growth factor (HGF), a heterodimer composed of the α-chain and β-chain, exerts multifunctional actions for tissue repair and homeostasis via its receptor, MET. HGF is cleaved by proteases secreted from inflammatory cells, and NK4 and β-chain remnant (HGF-β) are generated. Here, we provide evidence that HGF-β binds to a new receptor other than MET for promoting a host cell clearance system. By an affinity cross-linking, radiolabeled HGF-β was bound to liver non-parenchymal cells, particularly to Kupffer cells and sinusoidal endothelial cells, but not to parenchymal hepatocytes. The cross-linked complex was immunoprecipitated by anti-HGF antibody, but not anti-MET antibody, implying that HGF-β binds to non-parenchymal cells at a site distinct from MET. Mass spectrometric detection of the ligand receptor complex revealed that the binding site of HGF-β was the mannose receptor (MR). Actually, an ectopic expression of MR in COS-7 cells, which express no endogenous MR or MET, enabled HGF-β to bind these cells at a K D of 89 nM, demonstrating that MR is the new receptor for HGF-β. Interaction of HGF-β and MR was diminished by EGTA, and by an enzymatic digestion of HGF-β sugar chains, suggesting that MR may recognize the glycosylation site(s) of HGF-β in a Ca2+-dependent fashion. Notably, HGF-β, but not other MR ligands, enhanced the ingestion of latex beads, or of apoptotic neutrophils, by Kupffer cells, possibly via an F-actin-dependent pathway. Thus, the HGF-β-MR complex may provide a new pathway for the enhancement of cell clearance systems, which is associated with resolution of inflammation.

Inflammation is one of the host defense systems that removes damaged tissue or harmful foreign substances, and is required for inducing proper tissue regeneration. On the other hand, proteolytic enzymes released from inflammatory cells, such as neutrophil elastase, elicit apoptotic events in functional cells, thus indicating a dual role for controlling or accelerating inflammation (1). These proteases modulate tissue repair processes by degrading active cytokines, creating active growth factor from its precursor, and regulating the activity of the cell surface receptor of these cytokines (2, 3). Sometimes, proteolytic fragments of growth factors may display unique activities distinct from the original functions. For example, fragments of VEGF elicit inflammatory cell infiltration by interacting with their non-cognate receptors (4).

Hepatocyte growth factor (HGF)2 was originally discovered as a mitogen of rat hepatocytes in a primary culture (5, 6). HGF exerts a variety of biological activities through a high-affinity receptor, MET (7, 8), and plays a critical role in tissue homeostasis and regeneration (9, 10). HGF is a heterodimer composed of a 69-kDa α-chain and 34-kDa β-chain linked by a disulfide bond that contains one N-terminal hairpin domain (N) and four kringle domains (K1–K4) in the α-chain, and one serine protease-like domain in the β-chain. The HGF β-chain is structurally analogous to serine proteases, but the catalytic triad essential for serine protease activity is mutated and shows no enzyme activity. The α-chain of HGF has a high affinity for MET, but activation of MET is dependent on the subsequent binding of the β-chain (11).

HGF is synthesized and secreted as a single chain pro-HGF, and converted into the active form by a proteolytic cleavage at Arg494–Val495 (12). Proteolytic conversion by proteases also

