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HEPATOCYTE GROWTH FACTOR STIMULATES GROWTH OF HEMATOPOIETIC PROGENITOR CELLS

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SUMMARY: Hepatocyte growth factor (HGF), a mesenchymal-derived polypeptide, stimulates growth, motility and morphogenesis of various types of cells, most predominantly of epithelial cells. We have now identified an additional biological activity of HGF; this factor probably has a role in early hematopoiesis. We examined the effects of HGF on the growth of various murine hematopoietic progenitor tumor cell lines and found that HGF stimulated DNA synthesis in the myeloid leukemia cell line, NFS-60. The mitogenic effect of HGF on NFS-60 cells was additive with the effect of interleukin 3 (IL-3). On the other hand, HGF had no apparent effect on other myeloid leukemia cell lines examined, such as DA-3 and FDC-P1 cells. Scatchard analysis of specific binding of [^{125}I]HGF revealed expression of a high-affinity HGF receptor on NFS-60 and DA-3 cells, but not on FDC-P1 cells. Expression of *c-met* mRNA correlated well with the existence of a high-affinity HGF receptor. Since the myeloid leukemia cell lines used are cells in the early stage of myeloid differentiation, HGF may play a role in early hematopoiesis.

Hepatocyte growth factor (HGF) was first demonstrated as a mitogen for adult rat hepatocytes in primary culture present in the serum of partially hepatectomized rats (1) and in rat platelets (2), and purified from rat platelets as a disulfide-linked heterodimer composed of a 69 kDa α-chain and a 34 kDa β-chain

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Abbreviations: HGF, hepatocyte growth factor; IL-3, interleukin-3; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor.

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(3,4). Molecular cloning of HGF cDNA revealed that HGF is derived from a single-chain precursor with a structure similar to that of plasminogen in its N-terminal four kringle domains and C-terminal serine protease-like domain (5-8). Until recently HGF was thought to have narrow target cell specificity and to act only as a mitogen for liver regeneration, but there is now evidence that HGF has multiple biological activities, depending on target cell types (reviewed in refs. 9-11). HGF has mitogenic activity for a wide variety of cells, such as hepatocytes, renal-tubular epithelial cells (12), epidermal keratinocytes (13) and melanocytes (14), whereas it inhibits growth of tumor cells, including hepatoma, melanoma, and squamous cell carcinoma (15,16). Additionally, HGF markedly enhances cell motility, which results in scattering of cohesive epithelial cells (17-19). Moreover, HGF acts as a morphogen inducing tubule formation of renal epithelial cells in collagen gels (20).

A high-affinity HGF receptor with a Kd value of 20-30 pM is widely distributed in adult rat tissues and in various epithelial cells (21-23). The HGF receptor was recently found to be the *c-met* proto-oncogene product, a member of the tyrosine kinase receptor family (24-26). Transfection of *c-met* cDNA in COS-7 cells is linked to expression of the high-affinity HGF receptor and accessibility of mitogenic stimulus induced by HGF (26).

Whereas it is generally accepted that the principal targets for HGF are epithelial cells, our extensive studies on cell distribution of high-affinity HGF receptor, by monitoring the specific binding of [^{125}I]-labeled HGF, showed that a limited number of hematopoietic cells express the high-affinity HGF receptor, therefore expanding the putative target cells for HGF (23). We found that HGF stimulates the growth of J-111 (human monocytic leukemia) and CCRF-CEM (human T-lymphoblasts) cells, and inhibits the growth of IM-9 (human B-lymphoblasts) cells (23). In addition, expression of *c-met* mRNA in several hematopoietic progenitor cell lines suggested a role for HGF in early hematopoiesis (27). To further address this issue, we examined the mitogenic effect of HGF on murine hematopoietic progenitor cell lines and analyzed the HGF receptor present on these cells. We obtained evidence for the expression of a high-affinity HGF receptor on several hematopoietic progenitor cell lines and the mitogenic effect of HGF on one of these lines, NFS-60. These findings indicate that HGF is involved in early hematopoiesis.

