



# Hepatocyte growth factor levels in bone marrow plasma of patients with leukaemia and its gene expression in leukaemic blast cells

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**Summary** Hepatocyte growth factor (HGF) has been known as a multiple function factor, which also stimulates early haematopoiesis. In this study, we found that HGF was expressed at both the RNA and protein levels in acute myeloid leukaemia (AML) and chronic myeloid leukaemia (CML). In patients with AML ( $n = 20$ ) and CML ( $n = 5$ ), bone marrow plasma HGF concentrations were  $20.44 \pm 6.26$  (mean  $\pm$  s.e.)  $\text{ng ml}^{-1}$  and  $7.17 \pm 0.53 \text{ ng ml}^{-1}$  respectively. These were significantly higher ( $P < 0.01$ ) than the value for normal subjects ( $n = 26$ ): mean  $0.92 \pm 0.09 \text{ ng ml}^{-1}$ . Constitutive HGF production was observed in freshly prepared leukaemic blast cells from three patients with high HGF levels of bone marrow plasma. Expression of HGF mRNA was correlated with bone marrow plasma HGF levels. After complete remission was obtained in six patients, bone marrow plasma HGF levels were significantly decreased. In contrast, the HGF mRNA was less abundantly expressed in acute lymphoid leukaemia (ALL). In patients with ALL ( $n = 5$ ), bone marrow plasma HGF concentration ( $0.69 \pm 0.14 \text{ ng ml}^{-1}$ ) remained low within the value for normal subjects. These results suggest that some populations of myeloid lineage cells have the ability to produce HGF.

**Keywords:** hepatocyte growth factor; leukaemia; bone marrow

Hepatocyte growth factor (HGF), also known as a scatter factor (Weidner *et al.*, 1990), was initially identified as a mitogen for primary cultured adult rat hepatocytes, from rat platelets (Russell *et al.*, 1984) and from the plasma of patients with fulminant hepatic failure (Gohda *et al.*, 1986). It was purified from rat platelets as a disulphide-linked heterodimeric molecule composed of a 69 kDa  $\alpha$ -chain and a 34 kDa  $\beta$ -chain (Nakamura *et al.*, 1986, 1987). Molecular cloning of HGF cDNA revealed that both chains of HGF are encoded in a single gene (Miyazawa *et al.*, 1989; Nakamura *et al.*, 1989; Seki *et al.*, 1990; Tashiro *et al.*, 1990). Accumulating evidence indicates that, in addition to its effect on hepatocytes, HGF is a unique multifunctional cytokine acting on a wide variety of cells as a mitogen (Igawa *et al.*, 1991; Kan *et al.*, 1991; Rubin *et al.*, 1991), a motogen (Gherardi *et al.*, 1989; Gherardi and Stoker, 1991), a morphogen (Montesano *et al.*, 1991), an angiogenic factor (Bussolino *et al.*, 1992; Grant *et al.*, 1993) and a tumour cytotoxic factor (Higashio *et al.*, 1990; Tajima *et al.*, 1991). Indeed, HGF mRNA is expressed in various tissues, including kidney (Tashiro *et al.*, 1990; Nagaïke *et al.*, 1991), heart (Tashiro *et al.*, 1990), lung (Tashiro *et al.*, 1990; Yanagita *et al.*, 1992, 1993) and brain (Tashiro *et al.*, 1990), as well as in injured liver (Kinoshita *et al.*, 1989).

Previous reports have shown that HGF stimulates growth of haematopoietic progenitor cells derived from mouse (Kmieciak *et al.*, 1992; Nishino *et al.*, 1995) and human (Galimi *et al.*, 1994). Of particular interest is HGF receptor (HGFR)/c-met mRNA expression (Bottaro *et al.*, 1991; Naldini *et al.*, 1991a,b; Giordano *et al.*, 1993) has been reported in several murine haematopoietic progenitor cell lines (Kmieciak *et al.*, 1992; Mizuno *et al.*, 1993) and human haematopoietic progenitor cells (Galimi *et al.*, 1994). Recently, we reported that the human promyelocytic leukaemia cell line, HL-60, produces HGF (Nishino *et al.*, 1991; Inaba *et al.*, 1993). More recently, Nakamura *et al.* (1994a) reported that a high level of HGF was detected in blood and bone marrow plasma of leukaemia patients. These

data strongly suggest the possibility that HGF may play an important role in haematopoiesis and that it may act as an autocrine or paracrine growth factor in the development of leukaemia.

