

Hepatocyte growth factor has potent anti-proliferative activity in various tumor cell lines

Hisao Tajima, Kunio Matsumoto and Toshikazu Nakamura

Department of Biology, Faculty of Science, Kyushu University, Fukuoka 812, Japan

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Hepatocyte growth factor (HGF) has potent mitogenic activity for mature hepatocytes and various normal epithelial cells. We now have evidence that HGF at 1–10 ng/ml, strongly inhibits the growth of HepG2 hepatocellular carcinoma cells, B6/F1 melanoma cells and KB squamous carcinoma cells. These tumor cells express high affinity receptors for HGF with a K_d of 25–28 pM, similar to findings with hepatocytes. HGF at 1–100 ng/ml had no significant cytolytic effect on tumor cells. Therefore, the anti-proliferative effect of HGF on tumor cells seems to be cytostatic, not cytolytic. As HGF apparently has bidirectional effects on cell growth, the possibility that it can serve as an anti-tumor agent merits attention.

Hepatocyte growth factor (HGF), Anti-tumor agent, Growth inhibition, HGF receptor

1. INTRODUCTION

Hepatocyte growth factor (HGF) is a novel growth factor composed of a 69 kDa α -subunit and a 34 kDa β -subunit [1–3]. HGF cDNA cloning revealed that HGF derives by proteolytic processing from a single precursor protein consisting of 728 amino acids, and the homology with plasminogen is 38% [4,5]. HGF at 5–8 ng/ml (60–90 pM) promotes the growth of mature parenchymal hepatocytes in primary culture. HGF activity and expression of HGF mRNA were markedly increased in livers or plasma of rats with various liver injuries [6–9] and elevated levels of HGF were noted in ascites from patients with cirrhosis [10]. Mature hepatocytes in primary culture express a high affinity receptor for HGF, exhibiting K_d =20–30 pM and 500–600 sites/hepatocyte [11]. HGF receptor is widely distributed in various epithelial cells, including mature hepatocytes, but apparently not in mesenchymal cells such as fibroblasts and hematopoietic cells. HGF enhances the growth of various epithelial cells such as renal tubular cells [12], keratinocytes [13] and melanocytes [14], but it has no evident effect on growth of mesenchymal cells. Scatter factor first described by Stoker and his colleagues is a fibroblast-derived factor which enhances cell motility and was found to be identical to HGF [15,16]. Indeed, recombinant human HGF markedly enhances the migration of various species of epithelial cells, including MDCK canine renal epithelial cells, at physiological concentrations [17]. We have now

obtained evidence that HGF has cytostatic anti-proliferative effects on various species of tumor cells, hence may be a bidirectional growth regulator, and possibly a putative anti-tumor agent.

2 MATERIALS AND METHODS

2.1 Cell cultures

HepG2 (human hepatocellular carcinoma, a gift from Genentech Co.) and KB (human epidermoid carcinoma, JCRB 90) cells were cultured in Dulbecco's modified Eagle's (DME) medium supplemented with 10% fetal calf serum (FCS). B6/F1 (mouse melanoma, a gift from Genentech Co.) cells were cultured in a mixture of DME and Ham's F12 medium (1:1) supplemented with 10% FCS.

2.2 Measurement of DNA synthesis and cell growth

Cells were seeded at a density of 1×10^4 cells/cm² on a 24-well plastic plate (Corning), and cultured in medium containing an appropriate amount of HGF. After 4 days of culture, the cells were pulse-labeled with 0.3 μ Ci/ml [¹²⁵I]deoxyuridine (2200 Ci/mmol, New England Nuclear) for 4 h, then were washed twice with phosphate-buffered saline and once with 10% (w/v) ice-cold TCA and solubilized with 1 M NaOH. The radioactivity incorporated to the cells was counted in a γ -counter.

To measure the cell growth, the cells were seeded (8×10^4 cells/well for B6/F1 cells and KB cells, and 4×10^4 cells/well for HepG2 cells) on 6-well plates (Corning) and cultured in the presence of various concentrations of HGF for 4 days. The cells were dissociated by trypsin-treatment and the number of cells was determined using a hemocytometer.