MATERIALS AND METHODS

Cell culture and growth factors: The origin of most of cells used in this study is given in Table 1. NFS-60, NFS-107, DA-1, DA-3, and FDC-P1 cells were isolated from primary retrovirus-induced leukemias and maintained as described elsewhere (28,29). IL-3 was obtained from conditioned medium of WEHI-3 cells (30). Human recombinant HGF was purified from the culture medium of C-127 cells transfected with plasmid containing human HGF cDNA (5,7).

Assay for DNA synthesis: To examine DNA synthesis, the cells were adjusted to a density of 5 x 10^5 cells/ml on 96-well plates and cultured in RPMI-1640 medium containing an appropriate amount of HGF in the presence or absence of 20 units/ml of IL-3. After 24 h of culture, the cells were pulse-labeled with 0.3 μCi/ml [^{125}I]deoxyuridine (2200 Ci/mmol, New England Nuclear) for 8 h. These cells were washed with phosphate-buffered saline (PBS) and then with ice-cold 10%(w/v) trichloroacetic acid. The cells were solubilized with 1 N NaOH and the radioactivity incorporated into DNA was counted in a γ-counter.

Analysis of [^{125}I]HGF binding to the receptor: Recombinant human HGF was radioiodinated by the chloramine-T method as described elsewhere (21). The [^{125}I]HGF we used had a specific activity of 70-160 μCi/μg protein. Cells (1 x 10^6 cells/tube) were preincubated at 10°C for 30 min with binding buffer (Hanks' solution containing 20 mM Hepes, 2 mg/ml bovine serum albumin, pH 7.0). After spinning down, ice-cold binding buffer (100 μl) containing various concentrations of [^{125}I]HGF, with or without a 100-fold excess amount of unlabeled HGF was added to the cells and the preparation was incubated at 10°C for 1 h (21). The cells were overlaid onto an oil cushion composed of di-*n*-butyl phthalate and di(2-ethylhexyl)-phthalate (3:2) and centrifuged for 5 min at 12,000g at 4°C. After discarding the aqueous and oil phases [^{125}I]HGF specifically bound to the cell pellets was counted in a γ-counter.

Northern blot analysis: Total RNA was isolated from cell lines using the guanidinium/cesium chloride method, as described (27). Poly(A)$^+$ RNA was isolated using an oligo-dT cellulose column. Northern blot analysis was performed as described (27). About 10 μg of poly(A)$^+$ RNA were electrophoresed on 1.2% formaldehyde agarose gel and transferred to nitrocellulose. Hybridization was done with a [^{32}P]-labeled 6.7 kb XhoI fragment of mouse *c-met* cDNA (27).

RESULTS

Effects of HGF on DNA synthesis in hematopoietic progenitor cell lines: We first examined the effects of HGF on DNA synthesis in various IL-3-dependent murine myeloid progenitor tumor cell lines. As shown in Fig. 1A, HGF dose-dependently stimulated DNA synthesis of one of these lines, NFS-60, under conditions of the absence of IL-3. Maximal stimulation was seen with 120 pM HGF and this corresponds to the concentration required for proliferation of other HGF responsive cells. This growth stimulatory effect of HGF on NFS-60 cells was also observed additively, even in the presence of 20 units/ml of IL-3, but in the presence of IL-3 the maximal stimulation was seen with 30-60 pM HGF and the stimulatory effect of HGF was not so marked at 120 pM. On the other hand, HGF had no apparent effect on the DNA synthesis of other IL-3-dependent myeloid leukemia cell lines examined, such as DA-3, DA-1, FDC-P1, and NFS-107 cells, under conditions of either the presence or absence of IL-3, although IL-3 did stimulate DNA synthesis in these cell lines (Fig. 1B, 1C, and Table 1).