In this study, we determined HGF production and its gene expression in leukaemic blast cells from myeloid leukaemic patients and the drastic diminution of HGF levels in bone marrow plasma by inducing remission.

## Materials and methods

### Patients

Thirty patients with leukaemia were studied. Mean age was 49.1 years, with a range from 21 to 75. The diagnosis was based on cell morphology and genetic markers. The clinical data for the patients are shown in Table I. The subtypes and numbers of cases were: 20 cases of acute myeloid leukaemia (AML) [M1 ( $n = 3$ ), M2 ( $n = 4$ ), M3 ( $n = 4$ ), M4 ( $n = 3$ ), M5 ( $n = 2$ )], four cases of myeloid leukaemic transformation from myelodysplastic syndrome (LT-MDS), five cases of chronic myeloid leukaemia (CML) and five cases of acute lymphoid leukaemia (ALL). Liver function was normal in all cases. Samples were also obtained from four patients with iron deficiency anemia (IDA) and 26 haematologically normal subjects without any disease. All samples were taken with informed consent.

### Determination of HGF concentration

Bone marrow samples were obtained in polypropylene tubes containing disodium ethylenediaminetetraacetate (EDTA) from iliac bones by aspiration; they were immediately centrifuged at 4°C. The supernatants were then stored at -40°C until assayed. HGF concentrations in blood and bone marrow plasma were determined by using enzyme-linked immunosorbent assay (ELISA) as described previously (Nishino *et al.*, 1991). Briefly, standard human HGF or samples with unknown concentrations of HGF were dispensed into a 96-well microtitre plate coated with a monoclonal antibody against human HGF. After incubation for 1 h, it was washed three times with phosphate-buffered saline plus 0.05% Tween-20 (PBS-T). After the addition of a 0.1 ml aliquot of a polyclonal antibody against human HGF,

**Table I** Clinical features of patients and bone marrow plasma HGF concentrations

<i>FAB classification</i>	<i>Case</i>	<i>Age/Sex</i>	<i>Bone marrow plasma HGF concentration (ng ml<sup>-1</sup>)</i>
<b>AML</b>			
AML M1	M01	64/M	30.33
AML M1	M06	58/M	0.56
AML M1	M17	44/F	0.62
AML M2	M03	38/F	15.61
AML M2	M04	34/M	3.19
AML M2	M05	51/M	1.34
AML M2	M16	25/F	2.99
AML M3	M10	49/M	83.40
AML M3	M11	57/F	10.96
AML M3	M12	44/M	20.00
AML M3	M15	61/M	15.20
AML M4	M02	54/F	7.12
AML M4	M13	29/F	44.00
AML M4	M18	44/M	4.52
AML M5	M19	58/F	102.6
AML M5	M20	23/M	4.64
LT-MDS	M07	62/M	6.12
LT-MDS	M08	55/M	14.70
LT-MDS	M09	71/F	39.15
LT-MDS	M14	64/M	1.72
<b>ALL</b>			
ALL L1	L02	75/F	0.20
ALL L1	L03	57/M	0.86
ALL L1	L04	42/M	0.74
ALL L1	L05	58/M	0.64
ALL L3	L01	66/M	1.01
<b>CML</b>			
CML	CM01	51/F	6.45
CML	CM02	22/F	7.06
CML	CM03	41/F	8.09
CML	CM04	64/F	8.58
CML	CM05	21/M	5.68

the plate was incubated for 1 h, then washed three times with PBS-T. After the addition of 0.2 ml of diluted goat (anti-rabbit immunoglobulin) IgG-peroxidase conjugate, the plate was incubated for 1 h and then washed. An aliquot (0.1 ml) of 0.25% *o*-phenylenediamine was added and the plate allowed to stand for 10 min. After the reaction was stopped by the addition of 0.1 ml of 1.0 N sulphuric acid, the absorbance was measured at 492 nm by an automatic plate reader with a reference wavelength of 690 nm. The detection limit of this assay is 0.10 ng ml<sup>-1</sup>.