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2 The abbreviations used are: HGF, hepatocyte growth factor; HGF-β, hepatocyte growth factor β-chain remnant; NPCs, non-parenchymal cells; KCS, Kupffer cells; MR, mannose receptor; Endo-F2, endoglycosidase F2; LSECs, liver sinusoidal endothelial cells; CFSE, carboxyfluorescein succinimidyl ester; CTLD, C-type lectin-like domain; HSPG, heparan sulfate proteoglycan; BS3, bis-(sulfosuccinimidyl) suberate.
occurs at different regions of HGF. Indeed, elastase cleaves HGF between Val$_{378}$ and Asp$_{479}$, resulting in the generation of NK4, a fragment of the α-chain N-terminal hairpin and K1–K4 domains, and the β-chain containing the C-terminal 16 residues of the α-chain linked by a disulfide bond, designated “HGF-β” (13) (supplemental Fig. S1). NK4 can bind to MET without its activation, and thus NK4 acts as a full antagonist of HGF (14). Interestingly, NK4 can inhibit FGF2- or VEGF-mediated endothelial cell proliferation (15), thus suggesting the presence of a functional receptor other than MET. We recently reported that NK4 interacts with perlecan, resulting in the inhibition of surface fibronectin assembly and integrin-dependent endothelial growth (16). In addition to pancreatic elastase (11, 13), leukocyte proteases and skin kallikrein also cleave HGF and generate an NK4-like fragment (17, 18). In contrast to the unique functions of NK4, it is still unknown whether another HGF fragment, HGF-β, has a biological function(s).

Purified HGF-β is reported to bind to "soluble" MET by bridging to the Sema-domain of MET in a direct interaction (19). However, HGF-β alone (i.e. absent of another partner, NK4) may not bind to the "cell surface-anchored" MET (11, 20). This background prompted us to hypothesize that HGF-β exerts a biological function via a MET-independent mechanism(s), as did NK4 (15, 16). To test this hypothesis, we attempted to identify a functional receptor of HGF-β by mass spectrometry. Herein, we provide evidence that HGF-β enhances phagocytosis via a mannose receptor (MR)-dependent pathway. We discuss the significance of the HGF-derived fragment receptor identification for understanding a cell clearance system.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human recombinant HGF was purified from a medium of CHO cells transfected with human HGF cDNA (11, 13). Human recombinant NK4 was also purified from cultured medium of CHO cells (13). The following antibodies were used: anti-MET (number sc-8057, Santa Cruz Biotechnology, Santa Cruz, CA), anti-SE-1 (number 10078, IBL, Gunma, Japan), anti-MR (number ab64693, Abcam, Cambridge, UK), anti-ED2 (number MCA342R, AbD Serotec, Oxford, UK), and anti-β-actin (number A1978, Sigma). Anti-human HGF rabbit antibody was prepared in our laboratory (21). Mucin type III from porcine stomach, thyroid stimulating hormone from bovine body was prepared in our laboratory (21). Mucin type III from porcine stomach, thyroid stimulating hormone from bovine body was prepared in our laboratory (21). Mucin type III from porcine stomach, thyroid stimulating hormone from bovine body was prepared in our laboratory (21). Mucin type III from porcine stomach, thyroid stimulating hormone from bovine body was prepared in our laboratory (21). Mucin type III from porcine stomach, thyroid stimulating hormone from bovine body was prepared in our laboratory (21). Mucin type III from porcine stomach, thyroid stimulating hormone from bovine body was prepared in our laboratory (21). Mucin type III from porcine stomach, thyroid stimulating hormone from bovine body was prepared in our laboratory (21). Mucin type III from porcine stomach, thyroid stimulating hormone from bovine body was prepared in our laboratory (21). Mucin type III from porcine stomach, thyroid stimulating hormone from bovine body was prepared in our laboratory (21). Mucin type III from porcine stomach, thyroid stimulating hormone from bovine body was prepared in our laboratory (21). Mucin type III from porcine stomach, thyroid stimulating hormone from bovine body was prepared in our laboratory (21). Mucin type III from porcine stomach, thyroid stimulating hormone from bovine body was prepared in our laboratory (21). Mucin type III from porcine stomach, thyroid stimulating hormone from bovine body was prepared in our laboratory (21). Mucin type III from porcine stomach, thyroid stimulating hormone from bovine body was prepared in our laboratory (21). Mucin type III from porcine stomach, thyroid stimulating hormone from bovine body was prepared in our laboratory (21). Mucin type III from porcine stomach, thyroid stimulating hormone from bovine body was prepared in our laboratory (21). Mucin type III from porcine stomach, thyroid stimulating hormone from bovine body was prepared in our laboratory (21). Mucin type III from porcine stomach, thyroid stimulating hormone from bovine body was prepared in our laboratory (21). Mucin type III from porcine stomach, thyroid stimulating hormone from bovine body was prepared in our laboratory (21). Mucin type III from porcine stomach, thyroid stimulating hormone from bovine body was prepared in our laboratory (21). Mucin type III from porcine stomach, thyroid stimulating hormone from bovine body was prepared in our laboratory (21). Mucin type III from porcine stomach, thyroid stimulating hormone from bovine body was prepared in our laboratory (21). Mucin type III from porcine stomach, thyroid stimulating hormone from bovine body was prepared in our laboratory (21). Mucin type III from porcine stomach, thyroid stimulating hormone from bovine body was prepared in our laboratory (21). Mucin type III from porcine stomach, thyroid stimulating hormone from bovine body was prepared in our laboratory (21). Mucin type III from porcine stomach, thyroid stimulating hormone from bovine body was prepared in our laboratory (21).
by silver staining. After tryptic digestion, the peptides were extracted, separated by liquid chromatography (Prominence nano; Shimadzu, Kyoto, Japan), and analyzed by MALDI-TOF/TOF mass spectrometry (4700 Proteomics Analyzer; Applied Biosystems, Foster City, CA).

