Rat Placental Hepatocyte Growth Factor/ Scatter Factor: Purification, Characterization, and Developmental Regulation (43637)

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Abstract. **In view of significant species-specific sequence differences between human and rat placental hepatocyte growth factor (HGF)/scatter factor (SF), the rat placental HGF/SF (rpSF) was purified, and its properties compared with human placental HGF/SF (hpSF). Like hpSF, rpSF scattered Madin-Darby canine kidney cells at 1–2 ng/ml and is composed of two subunits of 60 kDa and 30 kDa. Higher amounts (>50%) of uncleaved 90-kDa form was present in the HGF/SF preparations from both human and rat placentas. Rat placental SF reacts with antibodies raised against hpSF in rabbits and chickens. The SF activity when expressed per gram rat placental tissue rises rapidly up to 9 days and then levels off. When expressed per milligram tissue protein it also increases rapidly up to 9 days and then declines. The expression of HGF/SF mRNA during development parallels that of HGF/SF activity. The specific activity of HGF/SF receptor (c-*met*) mRNA also appears to peak at 6 days. These findings suggest that (i) in spite of significant (>10%) sequence differences between rpSF and hpSF, they exhibit similar structural, biologic, and immunologic characteristics and (ii) HGF/SF and its receptor are expressed in high amounts on Day 6 and then decline in developing placenta.**

[P.S.E.B.M. 1993, Vol 204]

Hepatocyte growth factor (HGF)/scatter factor (SF) is a heterodimeric glycoprotein produced by fibroblasts (1–3), smooth muscle cells (4), and human placenta (5, 6) that causes epithelial cell colonies to spread out and scatter. SF has been shown to be identical to HGF (7–9). In addition to scattering, HGF/SF stimulates migration and DNA synthesis (10, 11) in specific cell types. HGF/SF from rat and human tissues have been cloned, sequenced, and expressed (10, 11). They exhibit approximately 10% sequence differences (10, 11). In human placenta, SF activity appears to be high in second trimester and decreases slightly in the term placentas (5). In view of sequence differences between human and rat placental HGF/SF, we have purified the HGF/SF from rat placenta, compared its properties with human placental SF (hpSF) and determined the HGF/SF expression in placenta obtained from different stages of pregnancy in rat. Since c-met proto-oncogene product is receptor for HGF/SF (12) and is required for its action, we also determined its expression during placental development.

Materials and Methods

Cell Cultures. Madin-Darby canine kidney (MDCK) cells, purchased from American Tissue Culture Collection, Rockville, MD, were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Bethesda, MD) supplemented with 0.1 m*M* nonessential amino acids, 5.0 mg/ml D-glucose, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum (Gibco) in 5% CO_2 and 95% air as described before (7). The cells used in the study were free of mycoplasma contamination.

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Received March 17, 1993. [P.S.E.B.M. 1993, Vol 204]
Accepted June 2, 1993.

0037-9727/93/2041-0075$3.00/0
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Scatter Assay. Scatter activity was determined as reported previously (5, 13). Samples to be tested were serially diluted in serum-free DMEM in a 96-well titer plate (Falcon 3072) and added to MDCK cells in DMEM-10% fetal calf serum. Plates were incubated at 37°C with 5% CO_2 and 95% air for 20 hr, stained with crystal violet, and examined by light microscopy. The assay titer of a sample is the highest dilution at which significant scattering effect is observed. The activity present at the limiting dilution is defined as 0.5 scattering units/ml.