#### Northern blot analysis

Mononuclear cells were prepared from heparinised fresh peripheral blood or bone marrow samples by density gradient centrifugation on Ficoll-Metrizoate (Nyegaard, Norway; density = 1.077 g ml<sup>-1</sup>). Total cellular RNA was extracted from these cells by the acid guanidium-thiocyanate phenol-chloroform method (Chomczynski and Sacchi, 1987). For Northern blot analysis, 20 µg of total RNA was separated by electrophoresis on 1% agarose gel containing formaldehyde, transferred to nylon membranes (Hybond-N, Amersham International, Buckinghamshire, UK) by capillary action, and fixed. Human HGF (Nishino *et al.*, 1991) or rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Fort *et al.*, 1985) cDNA were labelled with [ $\alpha$ -<sup>32</sup>P]dCTP (sp. act. 111 TBq mmol<sup>-1</sup>; NEN Research Products, Boston, MA, USA) using hexadeoxynucleotide random primers (Amersham International). The membranes were hybridised with <sup>32</sup>P-labelled HGF or GAPDH cDNA as probes in 50% formamide, 3 × SSC (1 × SSC, 0.15 M sodium chloride plus 0.015 M sodium citrate, pH 7.4), 50 mM Tris-HCl (pH 7.5), 0.1% sodium dodecyl sulphate (SDS), 20 µg ml<sup>-1</sup> tRNA, 20 µg ml<sup>-1</sup> boiled salmon sperm DNA, 1 mM EDTA and 1 × Denhardt (0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone and 0.02% Ficoll) for 40 h at 37°C. The nylon membranes were washed with 2 × SSC, 1% SDS, and

1 × Denhardt; at 37°C for 1 h, followed by 0.1 × SSC and 1% SDS at 50°C for 1 h and then autoradiographed using intensifying screens at -80°C.

#### Primary cell culture

Mononuclear cells from peripheral blood or bone marrow samples were inoculated at 2 × 10<sup>6</sup> cells per ml in RPMI-1640 medium (Flow Laboratories, Irvine, UK) supplemented with 10% heat-inactivated fetal calf serum (FCS; Gibco, Grand Island, NY, USA) in 5% carbon dioxide-water-saturated atmosphere at 37°C. The culture supernatant was collected every day for the measurement for its HGF levels. HGF concentrations were determined by using ELISA.

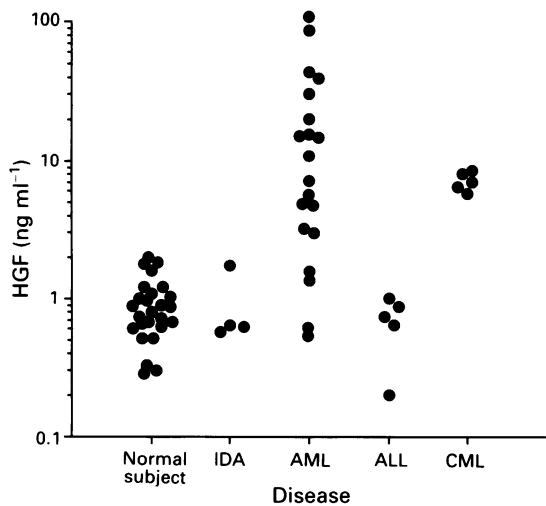
#### Statistics

All data were presented as means ± standard error (s.e.). Statistical analysis of HGF concentrations among the series of patients was performed using one-way analysis of variance (ANOVA) and multiple comparison (Scheffe type) for the assessment of means. HGF concentrations on the initial leukaemic state and the remission state were compared with the two-tailed Student's *t*-test for paired data. Differences were considered to be significant when *P*-values were less than 0.05. Analysis was carried out using the Stat View program (Abacus Concepts, Berkeley, CA, USA).