**Knockdown of MR on Primary Liver NPCs—**Three types of siRNA oligonucleotides for rat mrc1 (Stealth Select RNAi siRNA) were obtained from Invitrogen (catalog numbers: RSS306981 for siMR1, RSS306982 for siMR2, and RSS306983 for siMR3). NPCs were transfected with each RNAi at a concentration of 50 nM, using Lipofectamine 2000® reagent. The Stealth RNAi for GFP (Invitrogen) was used as a negative control.

**Real Time RT-PCR—**Total RNA was isolated from cultured cells using ISOGEN® (Nippon Gene, Tokyo, Japan), and reverse transcribed into first-strand cDNA using Superscript III reverse transcriptase (Invitrogen). The amount of target cDNA was normalized by comparison with the amplification of the β-actin gene. Primer sequence were as follows: rat mrc1 sense, 5’-CAA GGA AGG TGG GCA TTT GT-3’ and antisense, 5’-GGA AGC TGT GCT GTG ATG T-3’, and rat β-actin sense, 5’-AGT GTG AGG TGG ACA TCC GTA-3’ and antisense, 5’-GCC AGA AGG TTG GCA TTT GT-3’.

**Induction of Acute Hepatitis in Rats—**Male SD rats (Japan Slc, Hamamatsu, Japan) were administered with a single dose of carbon tetrachloride (CCl₄) (1.0 ml/kg, intraperitoneally). After 0, 3, 6, and 9 days of CCl₄ administration, the NPCs of rats were enriched from the total liver cell population by removing hepatocytes through differential centrifugation. The cell suspension of NPC (≈95% purity) was plated in a 96-well plate at 2 × 10⁵ cells/well, and the binding of 5 nM ¹²⁵I-HGF-MET-positive control cells) regardless of high doses, whereas HGF bound to the MET-positive cells in a dose-dependent manner. As a result, most cell lines and primary hepatocytes tested in this study showed little binding to HGF-MET, except for TMNK-1, a cell line of human liver endothelial cells (Fig. 2A). In addition, KCs and LSECs in the NPCs strongly bound to HGF-MET (Fig. 2A).

It is important to note that radiolabeled HGF-MET did not bind to MET-positive cells, such as hepatocytes and A549 lung carcinoma. Indeed, HGF-MET did not bind to BaF3-hMet cells (i.e. MET-positive control cells) regardless of high doses, whereas HGF bound to the MET-positive cells in a dose-dependent manner (supplemental Fig. S3). In contrast, purified HGF-MET and soluble MET likely form a tight complex under cell-free conditions (19). The difficulty in the binding of HGF-MET to MET on living cells might be due to a structural interference by other surface molecules, which are known to bind to MET (28, 29).