Purification of Rat Placental Scatter Factor. Adult Sprague-Dawley pregnant rats were obtained from Taconic Farms, NY. Rats with different durations of pregnancy (6, 9, 12, 15, 18, and 21 days) were sacrificed using CO_2. The placentas were collected, washed with ice-cold Ca^{2+}-Mg^{2+}-free Tris-HCl buffer (20 m*M*, pH 7.5; containing 0.9% NaCl), and cut into small pieces, and were either sonicated (Sonicator, W-385; Heat Systems-Ultrasonics Inc.) or homogenized in a waring blender at 4°C for 3 min in ice-cold triethandamine-buffered saline (TBS) containing 1 m*M* phenylmethylsulfonyl fluoride, 2 m*M* ethylenediaminetetraacetate, and 25 μg/ml gentamicin (2.0 ml/g). The suspensions were centrifuged at 20,000*g* for 30 min in a Sorvall centrifuge at 4°C, and the supernatants were assayed for scatter activity and subjected to chromatography as described (13).

Briefly, the clear supernatant obtained from extracts of 100 g of placenta was applied to a Bio-Rex 70 column (30-ml bed volume). The column was washed with 5 bed volumes of TBS and eluted with 3 bed volumes of 0.3–1.2 *M* gradient NaCl in the Tris-HCl buffer (20 m*M*, pH 7.5). Active fractions were pooled and diluted to a final salt concentration of 0.25 *M* NaCl and then applied to the S-Sepharose column (9-ml bed volume). The column was washed with 5 bed volumes of 0.3 *M* NaCl and the bound rat placental SF (rpSF) was eluted with a 50-ml gradient of 0.3–1.2 *M* NaCl in Tris-HCl buffer. Fractions (3.0 ml) were collected and aliquots were assayed for scatter activity. Active fractions (eluted at 0.7–0.9 *M* NaCl) were pooled and concentrated to 1.0 ml using a Ym-30 membrane in the Amicon system. For further purification of homogeneous rpSF, an immunoaffinity chromatography (6, 14) was used. Antibodies raised in rabbits against purified hpSF were employed. IgG fraction from the antiserum was obtained using protein A-Sepharose and was then coupled to cyanogen bromide-activated Sepharose 6B. Rat placental SF from S-Sepharose column was dialyzed against Tris-HCl buffer (20 m*M*, pH 6.5) containing 150 m*M* NaCl and applied to the affinity column. The column was washed with the Tris buffer and the bound rpSF was eluted with the immunoaffinity elution buffer (Pierce, Rockford, IL).

Preparation of Human and Mouse Scatter Factors. Human placental scatter factor was purified by ion exchange, reverse phase, and immunoaffinity chromatographic procedures, as described (6, 14).

Mouse scatter factor was purified from the conditioned medium of *ras*-transformed D4 clone of NIH 3T3 cells, as described (3).

Protein Determination. Total protein was determined by the dye-binding method (Bio-Rad Laboratories, Rockville Center, NY) using bovine serum albumin as a standard.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis. Vertical slab gel electrophoresis was performed according to the system described by Laemmli. Separating gels had 10% acrylamide and stacking gels had 2.5% acrylamide. The gels were run for 3 to 4 hr at constant current of 40 mA. The gels were then silver stained (Bio-Rad) to visualize the protein bands. A mixture of prestained proteins (BIO-RAD, Melville, NY) phosphorylase B (mol wt 110,000), bovine serum albumin (mol wt 84,000), ovalbumin (mol wt 47,000), carbonic anhydrase (mol wt 33,000), soybean trypsin inhibitor (mol wt 24,000), and lysozyme (mol wt 16,000) were co-electrophoresed as molecular weight markers.

Western Immunoblotting. Purified rpSF preparations (10–100 ng) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10% acrylamide gels, and the proteins were electrophoretically transferred onto nitrocellulose membranes (Bio-Rad) (15). The membranes were treated with 5% dried milk, in 20 m*M* TBS (blocking buffer), and incubated with primary antibodies in blocking buffer (anti-hpSF antibody in chicken or rabbit or monoclonal antibody in mice) for 2 hr (6, 7, 14). The membranes were then washed with TBS and incubated with secondary antibodies (rabbit anti-chicken IgG, goat anti-rabbit IgG, or rabbit anti-mouse IgG) linked to alkaline phosphatase (Sigma) and color was developed using the chromogenic substrate 5-bromo-4-chloro-3-indolyl phosphate (Sigma).