Analysis of HGF receptor in hematopoietic progenitor cell lines: As the proliferative action of HGF seems to be transduced by the cell surface HGF

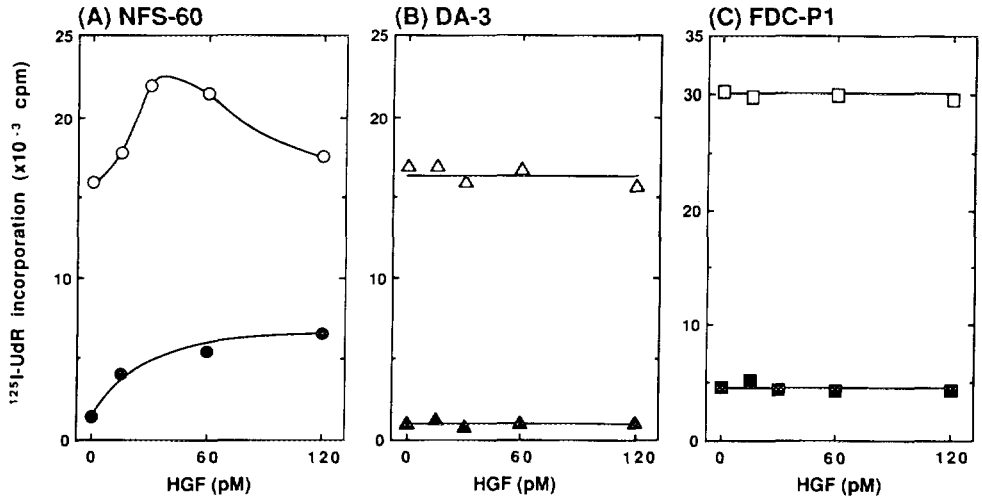

Fig. 1. Effects of HGF on the DNA synthesis of hematopoietic progenitor tumor cell lines. (A) NFS-60, (B) DA-3, and (C) FDC-P1 cells. Cells were cultured for 24 h with appropriate amounts of HGF in the presence (open symbol) or absence (closed symbol) of 20 units/ml IL-3 and pulse-labeled with [^{125}I]iododeoxyuridine for 8 h. Data represent the mean of triplicate measurements.

receptor, we next examined specific binding of [^{125}I]-labeled HGF to these hematopoietic progenitor cell lines. Scatchard plot analysis of the specific binding of [^{125}I]HGF to NFS-60 cells revealed the presence of two classes of receptors of high and low affinity (Fig. 2A). A high-affinity receptor had a Kd value of 47 pM, which is close to the half-maximal dose of HGF in stimulating DNA synthesis of NFS-60 cells and to the values of high-affinity HGF receptor on other HGF responsive cells, such as rat hepatocytes and renal tubular epithelial cells (21,23). The number of high-affinity binding sites per cell is 22, a value much smaller

Table 1. Expression of c-met mRNA and a high-affinity HGF receptor and response to HGF of IL-3 dependent myeloid leukemia cell lines

Cell line	Origin	Expression		Mitogenic response to HGF
		c-met mRNA	high-affinity HGF receptor	
NFS-60	(NFS x DBA/2)F1 myeloid leukemia	+	+	+
DA-3	BALB/c splenic lymphoma	+	+	-
DA-1	BALB/c splenic lymphoma	+	N.D.	-
FDC-P1	DBA/2 myeloid leukemia	-	-	-
NFS-107	NFS myeloid leukemia	-	N.D.	-

N.D., not determined.