Next, we examined the binding kinetics of HGF-MET to LSECs, which have high-affinity binding sites for HGF-MET. Specific HGF-MET binding to LSECs was seen in a saturable manner (Fig. 2B, left). Scatchard analysis showed that the Kᵢ value of HGF-MET in LSECs was 85.7 nM, and the number of binding sites (B_max) was 31,700 sites/cell (Fig. 2B, right).

To detect the complex formation between HGF-MET and its receptor, we carried out affinity cross-linking experiments in
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FIGURE 1. Protease resistance of HGF-β. Proteolytic digestion of HGF (left), NK4 (middle), and HGF-β (right) by elastase (A) or cathepsin G (B). HGF, NK4, and HGF-β were incubated with proteases for the indicated periods. Digested ligands were subjected to SDS-PAGE under nonreduced conditions and visualized by silver staining.

hepatic cells. After cross-linking with 125I-HGF-β to total NPCs, the ligand-receptor complex was precipitated with anti-HGF antibody. As a result, the HGF-β cross-linked complexes were detected as an apparent molecular mass of ~230 and 460 kDa, and these specificities were confirmed by addition of an excess amount of cold HGF-β that diminished the radioactive bands (Fig. 2C). We further examined what types of liver cells could be targeted by HGF-β: the cross-linked complex with HGF-β was evident in LSECs and KCs, mild in hepatic stellate cells, and negligible in hepatocytes (Fig. 2C).

Binding of HGF-β on cell surface MET was very low in some cell lines. In a cross-linking assay, we ruled out the possibility that MET is involved in HGF-β-receptor complexes. Using radiolabeled HGF as a positive control, we detected the HGF-MET complex in hepatocytes (Fig. 2D, left). In contrast, a complex formation between HGF-β and MET was not seen in NPCs (Fig. 2D, right), meaning that the HGF-β-receptor complex on NPCs does not involve MET. Overall, it was shown that HGF-β bound to hepatic NPCs (such as LSECs and KCs), but not hepatocytes, via a MET-independent fashion, thus suggesting the presence of a new receptor for HGF-β.

Identification of HGF-β Receptor as MR by Mass Fingerprinting—To determine whether the HGF-β-binding activity is altered in livers post-injury, we used CCl4-treated rats as a model for the following reasons: (i) HGF production is up-regulated in hepatic NPCs in response to hepatic injury (9, 30); (ii) endogenous and exogenous HGF are essential for liver regeneration after hepatic injury including CCl4-induced hepatitis (31–33); and (iii) proteases, such as elastase and cathepsins, are also increased, along with the neutrophil influx within 2 days post-CCl4 challenge (34, 35).

The binding of 125I-HGF-β to enriched NPCs (purity ≥95%, 2 × 10⁵ cells) remained unchanged between 1 and 3 days after CCl4 administration, but increased significantly after 4 days and peaked at 6 days (Fig. 3A). To isolate the HGF-β receptors, HGF-β affinity cross-linked complex was purified from NPCs 5 days after CCl4 treatment. HGF-β was conjugated with biotin via a disulfide bond(s), and by use of this biotinylated ligand, 230- and 460-kDa complexes were trapped by streptavidin beads and eluted in the presence of DTT, because this reducing agent is useful for separation of the HGF-β-receptor complex from biotin-bound streptavidin beads (Fig. 3B and supplemental Fig. S2). The purified complex from HGF-β-treated NPCs showed specific protein bands under silver staining compared with that from vehicle-treated control NPCs, (Fig. 3C, asterisk).