Northern Blot Analysis. Total RNA from rat placenta was prepared as described by Chirgwin *et al.* (16). For Northern blot analysis, 20 μg of RNA was electrophoresed on agarose gels containing formaldehyde and blotted onto nitrocellulose membranes by capillary transfer (17). The 1.7-kb *Xba-Hin*ds III rat HGF/SF cDNA fragment obtained from pBluescript plasmid containing HGF/SF cDNA (obtained from Dr. Nakamura, Kagoshima University, Japan; 10, 18) was isolated, labeled with [^{32}P]dCTP using a multiprime labeling kit from Boehringer Mannheim (Indianapolis, IN), and used as an HGF/SF cDNA probe. For HGF/SF receptor (c-*met*) probe a 1.5-kb *Eco*R1 mouse met cDNA fragment obtained from Dr. M. Park, McGill University, Montreal, Canada (20) was used. The blots were washed to a final stringency of 0.6 SSC (0.15 *M*

NaCl and 0.015 *M* trisodium citrate, pH 7.4) at 48°C and exposed to Kodak X-Omat film for 24 to 48 hr. After exposure, Northern blots were stripped and re-probed with a [^{32}P]dCTP-labeled actin probe (Clontech, San Francisco, CA) to normalize for loading and transfer of RNA. Sizes (kb) were estimated using an RNA ladder as well as ribosomal RNA as internal standards.

Results

Purification and Properties of rpSF. Rat placental SF was purified using ion exchange and immunoaffinity chromatography. These procedures have been used for purification of hpSF. Like hpSF, most of the SF was bound to the Bio-Rex column and could be eluted as a sharp peak with 0.7–0.9 *M* buffered NaCl. The SF bound to the S-Sepharose column with high affinity and could be eluted with 0.7–0.9 *M* buffered NaCl. A thousand-fold purification was obtained using these steps. A further 5-fold purification was achieved using immunoaffinity chromatography (Table I). Rat placental SF exhibits similar electrophoretic mobility as that of the hpSF (~78 kDa). On reduction, it gives three bands around 90 kDa, 60 kDa, and 30 kDa. A major portion of the rpSF remains as uncleaved 90-kDa single chain protein (Fig. 1). The purified rpSF scatters MDCK cells (Fig. 2) and has a specific activity of 2400 units/μg protein.

When partially purified (after Bio-Rex step) rpSF preparations were subjected to immunoblotting, using hpSF antibody raised in rabbits or chickens as primary antibodies and alkaline phosphatase-linked secondary antibodies, an intense protein band at ~78 kDa was observed (Fig. 3). When placental extracts or partially purified HGF/SF preparations were overloaded, a minor reaction to other protein bands ranging in molecular mass from 75 to 92 kDa was observed.

SF Activity in Rat Placenta During Pregnancy. The total SF activity in rat placenta rises rapidly after conception, reaching highest levels at the ninth day, and then levels off (Fig. 4). The activity, when expressed per milligram of tissue protein, rises up to the ninth day and then declines.

HGF/SF and c-*met* on RNA Expression. A major, approximately 6-kb HGF/SF mRNA species was detected in total RNA extracted from placentas from 6-day-old pregnant rats; minor amounts of additional bands at approximately 4.2 kb and 3 kb were also observed in some gels (not shown here). This 6-kb band was not detectable in RNA extracted from placentas from 12- and 16-day pregnant rats (Fig. 5). A 6-kb band was also observed with adult male rat lung RNA (positive control). Similarly, a 7.5-kb c-*met* mRNA band was present in 6-day-old pregnant rats but was not detectable in placentas from 12- or 16-day pregnant rats (Fig. 5). An identical c-*met* mRNA band at 7.5 kb was observed with liver RNA (Fig. 5).

