Hepatocyte Growth Factor Specifically Binds to Sulfoglycolipids*

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Hepatocyte growth factor (HGF) is a heparin-binding pleiotropic factor that acts on a variety of epithelial cells. The interaction of human HGF with glycolipids was studied by overlaying them with [125I]-HGF on thin layer chromatograms and by a solid-phase assay using lipids adsorbed on microtiter plates. Among various glycolipids tested, HGF was found to bind to sulfoglycolipids, including galactosylceramide sulfate (SM4), lactosylceramide sulfate (SM3), and gangliotriaosylceramide bis-sulfate. In contrast, HGF failed to bind to gangliosides or neutral glycolipids. HGF binding to SM4 was strongly inhibited by dextran sulfate, heparin, and fucoidan, whereas neither keratan sulfate nor hyaluronic acid had any inhibitory activity.

When glycolipids from a renal cancer cell line, SMKT-R3, which overexpresses sulfoglycolipids, were developed on a thin layer chromatogram, SM4 and SM3 were the only glycolipids that bound HGF. We further examined the effect of the incorporation of glycolipids into SMKT-R3 cells on HGF binding to the cells. The incorporation of SM4 into the cells enhanced HGF binding to SMKT-R3 cells, while that of galactosylceramide, a precursor of SM4, had no effect. These observations indicated that SM4 exogenously incorporated into the cell membranes could react with HGF and suggested that endogenous sulfoglycolipids on SMKT-R3 cells might function as reservoirs for HGF.

Sulfoglycolipids are a class of acidic glycolipids containing one or two sulfate esters on their oligosaccharide chains and are responsible for some of the negative charge on the cell surface. Kidney and brain contain abundant sulfoglycolipids (1). In human renal cell carcinoma tissue, sulfoglycolipid content is markedly increased as compared with uninvolved tissue (2). Furthermore, the accumulation of sulfated glycolipids was also demonstrated in established cells from human renal cell carcinoma and associated with an elevated activity level of glycolipid sulfotransferase (3).

Although a variety of functions have been suggested for sulfoglycolipids (4), the actual functions in vivo remain to be clarified. Recently, sulfated glycolipids were found to bind specifically to several proteins, including laminin (5, 6), thrombospondin (7), von Willebrand factor (8), multicalcitic protease (9), and amphoterin (10). These bindings to sulfoglycolipids were inhibited by sulfated glycoconjugates such as heparin. HGF is a pleiotropic factor that is produced by mesenchymal cells and acts as a mitogen, motogen, and morphogen for various epithelial cells, including renal cells (11). In our recent study, it was demonstrated that HGF elevated glycolipid sulfotransferase activity in renal cell carcinoma cells. Since HGF is known to have an affinity for heparin (12), these observations prompted us to examine whether HGF bound to sulfoglycolipids on renal cancer cells.

EXPERIMENTAL PROCEDURES

Materials—Na[125I] (17 Ci/mg) was purchased from New England Nuclear. Human recombinant HGF was purified from culture medium of C127 mouse fibroblasts or Chinese hamster ovary cells transfectected with plasmid containing human HGF cDNA (13). HGF was radiolabeled by the chloramine-T method as described previously (14). The [125I]-HGF had a specific activity of 70-160 pCi/μg protein. Antisera were raised against human recombinant HGF in rabbits (15). Heparin, fucoidan, dextran sulfate, keratan sulfate, and hyaluronic acid were obtained from Sigma. DEAE-Sephadex A-25 was the product of Pharmacia-LKB. Other reagents were of analytical grade.

Cell Culture—SMKT-R3 cells were established from human renal cell carcinoma and cultured as described previously (16). Cell viability was estimated by the trypan blue exclusion test and was always greater than 95%.

Preparation and Analysis of Glycolipids from Renal Cancer Cells—Glycolipids were prepared from SMKT-R3 cells as described previously (3). Briefly, lipids of the cells were extracted with a mixture of chloroform/methanol/water (60:35:8) and 30:60:6, the ratio of the solvent mixture is expressed by volume). The extracts were subjected to mild alkaline hydrolysis to destroy ester lipids and then fractionated into neutral and acidic lipid fractions by DEAE-Sephadex A-25 column chromatography. Prepared glycolipids were chromatographed on aluminum-backed silica gel HPTLC plates (Merck) using the solvent system chloroform/methanol/0.2% CaCl2 (60:35:7) and detected with an orcinol reagent (17).

