Vol. 174, No. 1, 1991 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1991 Pages 331-337

Hepatocyte Growth Factor/Hepatopoietin A Stimulates the Growth of Rat Kidney Proximal Tubule Epithelial Cells (RPTE), Rat Nonparenchymal Liver Cells, Human Melanoma Cells, Mouse Keratinocytes and Stimulates Anchorage-Independent Growth of SV-40 Transformed RPTE

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Received November 7, 1990

Hepatocyte growth factor/hepatopoietin A is a mitogen for primary hepatocytes and may mediate regeneration after liver damage. To date, the activity of this novel factor has been restricted to hepatocytes. We now show that the factor is also a mitogen for a number of primary epithelial cells but is inactive with human foreskin fibroblasts, human endothelial cells and HEP3B cells. The factor also substitutes for HBGF-2 (basic FGF) in stimulating the anchorage-independent growth of SV-40 transformed rat kidney epithelial cells. Therefore, hepatocyte growth factor/hepatopoietin A appears to act on a variety of epithelial, but not mesenchymal, cells which respond to HBGFs.

The observation that serum factors may mediate the growth of hepatocytes following liver damage (1,2) has lead to the purification, characterization and cloning of a factor from serum which is a potent mitogen for primary cultures of human and rat hepatocytes (3-8). This novel factor is a high molecular weight (70-100 kd) heparin-binding protein which is a dimeric polypeptide containing a heavy (50-70 kd) and a light (30-35 kd) chain. HGF/HPTA is structurally distinct from the heparin-binding growth factor (HBGF) family (also called fibroblast growth factors; see ref. 9 for review) and does not cross-react with HBGF-receptors (M. Kan et al.

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Abbreviations used: Rat kidney proximal tubule epithelial cells, RPTE; human umbilical cord endothelial cells, HUV-EC; human melanoma cells, NEL-MI; human foreskin fibroblasts, HFF; mouse oral epithelium keratinocytes, MK; hepatic nonparenchymal epithelial cells, NPL; hepatocyte growth factor/hepatopoietin A, HGF/HPTA; heparin-binding growth factor, HBGF; fibroblast growth factor, FGF; simian virus-40, SV40; RPTE transformed with SV40 viral DNA, SV-RPTE.

0006-291X/91 $1.50

unpublished results). Though HBGFs are mitogens for primary hepatocytes (10), HGF/HPTA is a more potent mitogen for human and rat primary hepatocytes (11).

To date, the activity of HGF/HPTA has been studied only with hepatocytes. We have now characterized the mitogenic activity of HGF/HPTA with a variety of cells including RPTE, NEL-MI, NPL, HFF, MK and HUV-EC cells. The results show that HGF/HPTA is a mitogen for RPTE, NPL and mouse keratinocytes and a weak mitogen for NEL-MI, but is inactive with HUV-EC, HEP3B cells and HFF. These data suggest that HGF/HPTA may be a mitogen for some primary epithelial cells which also respond to members of the HBGF family but not for mesenchymal cells which respond to these same factors.

MATERIAL AND METHODS

HGF/HPTA was purified to homogeneity as described by Zarnegar and Michalopoulos (12) using rat hepatocytes as the target cells. MK cells were isolated from BALB/c mouse oral epithelium by the explant method and transferred at a 1:3 split ratio using MCDB 153 medium (13) supplemented with 5F (14), 10 ng/ml HBGF-1 and 10 µg/ml heparin. The cells were used at passage number 12. HFF cells were isolated as described previously (15). The cells were transferred at a split ratio of 1:3 and were used at passage 5. NPL, (16) HUV-EC (17), HFF, NEL-MI (18) and HEP3B (19) cells were plated on collagen (Vitrogen 100, 10 µg/ml; Collagen Corp., Palo Alto, CA) coated 24-well plates at a density of 2 X 10^4 cells/well, except for HUV-EC which were plated at a density of 1 X 10^4 cells/well. Cells were maintained in RITC80-7 medium (20; Kyokuto Pharmaceutical Co., Tokyo, Japan). RITC80-7 medium was supplemented with 1 mg/ml bovine serum albumin, 4 µg/ml oleic acid, 10 µg/ml transferrin, 1 µg/ml insulin, 5 µg/ml ethanolamine, 50 µM dithiothreitol and 30 µg/ml L-proline for experiments with NEL-MI, NPL, HFF, HEP3B and HFF. In experiments with HUV-EC, 10% fetal bovine serum (FBS) and 10 µg/ml heparin were added. In experiments with MK cells, low calcium RITC80-7 medium (30 µM calcium chloride) was used to prevent terminal differentiation. Rat kidney proximal tubules were prepared and seeded in collagen coated 12-well plates at a density of 150 µg protein/well and maintained in serum-free DMEM:F12 (50:50) as described (21) and modified (22). When HBGF-1 was added to RPTE, the medium was supplemented with 10 µg/ml heparin. HBGFs were obtained from Upstate Biotechnology (Lake Placid, NY). Insulin was obtained from Sigma Chem. Co. (St. Louis, MO). All other factors and reagent used for cultures were obtained from commercial sources and were used without further purification.