Discussion

HGF/SF activity in rat placenta rises rapidly, reaching a peak value around 9 days of gestation. The cell types in placenta involved in synthesis of HGF/SF or the factor(s) responsible for its increased expression during development has not been identified. Presence of HGF/SF in hematopoietic cells, somites, squamous epithelium of the esophagus and skin, ependyma, bronchial epithelium, and chondrocytes of 12- to 19-day-old rat embryos has been demonstrated by immunofluorescence techniques. After 19 days, immunoreactive HGF/SF was present in the hepatocytes, pancreas, submaxillary glands, renal collecting tubes, and neural tissues (21). Similar studies on human placenta have shown HGF expression in the villous syncytium, in extravillous trophoblasts, and in amniotic epithelium. Certain trophoblastic tumors were also reported to be positive (22).

Recent studies by Wolf *et al.* (21) have demonstrated that the cytoplasm of trophoblasts of the rat placental labyrinth stain strong for HGF/SF. Whether the dramatic increase in the placental HGF/SF in the early stages after conception is due to selective proliferation of trophoblasts or increased HGF/SF gene expression remains to be determined. The function of HGF/SF in placenta is unknown. Because of its mitogenic and motogenic properties, HGF/SF has been implicated in embryogenesis. Whether it contributes to development of fetus or to maternal adaptation during pregnancy remains to be investigated.

On reduction, rpSF, like the hpSF, dissociates into 90-kDa, 60-kDa, and 30-kDa bands. The band at ~30 kDa appears diffused presumably because it is heavily glycosylated (8). The 90-kDa band is more intense than the 60-kDa and the 30-kDa bands, which indicates that

Table I. Purification of Rat Placental Scatter Factor[a]

Step	Activity (units × 10^5)	Protein (mg)	SP (units/μg)	Recovery (%)	Purification (folds)
Placental extract	16.2	3600	0.4	100	1
Bio-rex 70 chromatography	10.4	11.7	89	64	222
S-Sepharose chromatography	6.3	1.42	443	39	1109
Immunoaffinity chromatography	1.67	0.069	2400	10.3	6000

[a] For details, see text.

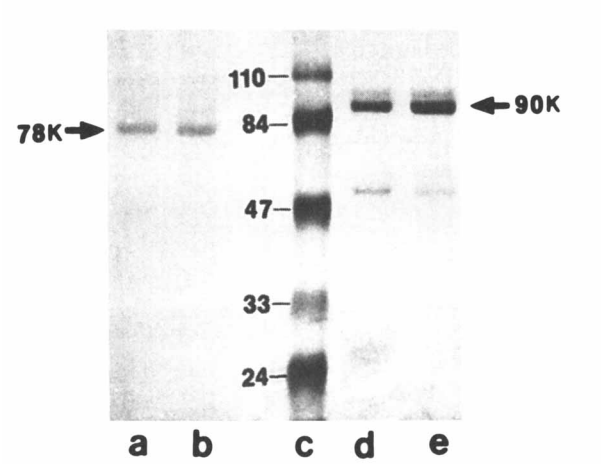

Figure 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified nonreduced rpSF (a) and hpSF (b), reduced rpSF (d), and hpSF (e). The molecular masses of the following prestained proteins which were co-electrophoresed are shown in Lane c: phosphorylase B (110 kDa), bovine serum albumin (84 kDa), ovalbumin (47 kDa), carbonic anhydrase (33 kDa), soybean trypsin inhibitor (24 kDa), and lysozyme (16 kDa).