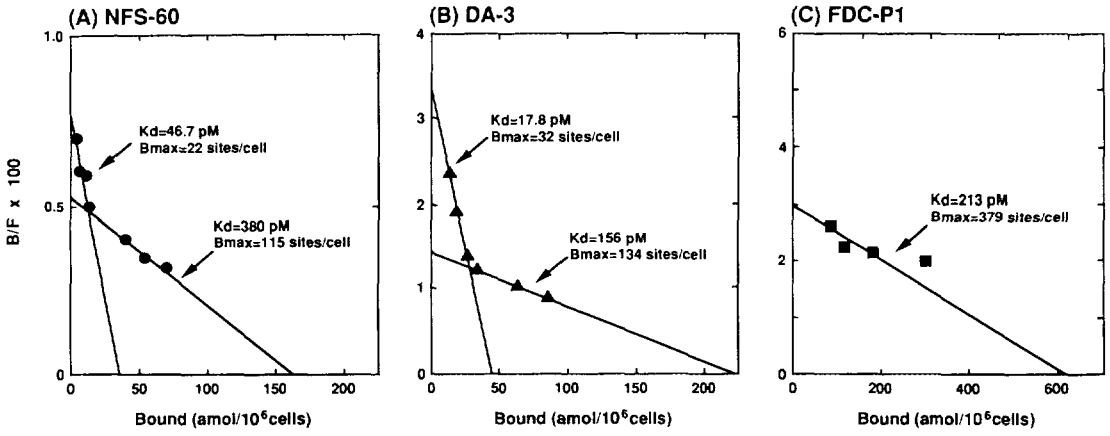

Fig. 2. Scatchard plot analysis of specific binding of [^{125}I]HGF to hematopoietic progenitor cell lines. (A) NFS-60, (B) DA-3, and (C) FDC-P1 cells. Cells were incubated for 1 h at 10°C with binding buffer containing increasing concentrations of [^{125}I]HGF with or without a 100-fold excess concentration of unlabeled HGF. Data represent the mean of triplicate measurements.

than that detected in various epithelial cells. The low-affinity receptor had a Kd value of 380 pM and the number of low-affinity binding sites per cell was 115. Scatchard analysis of the specific binding of [^{125}I]HGF for DA-3 and FDC-P1 cells, which do not respond to HGF, revealed that DA-3 cells express both high and low affinity binding sites, with a Kd value of 18 pM and 156 pM respectively (Fig. 2B), and FDC-P1 cells express only the low-affinity HGF receptor with a Kd value of 213 pM (Fig. 2C). It is noted that DA-3 cells, which do not respond to HGF, express a high-affinity receptor for HGF.

To examine the correlation between the expression of *c-met* mRNA and the expression of high-affinity HGF receptor, poly(A)$^+$ RNAs from various murine myeloid progenitor tumor cell lines were subjected to Northern blot analysis (Fig. 3). Some of these cell lines, including NFS-60, DA-3, DA-1, DA-34, and NFS-124 cells, expressed about 7.5-kb *c-met* mRNA, whereas the mRNA was not detected in cell lines such as FDC-P1 and NFS-107. Since the high-affinity HGF receptor was detected in NFS-60 and DA-3, but not in FDC-P1 cells, the expression of *c-met* mRNA seems to correlate well to the existence of high-affinity HGF receptors. Table 1 summarizes the expression of a high-affinity HGF receptor and *c-met* mRNA as well as the mitogenic response to HGF of several myeloid progenitor cell lines.

DISCUSSION

Self-renewal and differentiation into a variety of hematopoietic lineages of myeloid progenitor cells are regulated by the microenvironment and by several polypeptide factors produced by bone marrow stromal cells. As HGF is produced

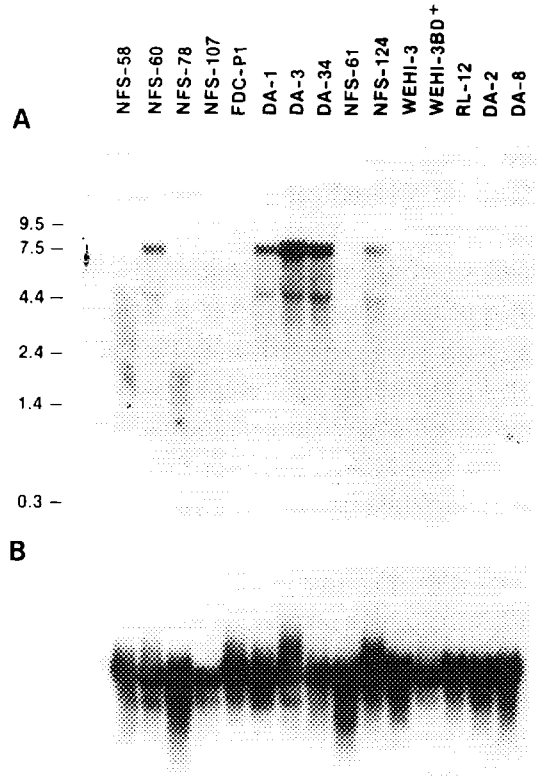

Fig. 3. Northern blot analysis of *c-met* mRNA in hematopoietic progenitor cell lines. (A) Ten micrograms of poly(A)$^+$ RNA purified from cells was electrophoresed on a 1.2% formaldehyde agarose gel, transferred to nitrocellulose, and hybridized with radiolabeled mouse *c-met* cDNA as a probe. (B) Same blot probed with control actin.