Cell growth was measured either by increases in cell number or by an increase in incorporation of [^{3}H]thymidine (New England Nuclear, Milford, MA) into acid insoluble material. Cell number was determined using a Coulter Counter (Coulter Electronics, Hialeah, FL). When [^{3}H]thymidine incorporation was measured, cells were plated at 2 X 10^4 cells/well in 24 well plates (see above) and maintained in hormone free medium (1 ml) overnight. [^{3}H]Thymidine was added (1 µCi/ml) and incorporation into acid insoluble material was determined 24 hr later. [^{3}H]Thymidine incorporation into RPTE was determined using cells grown in 12-well plates (see above) in 2 ml of medium in the presence or absence of factors for 4 days. [^{3}H]Thymidine was added to RPTE on day 4 and incorporation was determined 24 hr later. In experiments in which the effect of HBGF-1 on growth was determined, heparin (10 µg/ml) was added to the cultures.

SV-RPTE were transformed with a replication defective SV-40 viral vector (pX-8;23) as described (24). The vector was a generous gift of Dr. Catherine Reznikoff of the University of Wisconsin (Madison, WI). When

anchorage-independent growth was determined, cells were plated as a suspension in 0.5% agar in 6-well plates. The cells were maintained in DMEM:F12 (see above) in the presence of 10% FBS which is required for anchorage-independent growth (24). After 2 weeks, the growth was quantitated by manually counting the number of colonies/well and by determining the % area occupied by the colonies by videometric analysis using an Artek Model 980 cell counter (Artek Systems, Corp. Farmingdale, NY) as described (25).

RESULTS

Table 1 is a summary of the effect of HGF/HPTA and HBGF-1 (acidic FGF) on [^{3}H]thymidine incorporation for a variety of cells types. HFF cells are refractory to HPTA, but responded well to HBGF-1. NEL-MI cells do not respond to HBGF-1 but do shown a modest stimulation by HGF/HPTA. Like hepatocytes, NPL cells, MK cells and RPTE cells all respond to HBGF-1 and HGF/HPTA. The human hepatoma cell line HEP3B did not respond to HGF/HPTA, but already showed high levels of [^{3}H]thymidine incorporation. Thus, of the cell types studied, only the primary epithelial cells (NPL, rat hepatocytes, MK and RPTE) respond both to HGF/HPTA and HBGFs.

Not all cells which respond to HBGFs respond to HGF/HPTA. Both HBGF-1 and -2 are potent mitogens for endothelial cells in culture, however, HGF/HPTA was inactive with HUV-EC **(Table 2)**. Therefore, the activity of HGF/HPTA in primary epithelial cells cannot be accounted for by contamination of the preparation with HBGF-1 or -2 since HUV-EC cells are exquisitely responsive to HBGFs.

Table 1. Effect of HGF/HPTA and HBGFs on the incorporation of [^{3}H]thymidine in a variety of cell types

Cell Type	[^{3}H]Thymidine (cpm)		
	no addition	HGF/HPTA	HBGF-1
HFF	3,700	3,900	23,500
MK	1,200	19,800	24,200
NEL-MI	20,100	33,000	23,500
NPL	550	10,500	12,400
RPTE	14,138	116,728	*n.d.
HEP3B	38,700	35,300	*n.d.

Cells were grown as described in Materials and Methods. HBGF-1 (acidic FGF) was added at a concentration of 10 ng/ml along with 10 µg/ml heparin, HGF/HPTA was added at a concentration of 150 ng/ml. *n.d.= not determined, however, both HEP3B and RPTE do respond to HBGF-1 (see ref. 27 and 22, respectively). Values are for duplicate or triplicate determination of [^{3}H]thymidine incorporation from single experiments which are typical of three separate experiments. With the exception of the RPTE cells, the variation between experiments was greater than the variation within an experiment. HFF- human foreskin fibroblasts, MK- mouse keratinocytes, NEL-MI- human melanoma cell line, NPL- nonparenchymal liver epithelial cells, RPTE- rat proximal tubule epithelial cells, and HEPG3- human hepatoma cell line. RPTE are seeded at a higher density than the other cells, hence the increased baseline incorporation.