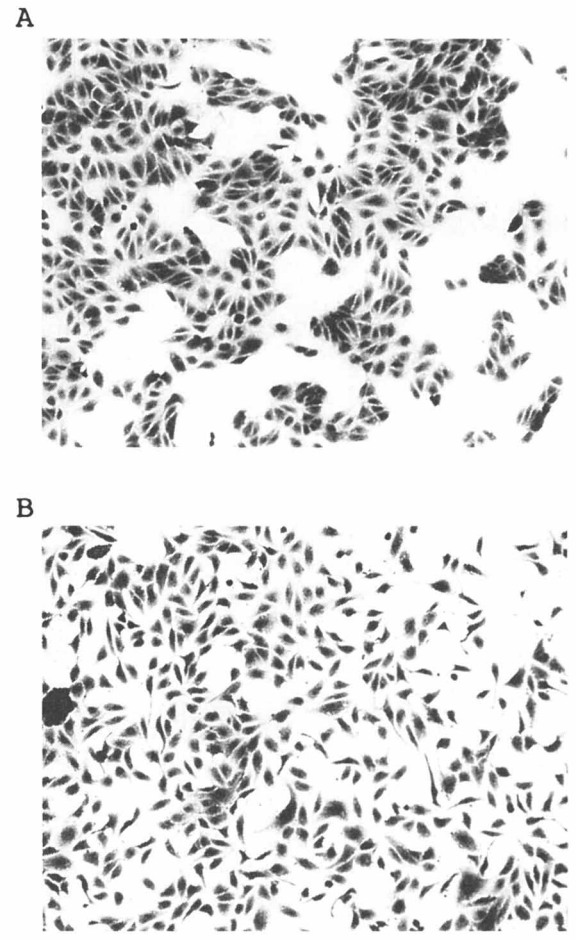

Figure 2. Effect of rat placental scatter factor on MDCK cells. Colonies of MDCK cells were incubated at 37°C for 20 hr without (A) and with (B) rpSF (approximately 10 units/ml), stained with crystal violet, and photographed (×130).

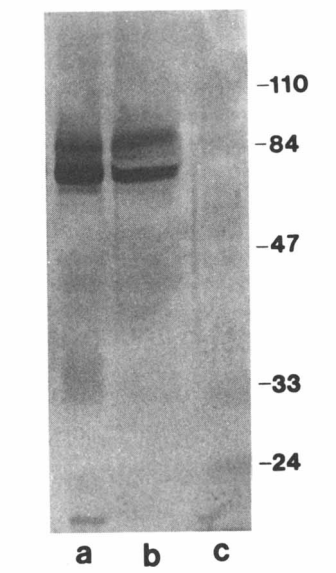

Figure 3. Western blot of rat placental scatter factor. Partially purified (after Bio-Rex step) rpSF (Lane a) and hpSF (Lane b) preparations were pooled, concentrated, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis by procedures described in the text. The primary antibody was raised against hpSF in rabbits and the secondary antibody was goat anti-rabbit IgG linked to alkaline phosphatase. The molecular masses of the following prestained proteins which were co-electrophoresed are shown in Lane c: phosphorylase B (110 kDa), bovine serum albumin (84 kDa), ovalbumin (47 kDa), carbonic anhydrase (33 kDa), soybean trypsin inhibitor (24 kDa), and lysozyme (16 kDa).

most of the rpSF exists as a noncleaved protein in placenta. Studies by Nakamura *et al.* (10) suggest that the mature form of HGF/SF (90 kDa) exists as a heterodimer of α (60 kDa)- and β (30 kDa)-subunits which arise by proteolytic cleavage from a single-chain form. As observed with hpSF preparations (14), the uncleaved lower band as well as the upper band (the cleaved form) are present in the doublet around the 90-kDa region in rpSF preparations, and both are biologically active when eluted from a nonreduced gel (data not shown).

Minor reaction with other protein bands was observed with the antibody preparation when high amounts of placental extract proteins or partially purified HGF/SF was applied to the gel (Fig. 3). At lesser concentrations of partially purified HGF/SF, immunoreaction was restricted to the 78-kDa band. The slight immunoreaction observed at higher concentrations could be due to the presence of alternatively spliced forms or different degrees of glycosylation, or due to the cleaved and uncleaved forms of HGF/SF. Identical results were obtained with HGF/SF antibodies raised in chickens (data not shown).