#### Results

##### Determination of HGF levels in bone marrow plasma and peripheral blood

HGF concentrations in bone marrow plasma are summarised in Figure 1. In bone marrow plasma from 26 normal subjects, HGF level was 0.92 ± 0.09 (mean ± s.e.) ng ml<sup>-1</sup> with a



**Figure 1** Bone marrow plasma HGF concentrations in normal subjects ( $n = 26$ ), patients with IDA ( $n = 4$ ), and patients with leukaemia (AML,  $n = 20$ ; ALL,  $n = 5$ ; CML,  $n = 5$ ). HGF concentrations were determined by ELISA as described in Materials and methods.

range from 0.29 to 1.99 ng ml<sup>-1</sup>. In patients with AML ( $n = 20$ ) and CML ( $n = 5$ ), HGF concentrations were  $20.44 \pm 6.26$  ng ml<sup>-1</sup> (range from 0.56 to 102.6 ng ml<sup>-1</sup>) and  $7.17 \pm 0.53$  ng ml<sup>-1</sup> (range from 5.68 to 8.58 ng ml<sup>-1</sup>) respectively. These values were both significantly greater than that for normal subjects ( $P < 0.01$ ). HGF concentrations in patients with ALL ( $n = 5$ ) and IDA ( $n = 4$ ) were  $0.69 \pm 0.14$  ng ml<sup>-1</sup> (range from 0.20 to 1.01 ng ml<sup>-1</sup>) and  $0.90 \pm 0.29$  ng ml<sup>-1</sup> (range from 0.58 to 1.76 ng ml<sup>-1</sup>) respectively. HGF levels were significantly lower in peripheral blood than those in bone marrow plasma, as reflected by the comparison of HGF levels in both specimens obtained at the same time in nine cases (data not shown).

*Northern blot analysis*

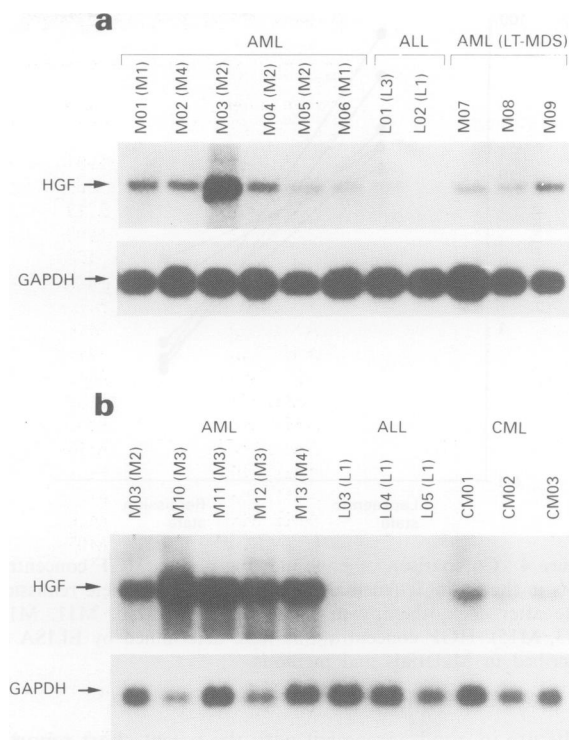
To confirm the possibility of HGF production in leukaemic blast cells from AML and CML patients, Northern blot analysis of total cellular RNA from leukaemic blast cells using a human HGF cDNA as a probe was performed. Figure 2 shows a single band of 6.0 kb HGF transcript in patients with AML and CML. Supporting the specificity of HGF production for the cells in the myeloid lineage, very low expression of the HGF transcript was obtained from ALL patients. Basal levels of HGF mRNA, as semiquantitated by laser densitometer, broadly correlated with its protein levels in bone marrow plasma (data not shown).