Using a trypic peptide mass fingerprinting, rat mrc1 (NCBI number gi 157822935) (i.e. MR, a recognition receptor (36)) was identified as an HGF-β receptor (Table 1 and supplemental Fig. S4). Both 230- and 460-kDa complexes contained MR-derived peptides. Indeed, the HGF-β-linked complex contained MR, as evidenced by Western blot analysis (Fig. 3D). The molecular mass of HGF-β and of MR is 34- and 175-kDa, respectively (6, 36). Thus, the molecular size of 230-kDa appears to reflect this complex. The 460-kDa protein is predicted to be a dimer of the 230-kDa complex, because liver MR is known to exist in the form of a “dimer” (37). In the rat model, protein and mRNA levels of MR were almost unchanged within 3 days after CCl4 treatment, but increased 6–9 days post-challenge (Fig. 3E), being similar to the result of HGF-β-NPCs binding assay (Fig. 3A).

MR Is Essential for HGF-β Binding—To demonstrate that MR is a binding site for HGF-β, forced expression of MR was induced in COS-7 cells, which did not express endogenous MR (not shown) or MET (8). COS-7 cells overexpressing MR (COS-7MR) showed significantly more HGF-β binding compared with empty vector-transfected cells (COS-7Mock) (Fig. 4A, left). Moreover, affinity-labeled HGF-β was detected on COS-7MR, but not COS-7Mock, via immunoprecipitation with an anti-
body to MR (Fig. 4A, middle). Scatchard plot analysis revealed that COS-7Mock cells had no obvious specific binding site for HGF-H9252 (Fig. 4A, right), whereas the binding property of COS-7MR cells to HGF-H9252 was similar to that of liver NPCs (Fig. 4A, right). Inversely, down-regulation of endogenous MR by a knockdown technique led to a decrease in the binding ability of HGF-H9252 (Fig. 4B). When NPCs were transfected with 3 different siRNAs for MR, expression levels of MR mRNA and protein were markedly decreased, as evidenced by real-time PCR and Western blotting, respectively (Fig. 4B, left). Under such an MR-decreased condition, HGF-H9252 binding to NPCs was clearly suppressed (Fig. 4B, right), indicating that MR is an HGF-β-binding receptor.

**Binding Characteristics of HGF-H9252 on MR**—MR binds to mannosylated-, fucosylated-, and N-acetyl-glucosaminylated molecules on its C-type lectin domain (CTLD) in a Ca\(^{2+}\)-dependent manner (38). Thus, we tested several compounds for the ability to interfere with HGF-β binding to NPCs. The addition of cold HGF or cold HGF-β inhibited the binding of \(^{125}\)I-HGF-H9252 to NPCs, whereas heparin did not (Fig. 5A, left). The fact that HGF competed with the HGF-β-MR complex suggests that HGF also may be a MR ligand. Indeed, HGF contains glycosylation sites in the β-chain (39). With regard to this, the binding affinity of HGF to heparan sulfate proteoglycans seems to be 40-fold higher (\(K_D = 2\) nM) (40) than that of HGF-H9252 to MR. HGF-H9252 has no hairpin-loop domain, indicating no binding to heparan sulfate proteoglycans (41). Thus, it is likely that HGF-β preferably binds to MR, whereas HGF binds to heparan sulfate proteoglycans (and MET) for conferring a slow release system, as reviewed (9).

Furthermore, competition experiments with α-mannose, chelator EDTA and EGTA, and known MR ligands, mucin type...
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III and thyroglobulin, showed that these competitors inhibited the binding of HGF-β to NPCs (Fig. 5A, right), thus suggesting that HGF-β binding to NPCs may be mediated via CTLD of MR. To verify the importance of the glycosylation-based structure of HGF-β on MR binding, we treated HGF-β with glycosidase Endo-F2, because Endo-F2, but not Endo-H or N-glycanase, cleaved the biantennary complex and high mannose-type oligosaccharides of N-linked glycoprotein (not shown). The enzyme-treated HGF-β showed a shift of electrophoretic mobility due to the loss of glycosylation (Fig. 5B, left). As a result, the binding ability to NPCs was diminished by Endo-F2 treatment (Fig. 5B, right), implying the importance of the HGF-β glycosylation site(s) for the HGF-β-MR interaction.