The rpSF mRNA and c-*met* mRNA levels follow a similar pattern during placental development. These mRNA reach maximal levels in 6 days and then decline. The physiologic significance of this finding remains to be determined.

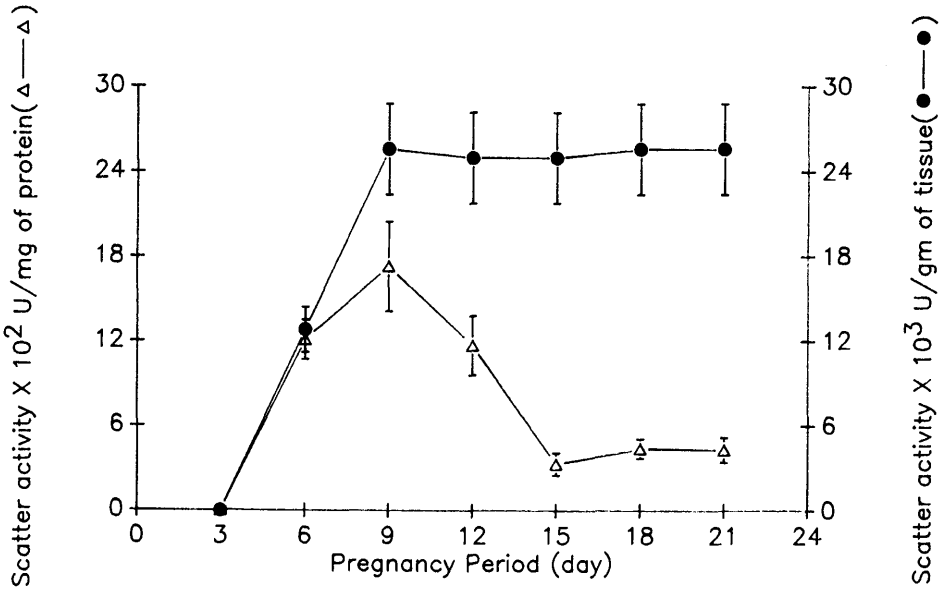

Figure 4. Scatter factor activity in placenta during pregnancy in rats. Placentas were obtained from rats at different pregnancy periods, homogenized in TBS (2 ml/g), and centrifuged, and supernatants were assayed for the scattering activity. See text for details.

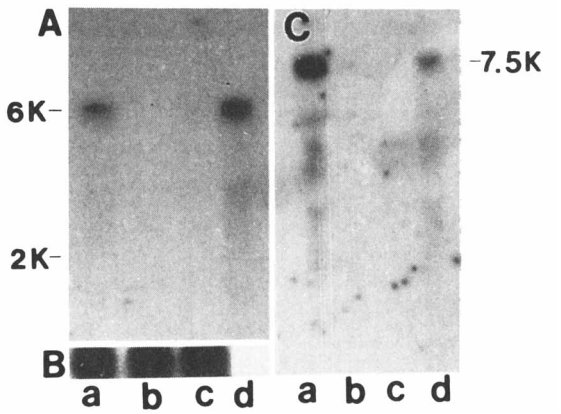

Figure 5. Northern blot analysis for HGF/SF and c-*met* mRNA in rat placental tissue during pregnancy. Twenty micrograms of total mRNA were isolated from placentas of 6-day (Lane a), 12-day (Lane b), and 16-day (Lane c) pregnant rats and from adult male rat lung (Panel A, Lane d) and liver (Panel C, Lane d), subjected to agarose gel electrophoresis, and transferred to nitrocellulose. The blots were hybridized with ^{32}P-labeled HGF/SF cDNA (Panel A) and c-*met* cDNA probes (Panel C). HGF/SF cDNA hybridized blots were stripped and rehybridized to actin-cDNA probe (Panel B). For details, see text.

This work was supported by the Joel Finkelstein Foundation. Dr. Rosen was supported by U.S. Public Health Grant CA-50516, American Cancer Society Grant BE7, and Established Investigator award from the American Heart Association.

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