2.3 Binding assay for HGF receptors

To characterize HGF receptors, binding of [¹²⁵I]HGF to cells was analyzed as follows. Cultures were equilibrated in binding buffer (Hank's solution containing 20 mM HEPES, 2 mg/ml bovine serum albumin, pH 7.0) for 30 min at 10°C. Ice-cold binding buffer containing increasing concentrations of [¹²⁵I]HGF, with or without a 100-fold excessive concentration of unlabeled HGF, was added and the preparation incubated for 1 h at 10°C. Subsequently cultures were rinsed three times with ice-cold binding buffer, and [¹²⁵I]HGF bound

Correspondence address: T. Nakamura, Department of Biology, Faculty of Science, Kyushu University, Fukuoka 812, Japan. Fax: (81) (92) 632 2741.

to cells was measured in a γ -counter after solubilization of the cells with 1 M NaOH

2.4 Measurement of cytolytic activity

Cytolytic activity was measured as follows: cells were plated at a density of 5×10^4 cells/well on a 24-well plate (Corning). The next day, the medium was replaced with fresh medium and viable cells were labeled with $70 \mu\text{Ci/ml}$ $\text{Na}^{51}\text{CrO}_2$ (250–500 mCi/mg Cr, Amersham) at 37°C for 2.5 h. Cultures were washed three times with fresh medium and cultured for 24 h in medium containing appropriate concentrations of HGF. The radioactivities in the culture media and cell lysates dissolved in 2% SDS were counted in a γ -counter, and the percent of cytolysis was calculated as follows:

$$\% \text{ of cytolysis} = 100 \times (A - A_0) / (A + B),$$

wherein A represents radioactivity in culture medium of target cells cultured with HGF for 24 h, B represents radioactivity remaining in target cells after cultivation, and A_0 represents radioactivity in culture medium of target cells cultured without HGF, which means the spontaneous release of ^{51}Cr .

3 RESULTS

3.1 Inhibition of DNA synthesis by HGF

HGF dose-dependently inhibited DNA synthesis of tumor cells (Fig. 1). There were no significant morphological changes seen with any concentration of HGF, as examined under a phase-contrast microscope (not shown). The maximal inhibition seen with 5–10 ng/ml HGF corresponds to 50–70% of inhibition. At higher concentrations (>10 ng/ml), there was no further inhibition of DNA synthesis by HGF. For purpose of comparison, the effect of HGF on DNA synthesis of mature parenchymal hepatocytes was examined. HGF at 5–8

Table 1

K_d and B_{\max} values of an HGF receptor on normal and tumor cells

Cells	Cell type	HGF receptor	
		K_d (pM)	B_{max} (sites/cell)
<i>Normal cells</i>			
Hepatocyte ^a	parenchymal liver cell (rat)	24	565
Renal tubular cell	epithelial (rabbit)	28	720
Melanocyte ^b	epidermal (human)	22	120
Keratinocyte ^c	epidermal (human)	17–45	700–1100
<i>Tumor cells</i>			
KB	epidermoid carcinoma (human)	25	750
B6/F1	melanoma (mouse)	24	350
HepG2	hepatocellular carcinoma (human)	25	1510

K_d and B_{\max} values in tumor cells were calculated from Scatchard analysis as shown in Fig. 3B.

^{a-c} The results were taken from our reported results. See references [11], [14] and [13], respectively.

ng/ml markedly stimulated DNA synthesis of hepatocytes. Therefore, HGF has bidirectional effects on DNA synthesis and the stimulatory or inhibitory effect of HGF seems to depend on the cell type, that is normal or transformed: HGF stimulates DNA synthesis of hepatocytes in primary culture, but inhibits that of HepG2 hepatocellular carcinoma cells. Similarly, HGF stimulates the growth of melanocytes [14], but inhibits that of B6/F1 melanoma cells.

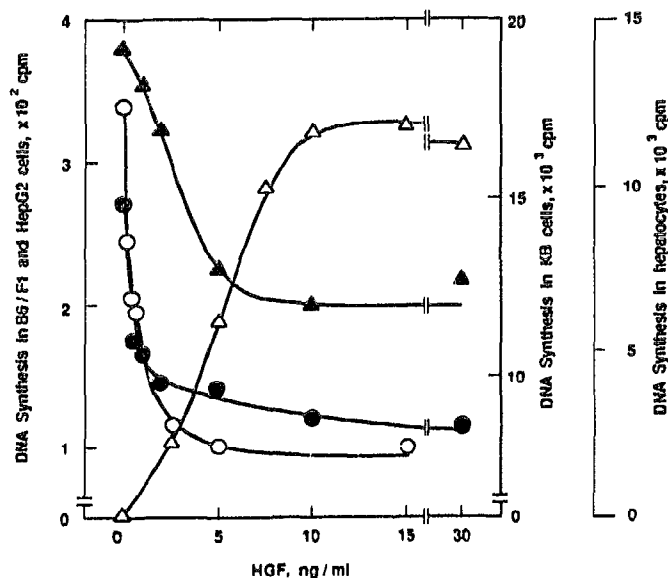


Fig. 1. Effect of HGF on the DNA synthesis of HepG2, B6/F1, KB cells and hepatocytes. Cells were plated in 24-well plates at a density of 1×10^4 cells/cm² and cultured in medium containing an appropriate amount of HGF. Cells were cultured for 4 days and pulse-labeled with [¹²⁵I] deoxyuridine for 4 h. The average of triplicate measurements is shown. (●) B6/F1 cells, (○) HepG2 cells, (▲) KB cells and (△) hepatocyte.