Enhancement of Ingestion Activity by HGF-β—Because clearance activity of KCs is one of the important functions for inflammation resolution, we determined whether HGF-β modulates the ingestion activity of KCs. After the incubation of KCs with the beads (size: 0.5 μm) for 60 min, HGF-β dose-dependently increased the ratio of KCs that ingested ≈10 beads (i.e. positive cells) to total KCs (Fig. 6A), and this was associated with the reciprocal decrease in KCs containing 4–9 beads in the cytosols (not shown), and this shift suggests the effect of HGF-β on bead intake. Actually, HGF-β-mediated increases in ingestive activity were also confirmed by flow cytometry (supplemental Fig. S5).

During the ingestion of small particles (≤1 μm), whether F-actin dependence is major or minor remains unclear (42–44). The effect of HGF-β on beads (0.5 μm) was abolished by cytochalasin-D, an actin inhibitor (Fig. 6B), thus suggesting that the HGF-β-promoted ingestion is mediated via the F-actin-dependent (i.e. phagocytosis-dominant) system (45).

Such an ingesting effect of HGF-β was attenuated by MR knockdown (Fig. 6C), indicating a significant role of MR during the bead intake. The inhibitory effect of MR siRNA, or of mannosone (Fig. 5A), is partial, probably due to an insufficiency of knockdown. We also cannot exclude the possibility that the MR-independent pathway may be involved in the phagocytic event. Of note, the enhancing effect on the bead intake was not observed in the case of other MR ligands, such as mucin type III and thyroid-stimulating hormone (Fig. 6D).

The fact that HGF-β enhanced bead intakes in an F-actin-dependent manner led us examine the possible phagocytic functions of HGF-β against dying cells. Therefore, we used an in vitro model of phagocytosis (i.e. apoptotic neutrophils used as an ingested target). Flow cytometry revealed that the enhancement of ingestive activity of KCs by HGF-β was reproducible in the co-culture model of apoptotic neutrophils and KCs (KCs/HGF-β (−) groups: 11.6% versus KCs/HGF-β (+) group: 25.8%) (Fig. 7A). As expected, this effect was abolished by cytochala-
### TABLE 1
Identified peptides by MALDI-TOF/MS analysis of purified HGF-β-receptor complex

| Band | Peptide | Measured mass | Calculated mass | Δ mass | Score | Peptide location in rat MRC1 |
|------|---------|---------------|-----------------|--------|-------|----------------------------|
| 1    | APRIL 13, 2012• VOLUME 287 • NUMBER 16 |

#### Band **

| Peptide | Measured mass | Calculated mass | Δ mass | Score | Peptide location in rat MRC1 |
|---------|---------------|-----------------|--------|-------|----------------------------|
| 1       | 1068.516      | 1068.513        | 0.003  | 25    | 964 KIFGFAEEKK             |
| 2       | 1112.471      | 1112.482        | -0.011 | 25    | 815 KDYQYYFSKE             |
| 3       | 1196.595      | 1196.608        | -0.013 | 58    | 964 KIFGFAEEKKK            |
| 4       | 1209.496      | 1209.530        | -0.034 | 42    | 118 RYTNWGGDKPL            |
| 5       | 1332.628      | 1332.610        | 0.018  | 68    | 118 KGTELYFNYGRQ           |
| 6       | 1461.766      | 1461.736        | 0.030  | 67    | 30 RQFLYINEDHCR            |
| 7       | 1653.892      | 1653.840        | 0.052  | 36    | 494 KMVSQIHTVPEGAEK      |

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**FIGURE 4. Effects of MR overexpression or knockdown on HGF-β binding.** A, left, 125I-HGF-β binding to NPCs, COS-7mock, or COS-7MR cells. Specific bindings are shown. Middle, affinity-labeled HGF-β on COS-7mock cells was immunoprecipitated with anti-MR antibody. Right, Scatchard analysis of 125I-HGF-β binding to COS-7mock and COS-7MR cells. B, left, transfection of MR siRNA resulted in down-regulation of MR mRNA and protein expression. Right, knockdown of endogenous MR diminished HGF-β binding to NPCs.
The effects of other MR ligands on apoptotic cell phagocytosis were not evident compared with those of HGF-MET (Fig. 7B), as discussed later.