*HGF production by fresh leukaemic blast cells*

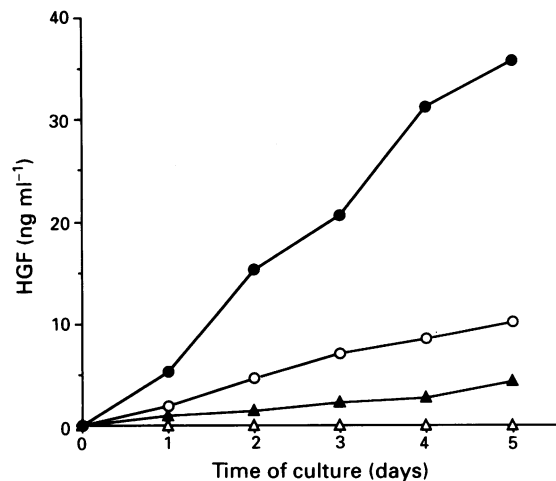
To further elucidate the HGF production by leukaemic blast cells, mononuclear cells, obtained from bone marrow and peripheral blood of four cases (M06, M10, M13, CM03), were cultured for 5 days. Figure 3 shows the HGF production of fresh leukaemic blast cells. The cells from three cases (M10, M13, CM03) produced HGF in a time-dependent manner. Furthermore, the rates of HGF production in these patients were correlated with their HGF levels in bone marrow plasma (data not shown).

*Diminution of HGF levels in bone marrow plasma by induction of remission*

We also examined the change of HGF levels by inducing remission. In six patients (M03, M10, M11, M12, M13, M15) with AML, morphologically complete remission was obtained after chemotherapy. Figure 4 shows a comparison of bone marrow HGF levels between the leukaemic and the



**Figure 2** Northern blot analysis of the human HGF mRNA in fresh leukaemic blast cells. Total RNAs (20  $\mu$ g) prepared from patients with leukaemia were electrophoresed, transferred and hybridised with human HGF cDNA or rat GAPDH cDNA as probes. The various lanes contained RNAs extracted from the indicated samples. (a) Cases M01, M02, M03, M04, M05, M06, M07, M08, M09 (AML) and L01, L02 (ALL). (b) Cases M03, M10, M11, M12, M13 (AML), L03, L04, L05 (ALL), and CM01, CM02, CM03 (CML).

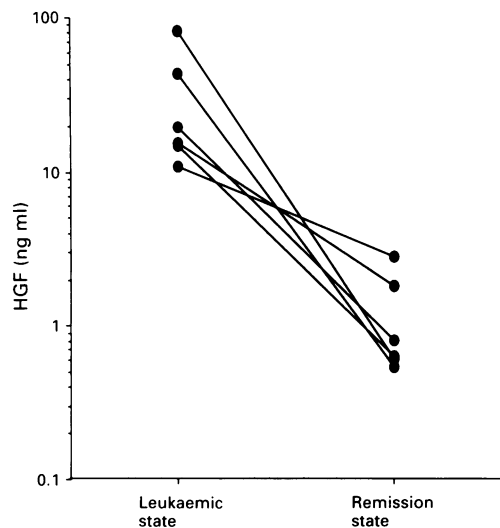


**Figure 3** Continuous HGF production of fresh leukaemic blast cells. Mononuclear cells from peripheral blood or bone marrow samples of patients with leukaemia were cultured at a starting cell density of  $2 \times 10^6$  cells/ml<sup>-1</sup> for 5 days. The amount of HGF in cultured medium was measured by ELISA as described in Materials and methods. The cases are: M10 (●), M13 (○), CM03 (▲), M06 (△).

remission state. Bone marrow HGF levels were significantly decreased in the remission state.

**Discussion**

In the present study, we have shown that HGF levels in bone marrow plasma from AML and CML patients were significantly higher than those for normal subjects. These



**Figure 4** Comparison of bone marrow plasma HGF concentrations in the initial leukaemic state and in the complete remission state after chemotherapy in six cases (M03, M10, M11, M12, M13, M15). HGF concentrations were determined by ELISA as described in Materials and methods.