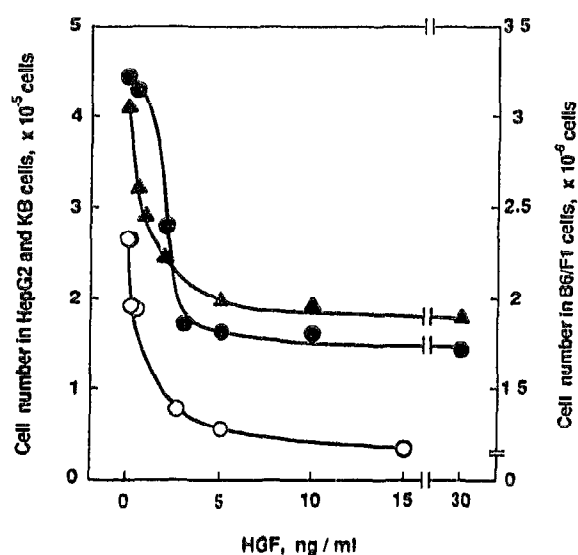


Fig. 2. Effect of HGF on growth of HepG2, B6/F1 and KB cells. Cells were plated in 6-well plates at a density of 8×10^4 cells/well for B6/F1 cells and KB cells, and 4×10^4 cells/well for HepG2 cells and cultured in the presence of various concentrations of HGF for 4 days. Cells were dissociated by trypsin-treatment and the number of cells was determined using a hemocytometer. The average of duplicate measurements is shown. (●) B6/F1 cells, (○) HepG2 cells and (▲) KB cells.

Table II
Effect of HGF on cytolytic release of ^{51}Cr from tumor cells

Cells	HGF ng/ml	^{51}Cr release in		% of cytotoxicity $((A-A_0)/(A+B)\times 100)$ (%)
		cultured medium (A) (cpm)	cells (B) (cpm)	
HepG2	0	11 876 \pm 0	35 722 \pm 1050	0
	5	11 905 \pm 29	39 196 \pm 367	0
	10	12 026 \pm 91	36 785 \pm 1163	0
	100	11 471 \pm 46	37 215 \pm 23	0
B6/F1	0	24 340 \pm 1411	54 108 \pm 642	0
	5	25 116 \pm 722	55 603 \pm 824	0.2
	10	25 482 \pm 220	54 806 \pm 729	0.4
	100	29 163 \pm 521	52 288 \pm 298	4.8
KB	0	21 900 \pm 59	60 297 \pm 15	0
	5	22 139 \pm 796	60 391 \pm 1199	0.2
	10	23 638 \pm 509	57 672 \pm 1866	2.5
	100	22 278 \pm 444	53 161 \pm 997	2.9

Each value is the mean \pm SE of triplicate measurements. A_0 is ^{51}Cr release (cpm) in cultured medium of target cells in the absence of HGF

3.2 Inhibition of cell growth by HGF

Inhibitory effects of HGF on DNA synthesis of tumor cells were evident when proliferation of the tumor cell lines was examined (Fig. 2). HGF dose-dependently suppressed the growth of tumor cells, and the maximal inhibition by 50–70% was seen with 5–10 ng/ml HGF, findings consistent with the effects on DNA synthesis.

3.3 HGF receptors on tumor cells

Analysis of HGF receptors showed that [^{125}I]HGF binds to these tumor cells in a concentration-dependent manner and that the binding is saturable (Fig. 3A). As shown in Fig. 3B, Scatchard plots of the binding indi-

cated a single class of high affinity binding sites expressed on these tumor cells. K_d and B_{max} values of tumor cells and other normal cells are summarized in Table I. The tumor cell lines tested express approximately 300–1500 binding sites/cell with K_d of 25–28 pM. Therefore, there were no significant differences in HGF receptors between normal and tumor cell lines.