**DISCUSSION**

In most organs, stroma-derived HGF elicits multiple biological functions in parenchymal cells via binding to its receptor, MET (9, 10). Such a stromal-to-epithelial interaction by HGF-MET is necessary for embryogenesis, tissue protection, and repair (9, 10). Under inflammation, HGF is digested by leukocyte-derived proteases, such as elastase, leading to generations of NK4 and HGF-MET (i.e. HGF-daughter molecules) (13, 17). We identified perlecan as a new receptor of NK4: the NK4/perlecan complex inhibits assembly of fibronectin, a key integrin ligand for supporting endothelial cell growth, resulting in the arrest of tumor angiogenesis (16). Herein, we identified “MR” as a receptor of another HGF fragment, the HGF/MET-binding site, in stromal cells. We found that HGF-MET enhanced phagocytosis of apoptotic cells by KCs in an MR-dependent manner. This is the first report to show that an HGF fragment promotes clearance of dying cells via a functional receptor.

Clearance of dying/dead cells by macrophages plays a significant role in the resolution of inflammation and tissue protection from harmful exposure to inflammatory contents of dead cells (46). In our rat model of hepatitis, MR (corresponding to the HGF/MET-binding site) began to increase, especially in the recovery phase (i.e. 6 days after CCl4 challenge), where cell debris could be cleared by resident and infiltrated macrophages. To date, little information was available for the role of MR in apoptotic cell clearance. Dini et al. (47) reported that uptake of apoptotic cells by liver cells could be inhibited by mannose. Kawao et al. (48) found an increase in MR-expressing macrophages at the borderline of dead cells in a rat model of hepatitis. Although further fundamental studies are required for elucidating the molecular basis of MR-related phagocytosis, we at least emphasize that HGF-MET is the first MR ligand to drive a clearance system of dying/dead cells.

Another key result is that HGF-MET binding leads to enhanced ingestion of small beads, even if MR ligands are not coated, hence suggesting “nonspecific” ingestion. Such a similar finding is seen in a previous report: calcitonin gene-related peptide enhances “non-coated” bead intakes in a culture of peritoneal macrophages, and this enhancing effect is diminished by an excess amount of mannose (49). Given that MR is a key receptor for clearance of glycoproteins via endocytosis (36), HGF-MET itself could be internalized through the endocytic (rather than phagocytic) pathway. With regard to this, it was reported that endocytic internalization of a ligand-receptor
promoted phagocytosis through endolysosomal trafficking (50), where small GTPase molecules are considered critical for both endomembrane trafficking and F-actin extension, required for phagocytosis. In the KC culture, HGF-β/H9252 was in part located in the endosome, and this was associated with F-actin extension. We are now elucidating the role of GTPase Rho, a candidate bridge between the ligand-receptor internalization and subsequent (nonspecific) phagocytosis.