Table 2. Effect of HGF/HPTA and HBGF-1 on the growth of human umbilical cord endothelial cells

Factor	Cell Number/well
No Addition	6950 ± 950
HGF/HPTA (150 ng/ml)	6250 ± 750
HBGF-1 (10 ng/ml)	64600 ± 17000

Human umbilical cord endothelial cells were prepared as described (17). Cells were seeded at a density of 1 X 10^4 cells in each well of a 24 well plate containing 1 ml of RITC80-7 medium supplemented with 10% fetal bovine serum. After overnight culture in the absence of factors, various factors were added and cell number was determined on day 6 after seeding. The data are the average ± the range of duplicate determinations from a single experiment and are representative of three separate experiments since variation between experiments was greater than variation within an experiment.

The response of the MK and RPTE cells to HGF/HPTA has not been previously reported, therefore we further characterized the dose response relationship for HGF/HPTA with both cell types. In RPTE, HGF/HPTA produced a dose dependent increase in cell number and 15-45 ng/ml produced a response comparable to the maximal response observed with 10 ng/ml of HBGF-2 **(Table 3)**. A similar situation was seen with the MK cells, however, 75 ng/ml HGF/HPTA were required to produce a maximal response equivalent to HBGF-2 at 10 ng/ml **(Table 3)**. Since the molecular weights of HGF/HPTA and HBGFs are

Table 3. Concentration dependence of HGF/HPTA on the growth of MK and RPTE cells

Growth Factor	Relative Cell Growth	
HGF/HPTA	RPTE Cells	MK Cells
0	1.0 ± 0.15	1.0 ± 0.11
5	n.d.	2.4 ± 0.08
7.5	2.1 ± 0.04	n.d.
15	2.8 ± 0.01	6.0 ± 0.19
45	4.3 ± 0.18	12.4 ± 0.06
75	n.d.	16.5 ± 0.01
HBGF (10 ng/ml)	3.9 ± 0.01	20.2 ± 0.06

Cells were grown as described in Materials and Methods. Since both HBGF-1 and -2 are equipotent in stimulating rat kidney proximal tubule epithelial cell (RPTE) growth only HBGF-2 (10 ng/ml) was tested, while HBGF-1 was used for the mouse keratinocytes (MK). The data are presented as the relative increase in growth over cells to which no factors were added; a value of 1.0 indicates no increase in growth. Growth was assessed by increases in cell number for RPTE cells while growth increases for MK cells were assessed by incorporation of [^{3}H]thymidine. The control values (listed as 0 in the table) were 6.7 X 10^4 cells/well for RPTE and 1200 cpm/10^4 cells for MK cells. The values for RPTE are the mean ± standard deviation for three separate experiments (n=3) while the MK data are the average ± the range of duplicate determinations from one experiment representative of 3. The variation between experiments with the MK cells was greater than the variation within an experiment.

approximately 100 kd and 20 kd, respectively, the molar potencies are similar for both factors in both cell types.

Recently, we observed that HBGFs are potent inducers of anchorage-independent growth for RPTE transformed by SV40 (SV-RPTE; 24). Therefore, we determined whether HGF/HPTA could also support the anchorage-independent growth of SV-RPTE. **Figure 1** shows representative supsension cultures of SV-RPTE in the presence of HBGF-2 or HGF/HPTA. HGF/HPTA stimulated growth in soft agar, but at this concentration the colony number and size were reduced compared to HBGF-2 **(Table 4)**. Therefore, both HGF/HPTA and HBGFs are able to induce the transformed phenotype in SV-RPTE.