by fibroblasts, macrophages, and endothelial cells, all constituents of stromal cells, and because the *c-met*/HGF receptor mRNA is expressed in several murine myeloid cell lines, we entertained the notion that HGF may be a candidate for hematopoietic regulatory factors. We obtained the first evidence that HGF has mitogenic activity on one of the myeloid progenitor cell lines, NFS-60, and a high-affinity HGF binding site is expressed in several myeloid leukemia cell lines, including NFS-60. Since the cell lines represent relatively early stages of myeloid differentiation, these findings suggest that HGF plays a role in early hematopoiesis. Kmiecik *et al.* have just reported that the *c-met* mRNA and protein is expressed in the progenitor-enriched (lineage negative [lin$^-$]) murine bone marrow cells and that HGF has a synergistic effect with IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF) to stimulate colony formation of lin$^-$ bone marrow cells (31). These results further support the notion that HGF is a hematopoietic modulator in early hematopoietic process.

Kmiecik *et al.* also reported that HGF synergized with IL-3 to stimulate DNA synthesis in NFS-60 cells while HGF alone was inactive (31). Their finding differs from ours described in the present paper. While we have no definite explanation for the discrepancy, we did consistently observe the mitogenic activity of HGF alone on NFS-60 cells, in several independent experiments in two different laboratories.

Scatchard analysis of specific binding of [^{125}I]HGF on various cell lines revealed the presence of two classes of HGF receptors of high and low affinity. Low-affinity binding sites were detected in all the cell lines we examined. As the binding of HGF to a low-affinity HGF receptor was almost displaced by heparin (32), and the number of low-affinity binding sites was relatively low in hematopoietic cells, the low-affinity receptor for HGF probably is a heparin-like proteoglycan in the extracellular matrix. In contrast, a high-affinity HGF receptor was detected in myeloid cell lines such as NFS-60 and DA-3 cells, but not in others such as FDC-P1 cells. Expression of a high-affinity HGF receptor correlated well with the expression of *c-met* mRNA, as measured by Northern blot analysis. Since the Kd value of the high-affinity HGF receptor is in good accord with the half-maximal dose for HGF activities, the binding of HGF to the high-affinity receptor, but not to the low-affinity binding sites, seems specifically linked to the signal transduction of HGF.

It is to be noted that several myeloid leukemia cell lines (DA-3, DA-1 *etc.*) except for NFS-60 cells, all of which express *c-met* mRNA, do not mitogenically respond to HGF. Similar results were obtained for the response to granulocyte colony-stimulating factor (G-CSF). Among the various myeloid cell lines expressing the receptor for G-CSF, only NFS-60 cells were found to proliferate in response to G-CSF (33). It is likely that an unknown molecule that is involved in the mitogenic signal transduction through *c-met* and G-CSF receptor is expressed in NFS-60 cells, but not in other myeloid leukemia cell lines. Since these cell lines proliferate in response to IL-3, it is speculated that this unknown molecule does not participate in signaling pathway through IL-3 receptor. Another explanation is that activation of the *c-met* receptor of these myeloid cell lines except for NFS-60 cells may stimulate cell motility, as is the case for MDCK (Mardin-Darby canine kidney epithelia) cells (23), or cell survival, as found for PC-12 rat pheochromocytoma cells (unpublished data), in place of stimulation of growth. Studies on mechanisms of distinct responses between NFS-60 and other myeloid cell lines and on the action of HGF on normal bone marrow progenitor cells are expected to elucidate the roles of HGF in hematopoiesis.

We have data that the HGF level in sera of patients with aplastic anemia is low compared with that of healthy controls (unpublished data). This too suggests a role for HGF in human hematopoiesis and hematopoietic disorders. HGF may prove to be an effective agent to overcome the decrease in bone marrow cells seen in patients on anti-cancer drugs such as mitomycin C and vinblastine.

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