3.4 Cytostatic effect of HGF on tumor cells

The cytotoxicity caused by HGF was less than 2.5% at 5–10 ng/ml, concentrations at which HGF strongly suppressed both cell growth and DNA synthesis (Table II). Moreover, the cytotoxicity was still less than 5% at concentration as high as 100 ng/ml HGF. Therefore, the anti-

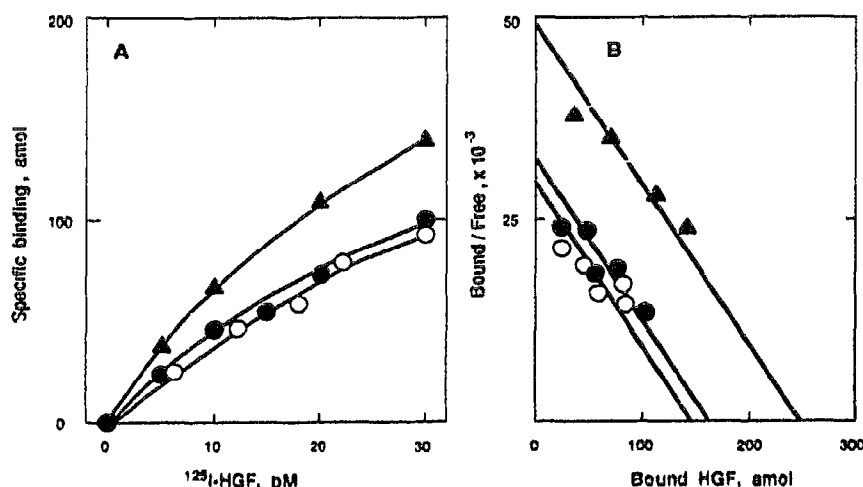


Fig. 3 Analysis of [^{125}I]HGF binding to the receptor on B6/F1, HepG2 and KB cells. Cells were incubated for 1 h at 10°C with ice-cold binding buffer containing increasing concentrations of [^{125}I]HGF with or without a 100-fold excessive concentration of unlabeled HGF. (A) Saturation curves of [^{125}I]HGF specific binding on cells exposed to increasing concentrations of [^{125}I]HGF are shown. (●) B6/F1 cells, (○) HepG2 cells and (▲) KB cells. (B) Scatchard plots of [^{125}I]HGF binding on these cells are shown. Symbols are the same as in (A).

proliferative effect of HGF on tumor cells is a cytostatic but not direct cytolytic effect, at physiological concentrations

4 DISCUSSION

HGF was seen to have a cytostatic effect on the growth of tumor cells. Because the half-maximal dose exhibiting growth inhibition is consistent with the K_d values (25–28 pM) of HGF receptor on tumor cells, cell growth inhibition induced by HGF may be functioning at physiological concentrations of HGF. Recently, Bottaro et al. reported that HGF receptor is identical to *c-met* proto-oncogene product [18]. As the *c-met* proto-oncogene encodes transmembrane-associated receptor tyrosine kinase, the primary event following HGF binding to its receptor seems to be autophosphorylation of the receptor. However, because these tumor cell lines express HGF receptor which is indistinguishable in affinity or the number from that of the normal cells, the bidirectional effects of HGF do not seem to be due to differences in HGF receptors between normal and tumor cells. It is likely that intracellular signalling pathways downstream from tyrosine phosphorylation of HGF receptor differ between normal and tumor cells.

More recently, Shiota et al. reported that the growth of FaO hepatocellular carcinoma cells stably transfected with an expression vector containing HGF cDNA linked to albumin promotor was markedly retarded compared to that of parental cells, and both tumorigenicity and size of tumors when implanted into nude mice were markedly decreased [19]. Taken together, these results indicate that HGF can inhibit growth of tumor cells both in vitro and in vivo.

Higashio et al. stated that tumor cytotoxic factor (TCF) purified from conditioned medium of IMR90 (human embryonic lung fibroblast) is identical or highly related to HGF [20], and Sone et al. found tumor cytolytic factor (TCF) is produced by human alveolar macrophages and blood monocytes [21,22]. They examined the cytotoxic effect of HGF/TCF at extremely higher concentrations. HGF has no significant cytolytic activity, rather it has cytostatic effects at physiological concentrations.

In our previous study, we noted that HGF inhibited the growth of normal human keratinocytes cultured under conditions of low Ca^{2+} (0.1 mM) [13]. In the presence of low Ca^{2+} (0.1 mM), keratinocytes proliferate rapidly, while the terminal differentiation of cells is suppressed in undifferentiated states, in contrast to cells cultured under physiological Ca^{2+} (1.8 mM). Since keratinocytes in low Ca^{2+} seems to have properties similar to those of tumor cells, growth inhibitory effects of HGF may be also present in normal cells when the growth is extraordinarily promoted without undergoing a normal differentiation.