DISCUSSION

Hepatocyte growth factors or hepatoptoietins represent a family of high molecular weight factors with heparin binding activity (3-8). Both HGF/HPTA and members of the HBGF family (9) are mitogens for hepatic parenchymal cells (10-12) and may be mediators of liver regeneration after partial hepatectomy or chemical damage. However, the results of this investigation demonstrate that the range of cell types acted on by HGF/HPTA includes other primary epithelial cells as well as a human melanoma cells and transformed RPTE. However, HGF/HPTA may interact with these cells through receptors other than the HBGF-receptor which binds HBGFs 1 and 2 since HGF/HPTA does not compete for the binding of ^{125}I-labelled HBGFS to HBGF-receptors on

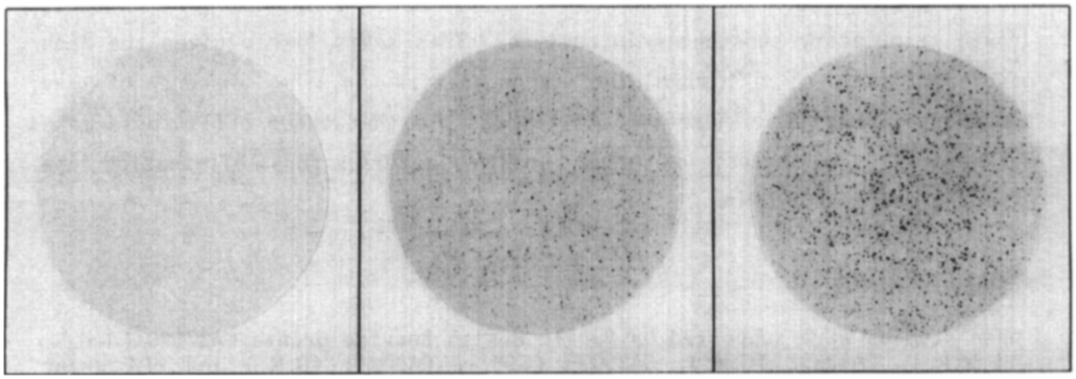

Figure 1. Anchorage-independent growth of SV40 transformed rat kidney proximal tubule epithelial cells by HGF/HPTA and HBGF-2.

SV-RPTE were seeded at a density of 1 X 10^5 cells/well in a 6 well plate in 0.5% agar. The cells were grown for 2 weeks and stained with (3-(4,5-dimethylthiazolyl)-2-2,5-diphenyltetrazolium bromide) (10 mg/ml in phosphate buffered saline). The photographs are of representative cells growing in wells in which the medium contained 10% fetal bovine serum alone, or 10% fetal bovine serum plus HGF/HPTA (75 ng/ml) or HBGF-2 (10 ng/ml). HBGF-1 and -2 are equipotent in inducing growth in soft agar, however, HBGF-1 requires the presence of heparin (22,24). Only data for HBGF-2 are shown.

Table 4. Stimulation of anchorage-independent growth of SV40 transformed RPTE by HGF/HPTA

Factor	Number of Colonies	Surface Area
no addition	0 ± 0	13 ± 0
HGF/HPTA (75 ng/ml)	1741 ± 178	226 ± 42
HBGF-2 (10 ng/ml)	3542 ± 438	614 ± 42

SV-RPTE were seeded at a density of 1 X 10^5 cells/well into 6-well plates as a suspension in 0.5% agar. All wells contained 10% FBS with or without the addition of factors. Surface area is in arbitrary units. Medium was changed every two days for two weeks whereupon colony formation was determined. The data are the average ± the range of two independent experiments (n=2).

HEPG2 cells (Kan et al. unpublished data). Therefore, HGF/HPTA may play a distinct role in the regulation of cell growth in tissues other than liver.

In a recent study using an anti-HGF/HPTA antibody, Zarnegar et al. (26) found that HGF/HPTA is present in large amounts in the exocrine pancreas, large cortical neurons of the brain, the thyroid gland and Brunner's gland of the duodenum. Given the widespread distribution of HGF/HPTA and the results of the present study, which demonstrates for the first time that HGF/HPTA may have activity in cell types other than hepatocytes, the role of this growth factor as an autocrine/paracrine or endocrine regulator of growth and function in other tissues should be further investigated.

The fact that HEP3B cells do not respond to HGF/HPTA even though HBGF-1 and -2 are mitogens for these cells makes sense if these cells produce growth stimulatory substances in culture. This seems likely given the high baseline level of [^{3}H]thymidine incorporation in the absence of any additions **(Table 1)**. Therefore, it is possible that these cells could make HGF/HPTA-like molecules, or other growth factors, thus eliminating the effect of added HGF/HPTA.

ACKNOWLEDGMENTS

These studies were supported by Public Health Service grants CA37589 (J.L.S. and W.M.), CA43632 (G.M.), CA35373 (G.M.), CA30241 (G.M.) and EPA grant CR814344 (G.M.).

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