results are in good agreement with the recent short report by Nakamura *et al.* (1994a). We have not observed any obvious correlation between HGF production and FAB classification. As shown in Table I, there was a large individual variation in bone marrow HGF levels even in the same FAB class. However, high HGF levels were observed in all M3, M4, and M5 cases that we tested. As the reason for high HGF levels, we have shown that leukaemic blast cells from patients with AML and CML produced HGF constitutively. In support of this hypothesis, we have observed a significant diminution of HGF levels in bone marrow plasma after eradication of leukaemic blast cells by successful treatment. Furthermore, we have found that bone marrow HGF levels in patients with ALL remained low within the value for normal subjects. These results together strongly suggest that HGF was produced specifically by myeloid lineage leukaemia (AML or CML) cells, but not by lymphoid lineage leukaemia (ALL) cells. This hypothesis was further supported by Northern blot analysis suggesting the positive staining of 6.0 kb HGF transcripts in the cells from AML and CML patients but not from ALL patients (Figure 2). We have previously reported that HGF production is induced in the promyelocytic leukaemia cell line, HL-60, by 12-*o*-tetradecanoyl phorbol 13-acetate (TPA), but not by dimethyl sulphoxide (DMSO) (Nishino *et al.*, 1991). It is well known that HL-60 cells are differentiated into macrophages in the presence of TPA and into granulocytes in the presence of DMSO. Noji *et al.* (1990) have shown by *in situ* hybridisation that endothelial cells and

Kupffer cells are HGF-producing cells in damaged liver. Kupffer cells are macrophages that reside in the liver and belong to the myeloid lineage. Recently, we reported release of HGF from rheumatoid synovial fluid cells, which contains a large number of polymorphonuclear cells (Yukioka *et al.*, 1994b). These results suggest that some populations of myeloid lineage cells may produce HGF. This notion is further supported by the recent report (Nakamura *et al.*, 1994) demonstrating the constitutive production of HGF by myeloid lineage leukaemic cell lines (KCL-22, KG-1A and KG-1), although all myeloid leukaemic blast cells do not produce HGF.

Various cytokines are produced by leukaemic cells. Some cytokines stimulate autocrine growth of leukaemic blast cells. Several reports have shown that HGFR/*c-met* mRNA is expressed in murine myeloid progenitor cell lines (Kmieciak *et al.*, 1992; Mizuno *et al.*, 1993). Therefore, HGF may also play a role as an autocrine growth factor in leukaemic blast cells. To explore this possibility, we have investigated the expression of the HGFR/*c-met* gene in cases (M03, M09, M10, M11, M12, M13 as AML and CM01 as CML), poly A RNAs of bone marrow blast cells from patients with AML and CML were analysed. However, HGFR/*c-met*-specific mRNA was not detected in any case (data not shown). Furthermore, recombinant human HGF had no effect on proliferation of blast cells from patients with AML (data not shown). Jucker *et al.* (1994) have reported that HGFR/*c-met* mRNA is overexpressed in some cases of leukaemia and lymphoma. Since they have demonstrated that expression of the HGFR/*c-met* gene is detected in only 1 AML case out of 29 leukaemias, HGF still might be an autocrine growth factor in a few cases of AML. Kmieciak *et al.* (1992) have reported that HGFR/*c-met* mRNA and protein are expressed in the progenitor-enriched murine bone marrow cells and that HGF has a synergistic effect with interleukin 3 (IL-3) and granulocyte-macrophage colony-stimulating factor (GM-CSF) to stimulate colony formation of bone marrow cells. Galimi *et al.* (1994) have reported that HGFR/*c-met* is expressed in human CD34-positive haematopoietic progenitor cells and that HGF stimulates erythroid and multipotent progenitor cells in the presence of cytokines such as erythropoietin, IL-3 and GM-CSF. During embryonic development, haematopoiesis originates in the yolk sac, then moves to the liver and spleen and finally settles in the bone marrow. Of interest, HGF and/or HGFR/*c-met* are expressed in the fetal liver (Selden *et al.*, 1990; Hu *et al.*, 1993; Galimi *et al.*, 1994) and yolk sac (Chan *et al.*, 1988). Recently, Nishino *et al.* (1995) have reported that both HGF and HGFR/*c-met* mRNA are expressed in the mouse fetal liver in the middle and late stages when haematopoiesis is most active. These results suggest that HGF may be a modulator in early haematopoietic processes. Our findings and the findings of others indicating HGF production by some populations of myeloid lineage cells, HGF may have positive-feedback effects on the growth of haematopoietic progenitors.

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