Assay of [125I]-HGF Binding to Glycolipids—Binding of [125I]-HGF to glycolipids on HPTLC plates was performed as previously described for [125I]-thrombospondin binding (7) except that the incubation with [125I]-HGF was done for 2 h at room temperature. The labeled HGF bound to glycolipids was visualized by radiography and quantified by densitometry of the autoradiograms. Solid-phase radioassays of HGF binding to glycolipids adsorbed on 96-well microtiter plates (Falcon 3012) were carried out based on the previously described method (7).

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1 The abbreviations used are: HGF, hepatocyte growth factor; FGF, fibroblast growth factor; DMEM, Dulbecco's modified minimal essential medium; GalCer, galactosylceramide; LacCer, lactosylceramide; Gb3Cer, globotriaosylceramide; Gb4Cer, globotetraosylceramide; SB2, bis-sulfoglycosylalpha3-galactosylceramide; SM2, gangliotriaosylceramide 3-sulfate; SM3, lactosylceramide 3-sulfate; SM4, galactosylceramide 3-sulfate; HPTLC, high performance thin layer chromatography.

2 T. Kobayashi, K. Honke, S. Gasa, T. Miyazaki, H. Tujima, K. Matsumoto, T. Nakamura, and A. Makita, manuscript submitted.
Fig. 1. Binding of $^{125}$I-HGF to glycolipids separated on HPTLC plates. Authentic glycolipids and glycolipids extracted from SMKT-R3 cells were chromatographed on HPTLC plates. After development, each plate was divided into two parts to visualize glycolipids with an orcinol reagent (A, lanes 1–3; B, lanes 1–5) or with $^{125}$I-HGF (A, lanes 4–6; B, lanes 6–8) as described under “Experimental Procedures.” Plate A, lanes 1 and 4, neutral glycolipid standards: GalCer, LacCer, Gb3Cer, and Gb4Cer from top to bottom; lanes 2 and 5, sulfoglycolipid standards: SM4, SM3, SM2, and SB2 from top to bottom; lanes 3 and 6, ganglioside standards: GM4, GM3, GM2, and GM1 from top to bottom. Plate B, lanes 1 and 6, neutral glycolipid fraction of SMKT-R3 cells; lanes 2 and 7, acidic glycolipid fraction of the cells; lanes 3 and 8, sulfoglycolipid standards as in plate A; lanes 4 and 5, ganglioside and neutral standards, respectively, as in plate A. Glycolipids that correspond to 1 mg of cell protein were applied on lanes 1, 2, 6, and 7.

Glycolipid Incorporation into Renal Cancer Cells—In order to incorporate glycolipids into SMKT-R3 cells, GalCer or SM4 dissolved in a small amount of dimethyl sulfoxide was added exogenously to the cells. The cells were incubated with glycolipids in serum-free DMEM at 37 °C for 10 min.

Cytfluorometric Analysis—Flow cytometry was performed by an indirect immunofluorescence method as described previously (3). In short, SMKT-R3 cells were harvested, washed, and reacted on ice for 30 min with a monoclonal antibody to sulfoglycolipid Sulph-I (18) or with a polyclonal antibody to HGF as the first antibody, and subsequently with a fluorescein isothiocyanate-conjugated F(ab’)2 fragment of rabbit anti-mouse IgG (Dako) or of goat anti-rabbit IgG (Cappel) as the second antibody, respectively. For some experiments, the cells into which glycolipids had been incorporated as above were preincubated with 0.4 mg/ml HGF on ice for 1 h before the immunofluorescence staining. Fluorescence profiles were determined with a FACScan (Becton Dickinson).

RESULTS

Binding of HGF to Sulfoglycolipids—To examine the ability of glycolipids on renal cancer cells to serve as receptors for HGF, binding to glycolipids was investigated in two ways (19). In the first assay technique, glycolipids were separated on a thin layer plate and then overlaid with radiolabeled HGF. As shown in Fig. 1A, HGF bound to authentic sulfoglycolipids SM4, SM3, and SB2, but not to either neutral glycolipid or ganglioside standards. Likewise, HGF specifically bound to SM4, SM3, and SB2, but not to either neutral glycolipid or ganglioside standards. Likewise, HGF specifically bound to SM4 and SM3 extracted from a renal cancer cell line, SMKT-R3, which expresses SM4, SM3, and SM2 (3), but not to other acidic or neutral glycolipids from the cells (Fig. 1B). As shown in Fig. 2A, HGF binding was dependent on the amount of SM4, detecting as little as 7.5 ng of SM4. When binding of HGF to increasing amounts of authentic SM4 was quantified by densitometric analysis of the autoradiogram, a dose-dependent curve was obtained (Fig. 2B).