It is interesting to note that only HGF-β among the MR ligands tested herein can enhance the phagocytic response of macrophages in vitro. Thus, the recognition pattern of HGF-β by MR should be discussed. CTLD in MR are known as critical extracellular regions for trapping sugars terminated in the mannose, fucose, or N-acetylglucosamine of MR ligands (36, 51). The trapping of MR ligands by CTLD occurs in a Ca²⁺-dependent fashion (38, 51), whereas binding of other domains (such as cysteine-rich domain) of the MR are Ca²⁺-independent (52). In our experiment, Ca²⁺-chelating agents (such as EDTA) abolished the binding of MR to HGF-β, suggesting that MR-CTLD might be a critical site for trapping HGF-β. Inversely, thyroid stimulating hormone (28-kDa), a ligand that binds to the MR cysteine-rich domain (52), did not alter phagocytic activities. Thyroglobulin is known to bind to MR via CTLD (53), but this molecule also did not modify phagocytic activity. The molecular size of thyroglobulin is 660-kDa (i.e., 20-fold of HGF-β), suggesting a possible structural interference. Taken together, we predict that MR-mediated phagocytosis is dependent on receptor domain site(s) and ligand sizes. Whether HGF-β has a peptide sequence(s) specific for MR-mediated phagocytosis warrants further attention.

The difference in the possible binding site between HGF-β/MR and HGF-MET should be discussed. As its name indi-

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FIGURE 7. Enhanced phagocytosis of apoptotic neutrophils by HGF-β-stimulated KCs. A, CFSE-labeled rat apoptotic neutrophils were coincubated with KCs with or without HGF-β for 1 h, and then these cells were analyzed by flow cytometry. B, KCs were stimulated with LPS with and without HGF-β, or with various MR ligands for 16 h, and then phagocytosis of the rat apoptotic neutrophils was measured. The phagocytosis index was calculated as described under “Experimental Procedures.”
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cates, MR recognizes mannose-containing sugar chains in the target molecule (36, 51). HGF-β includes N-linked glycosylation sites (i.e. Asn261 and Asn648) (39, 54). Of note, enzymatic digestion of sugar chains by Endo-F2 led to the significant decrease in the HGF-β-MR complex, thus indicating that these N-linked sugar chains seem to be required for the HGF-β chain to bind tightly to MR. In contrast, these glycosylation structures are not important for HGF to bind to MET; a deletion mutant of the HGF sugar chains can activate MET signaling, as did the wild-type HGF (54). With regard to this, structural analysis revealed that other sites in HGF-β (i.e. catalytic triad residues such as Gln534 and Tyr673) are critical for tight assembly of the MET Sema domain by the HGF-β chain (19, 55), possibly in the presence of the HGF-α chain (11). Overall, the HGF-β-MR complex seems to be dependent on N-linked sugar chains, whereas HGF activates MET in a glycosylation-independent manner (54).

We further discuss the possible sequential effects of HGF and its fragment HGF-β in vivo. Endogenous HGF is required for recovery from hepatitis (30–33, 56), but its molecular cascades are yet to be fully investigated. In the early stage of hepatitis, HGF is produced by NPCs, or is sequestered from surface heparan sulfate proteoglycans, then supplied to injured hepatic sites (i.e. local system). NPC-delivered and matrix-released HGF targets directly hepatocytes for inhibiting cell death and promoting its mitogenesis (9, 56) (1st stage). During inflammation, HGF is converted into HGF-β (and NK4) by proteases, as implied by our ongoing studies. MR(+) macrophages appear in a recovery phase of hepatitis, as shown herein and elsewhere (48). HGF-β (and possibly in part, HGF) targets the macrophages for cell clearance (2nd stage). Future studies using an in vivo model will shed more light on this speculation.

In summary, we successfully identified MR as a functional receptor of HGF-β, using mass spectrometry. The possible binding of HGF-β N-linked glycosylation sites to MR-CTLD promotes phagoctytic activities. MR is now believed to be a critical receptor conferring a recognition system of the glycoprotein(s) and microorganism (36, 51). Possible generation of HGF-β by neutrophils (17) leads to enhanced phagocytosis in macrophages. Macrophages and neutrophils are both a major source of HGF (9, 57). Of note, macrophages highly produce HGF during/after phagocytosis of microorganism-ingesting neutrophils (57). Such a cross-network among infection, recognition, and self-repair systems, through a ligand cleavage and changes in target receptors, may provide new insights into anti-inflammation, infectious control, and tissue regeneration. Future studies will shed more light on this notion.

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