HGF is a potent mitogen for mature hepatocytes in primary culture and seems to have an important role as

hepatotropic factor in liver regeneration after hepatic insult [6–10]. In addition to stimulating of cell growth and tissue repair, HGF has an antiproliferative effect on several tumor cells, and normal cells, under extraordinary conditions. Therefore, HGF may function as a negative regulator to suppress growth of tumor cells or normal cells during hyperplasia. HGF has an important role to sustain homeostasis of tissue organization by its bidirectional effects on cell growth. The antiproliferative effect of HGF on tumor cells suggests that this factor may be a putative anti-tumor agent.

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REFERENCES

- [1] Nakamura, T., Nawa, K. and Ichihara, A. (1984) *Biochem Biophys Res Commun* 122, 1450–1459.
- [2] Nakamura, T., Teramoto, H. and Ichihara, A. (1986) *Proc Natl Acad Sci USA* 83, 6489–6493.
- [3] Nakamura, T., Nawa, K., Ichihara, A., Kaise, N. and Nishino, T. (1987) *FEBS Lett* 224, 311–316.
- [4] Nakamura, T., Nishizawa, T., Hagiya, M., Seki, T., Shimonishi, M., Sugiyama, A., Tashiro, K. and Shimizu, S. (1989) *Nature* 342, 440–443.
- [5] Tashiro, K., Hagiya, M., Nishizawa, T., Seki, T., Shimonishi, M., Shimizu, S. and Nakamura, T. (1990) *Proc Natl Acad Sci USA* 87, 3200–3204.
- [6] Kinoshita, T., Tashiro, K. and Nakamura, T. (1989) *Biochem Biophys Res Commun* 174, 831–838.
- [7] Noji, S., Tashiro, K., Kayama, E., Nohno, T., Oyama, K., Taniguchi, S. and Nakamura, T. (1990) *Biochem Biophys Res Commun* 173, 42–47.
- [8] Asami, O., Ihara, I., Shimizu, N., Shimizu, S., Tomita, Y., Ichihara, A. and Nakamura, T. (1991) *J Biochem* 109, 8–13.
- [9] Konishita, T., Hirao, S., Matsumoto, K. and Nakamura, T. (1991) *Biochem Biophys Res Commun* 177, 330–335.
- [10] Shimizu, I., Ichihara, A. and Nakamura, T. (1991) *J Biochem* 109, 14–18.
- [11] Higuchi, O. and Nakamura, T. (1991) *Biochem Biophys Res Commun* 176, 599–607.
- [12] Igawa, T., Kanda, S., Kanetake, H., Saitoh, Y., Ichihara, A., Tomita, Y. and Nakamura, T. (1991) *Biochem Biophys Res Commun* 174, 831–838.
- [13] Matsumoto, K., Hashimoto, K., Yoshikawa, K. and Nakamura, T. (1991) *Exp Cell Res* 196 (in press).
- [14] Matsumoto, K., Tajima, H. and Nakamura, T. (1991) *Biochem Biophys Res Commun* 176, 45–51.
- [15] Gherardi, E. and Stoker, M. (1990) *Nature* 346, 228.
- [16] Stoker, M. and Gherardi, E. (1991) *Biochem Biophys Acta* 1072, 81–102.
- [17] Furlong, R.A., Takehara, T., Taylor, W.G., Nakamura, T. and Rubin, J.S. (1991) *J Cell Sci* (in press).
- [18] Bottaro, D.P., Rubin, J.S., Faleto, D.L., Chan, A.M.I., Kmieciak, T.E., VandeWoude, G.F. and Aaronson, S.A. (1991) *Science* 251, 802–804.
- [19] Shiota, G., Rhoads, D.B., Nakamura, T. and Schmidt, E.V. (1991) *Proc Natl Acad Sci USA* (in press).
- [20] Higashio, K., Shima, N., Goto, M., Itagaki, Y., Nagao, M., Yasuda, H. and Morinaga, T. (1990) *Biochem Biophys Res Commun* 170, 397–404.
- [21] Sone, S., Tachibana, K., Ishii, K., Ogawara, M. and Tsubura, E. (1984) *Cancer Res* 44, 646–651.
- [22] Sone, S., Lopez-Berestein, G. and Fidler, I.J. (1986) *Cancer Immunol Immunother* 21, 93–99.