In the second assay technique, HGF binding to glycolipids was investigated by a solid-phase binding assay using lipids adsorbed on microtiter plates. Consistent with the data presented above, HGF bound to SM4, SM3, and SB2, but not to SM2, GalCer, or gangliosides (Fig. 3). When SM4 was decylated, HGF binding to lyso-SM4 was considerably decreased as compared with intact SM4. Dose dependence of HGF binding to

Fig. 2. Dose dependence of $^{125}$I-HGF binding to sulfoglycolipid separated on an HPTLC plate. Serial doses of SM4 were chromatographed on an HPTLC plate. After development, the plate was cut into two parts to detect glycolipids by $^{125}$I-HGF binding (panel A, left) or by orcinol staining (panel A, right) as described under “Experimental Procedures.” HGF binding was quantified by densitometry of the autoradiogram (panel B).
SM4 was also observed in this assay, and binding was abolished completely by excess amounts (100 μg/ml) of unlabeled HGF (data not shown). Nonspecific binding of HGF to uncoated wells was less than 1% of the HGF added.

**Inhibition of HGF Binding to Sulfoglycolipids**—In order to confirm whether the binding observed above was specific to HGF, the effect of anti-HGF antibody on the binding of HGF to SM4 was examined using a solid-phase radioassay. As shown in Fig. 4, the HGF binding was inhibited by coincubation with the antibody in a concentration-dependent manner.

To further define the interaction of HGF with sulfoglycolipids, several anionic polysaccharides were tested for their ability to inhibit HGF binding to SM4 (Fig. 5). A sulfated fucan, fucoidan, was the most potent inhibitor, and dextran sulfate and heparin were also effective. In contrast, keratan sulfate and hyaluronic acid were inactive as inhibitors.

**Incorporation of Sulfoglycolipid Enhances the HGF Binding to Renal Cancer Cells**—Glycolipids exogenously added to cell culture media are incorporated into plasma membranes (20). This approach was utilized to examine whether the HGF binding to SMKT-R3 cells was increased when sulfoglycolipids were incorporated into the cell membranes. The amount of sulfated glycolipids was analyzed by flow cytometry with a monoclonal antibody directed to a sulfoglycolipid, Sulph-I, which specifically reacts with SM3 and SM4 (18). In our previous study, flow cytometry with Sulph-I was able to detect sulfoglycolipids on the SMKT-R3 cell surface (3). After SM4 was incorporated into SMKT-R3 cells, flow cytometry with Sulph-I was performed. As shown in Fig. 6, the SM4-enriched cells considerably enhanced the reactivity with the antibody. To examine whether the incorporation of SM4 into the cells increased the binding of HGF to the cells, the cells were pretreated with SM4 or GalCer, a precursor of SM4 that lacks a sulfate group, incubated with HGF, and subjected to flow cytometry using an anti-HGF antibody (Fig. 7). The difference of fluorescence intensity between line 1 (mean fluorescence intensity, 96) and line 2 (mean fluorescence intensity, 546) was interpreted as the endogenous ability to bind HGF on the cells. Pretreatment with SM4 resulted in an increment of the reactivity with the anti-HGF antibody (mean fluorescence intensity of line 4, 1012), while GalCer had no significant effect (mean fluorescence of line 3, 566). Compared with line 1, pretreatment with SM4 or GalCer or dimethyl sulfoxide as the vehicle had no influence on the reactivity with an irrelevant antibody (data not shown). These observations indicated that SM4 incorporated into the cell membranes could react with HGF and suggested that exogenous SM4 could function as an HGF-binding site. It is, therefore, likely that endogenous sulfoglycolipids on SMKT-R3 cells play a role as HGF receptors.

**DISCUSSION**

In the present study, we have demonstrated that HGF bound to sulfoglycolipids on plastic plates, on thin layer chromatograms, and on renal cancer cells. The properties of HGF binding to sulfoglycolipids were similar to those of thrombospondin (7), antistasin (21), and properdin (22) in regard to half-maximum binding and the potency of sulfated glycoconjugates to inhibit binding among sulfatide-binding proteins (23). HGF bound to SM4 and SM3, but not to GalCer or LacCer, which are the respective precursor glycolipids. Moreover, additional GalNAc at the nonreducing terminal of SM3 resulted in the inability of HGF to bind to SM2. On the other hand, HGF could bind to SB2, which is sulfated at the nonreducing terminal residue of SM2. These observations suggest that a sulfate ester on the nonreducing terminal residue is critical for HGF binding to
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sulfoglycolipid. HGF also appeared to recognize the fatty acid moiety to some extent since there was a considerable decrease in the binding of HGF to lyso-SM4 compared with SM4. Alternatively, it is possible that the free amino group of lyso-SM4 affects the binding.

There are two classes of HGF-binding sites on the surfaces of target cells, one with high affinity called c-Met, and one with low affinity (24–29). We also observed the presence of both binding sites on SMKT-R3 cells.3 Our present observations suggest that sulfoglycolipids on renal cancer cells can act as low affinity binding sites. Since HGF was eluted from the low affinity sites by excess heparin, it was suggested that the binding site most likely corresponded to matrix- or cell-associated heparan sulfate proteoglycans (25, 26). HGF has an affinity for heparan (12), and the N-terminal hairpin domain and the second kringle domain are responsible for heparin binding.3 Heparin-binding sites are involved in sulfoglycolipid binding to laminin (30), thrombospondin (31), and antistasin (32). Thus, the N-terminal hairpin structure of HGF is possibly associated with sulfoglycolipid binding. It is necessary to further explore the role of sulfoglycolipids in the low affinity binding sites on renal cancer cells.

It is clear from previous studies that the high affinity receptor (c-Met) is needed for the biological response of target cells (29). Although there has been no evidence for the involvement of the low affinity receptor in HGF signal transduction, it is unknown at present whether cellular responses to HGF require only c-Met or its association with other molecules. Recent studies have demonstrated that basic FGF in the absence of cell surface heparan sulfate proteoglycans does not bind to its high affinity receptor and is not active, suggesting that basic FGF binding to cell surface heparan sulfate proteoglycans is a prerequisite for its high affinity binding (33). Furthermore, a deletion-mutant protein of HGF that lacked the N-terminal hairpin structure did not bind to heparin and lost its biological activities (34). It is, therefore, conceivable that low affinity binding sites such as heparan sulfate proteoglycans and sulfoglycolipids are required for the high affinity binding or exertion of biological activities of HGF. Moreover, sulfoglycolipids on renal cancer cells may play a role as binders and reservoirs for HGF to accumulate it on the cell surface, to protect it from degradation, or to transfer it to the high affinity receptors that initiate the cellular response. It is of importance to characterize these low affinity receptors for the elucidation of the HGF signal pathway.

Although there are a number of heparin-binding growth factors (35), including HGF, acidic and basic FGF, and interleukin-3, the presence of a heparin-binding site is not always sufficient for binding to sulfoglycolipids (30, 36). To our knowledge, the present study is the first report that sulfoglycolipids bind a growth factor. It is essential to further study the binding properties of HGF to sulfoglycolipids and to examine the possibility of binding of other growth factors to sulfoglycolipids.

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Fig. 6. Effect of incorporation of SM4 into renal cancer cells on the cell surface expression of sulfated glycolipids. After SMKT-R3 cells were preincubated at 37°C for 10 min in serum-free DMEM containing 100 μM SM4 or GalCer or 0.1% dimethyl sulfoxide as vehicle (none) and washed free of excess glycolipids, they were allowed to react with Sulph-I or control isotype IgG. The antigenic expression was analyzed by flow cytometry.

Fig. 7. Effect of incorporation of sulfoglycolipid into renal cancer cells on HGF binding to the cells. After SMKT-R3 cells were preincubated at 37°C for 10 min in serum-free DMEM containing 100 μM SM4 or GalCer or 0.1% dimethyl sulfoxide as vehicle (none) and washed free of excess glycolipids, they were allowed to react with HGF on ice for 1 h followed by flow cytometry with anti-HGF antibody or control antibody.

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