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PROMYELOCYTIC LEUKEMIA CELL LINE, HL-60, PRODUCES HUMAN HEPATOCYTE GROWTH FACTOR

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Summary; The human promyelocytic leukemia cell line, HL-60, stimulated with PMA, produced human HGF-like immunoreactivity (HL-60 HGF), which was detected with human HGF-specific ELISA. The purified HL-60 HGF was indistinguishable from human HGF in the plasma of patients with fulminant hepatic failure by studies of subunit constitution and amino acid composition. The HL-60 HGF mRNA corresponded to 6 kb, which was consistent with previous reported data in rat and human HGF mRNA, was detected in stimulated HL-60, by northern hybridization analysis using human HGF cDNA probe. These findings indicated that HL-60 HGF was identical to, or closely resembled, human plasma HGF. The HL-60 cell is an attractive model for studies of HGF-producing mechanisms, the manner of secretion and the nature of induction signals.

Hepatocyte growth factor (HGF), which was isolated from rat platelets and the plasma of patients with fulminant hepatic failure (FHF), is a hetero-dimer molecule composed of a large 60kDa (α-chain) and a small 32 or 34kDa subunit (β-chain) (1) (2). HGF cDNA clones were isolated from rat liver and human placenta cDNA library (3) (4) (5). The entire amino acid sequence deduced from the cDNA sequence indicated that mature HGF molecule is derived proteolytically from pre-pro HGF, and that HGF has four kringles in its α-chain and is highly homologous to plasminogen. HGF, which strongly stimulates the DNA synthesis of adult rat hepatocyte in primary culture, was produced in regenerating liver induced by hepatotoxins (6) and in the plasma of a patient with FHF (7) (8). It has been suggested that this factor plays an important role in a system of controls for liver regeneration.

There is little evidence of an HGF-producing organ or cell. Platelets are one of the major HGF-storage sites. in rats but not in humans. Kinoshita et al. demonstrated that rat HGF mRNA increased markedly in the non-parenchymal liver cells of rats, but not in the parenchymal hepatocytes when experimental

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hepatitis was induced by hepatotoxins (9). Furthermore, they assumed that the HGF found in the plasma of patients with FHF was derived from the injured liver itself (6) (9).

In order to investigate the HGF-producing mechamism, the manner of secretion and the nature of the induction signals, cultivation of HGF producing cells is the best means. Therefore, we surveyed established cell lines for production of HGF. Consequently, we found that the promyelocytic leukemia cell line, HL-60, was able to produce human HGF-like factor by phorbol ester stimulation. In this paper, we characterize HL-60 derived HGF and compare it with human plasma derived HGF.

MATERIALS AND METHODS

HL-60 cells and preparation of conditioned medium; HL-60 cells were obtained from American Type Culture Collection (GIBCO). The cells were cultured in tissue culture flasks (Falcon Plastic), in RPMI 1640 medium containing 2.5% heat-inactivated fetal calf serum (GIBCO), 100u/ml penicillin, 100μg/ml streptomycin. To prepare conditioned medium, cells were inoculated at 1×10^6 cells/ml. Phorbol 12-myristate 13-acetate (PMA, Sigma) was added as required.

Assay of HGF-like activity; The rate of DNA synthesis in primary cultures of adult rat hepatocyte was monitored with incorporation of ^{125}I-deoxyuridine into DNA according to a modification of the method of Lobb and Fett (10). Parenchymal hepatocytes were isolated by *in situ* perfusion using collagenase, and were cultured as reported previously (1). Test samples were added to cultures after 24 hr of primary seeding. After further incubation for 12 hr, ^{125}I-deoxyuridine (2,000Ci/mmol, Amersham) was added and the cultures were continued for 24 hr. Then the cells were washed twice with PBS, immersed in 10% TCA and solubilized in 1N NaOH. Incorporation of ^{125}I-deoxyuridine into DNA was determined from the radioactivity of the NaOH solution.

Assay of HGF-immunoreactivity using human HGF-specific ELISA; HL-60 derived HGF was measured by the ELISA system of Tsubouchi et al.(11). Briefly, standard human HGF or unknown samples were dispensed into a 96-well microtiter plate coated with a monoclonal antibody against human HGF. After incubation for 1 hr, it was washed 3 times with PBS containing 0.05% Tween 20. After the addition of a 0.1ml aliquot of a polyclonal antibody against human HGF, the plate was incubated for 1 hr then washed 3 times. After the addition of 0.2ml of diluted goat (anti-rabbit immunoglobulin) IgG-peroxidase conjugate, the plate was incubated for 1 hr and then washed. An aliquot (0.1ml) of 0.25% o-phenylenediamine was added and the plate allowed to stand for 10 min. The reaction was stopped by addition of 0.1ml of 1N H_2SO_4. The absorbance was read at 492nm by an automatic plate reader with a reference wavelength of 690 nm.

Purification of HL-60 derived HGF-like factor; HL-60 cells were cultured with 1 ng/ml PMA for 48 hr. The supernatant (25*l*) was precipitated with 70% saturated $(NH_4)_2SO_4$, and the precipitate was dialyzed against 50mM Tris-HCl containing 0.15M NaCl, pH 8.5 overnight. Then, the dialyzed supernatants were applied to a column (2 x 30cm) of S-Sepharose Fast Flow (Pharmasia) equilibrated with 50mM Tris-HCl containing 0.15M NaCl and 0.05% Tween 20 (pH 8.5). A linear gradient of 0.15-1M NaCl was applied to the column using the FPLC system. Active fractions with bio- and immunoreactivity were pooled and adjusted to pH 7.8. The preparation was applied to a column (0.7 x 4 cm) of heparin-Sepharose

(Pharmacia) and then eluted with a linear gradient to 2.0 M NaCl using the FPLC system. The active fractions from a heparin Sepharose chromatography were applied to a Hi-por PR 304 column (4.6 x 250mm Bio Rad) equilibrated with 0.1% trifluoroacetic acid. HGF-like activity was eluted with a linear gradient of 0-90% acetonitrile in 0.1%trifluoroacetic acid. Fractions of 1 ml were collected and promptly neutralized by adding 0.1% sodium bicarbonate soln.

Human HGF was purified from the plasma of patients with fulminant hepatic failure in the manner above.

RNA blot hybridization analysis; Poly $(A)^+$ RNA obtained from HL-60 cells was denatured with 1 M glyoxal / 50%(v/v) dimethylsulfoxide, electrophoresed on a 0.8% agarose gel. and transferred to a nylon membrane (BIODYNE). The hybridization probe, a 1.4 kb EcoRI fragment of human HGF cDNA cloned from a human placenta library and ß-actin DNA fragment (NIPPON-GENE), were labeled with α-^{32}P-dCTP by the random primer labeling method (12). Hybridization and washing were carried out according to the procedure described by Alwine et al.(13). The filters were washed and air-dried, then subjected to autoradiography on Fuji X-ray films.

RESULTS

Secretion of HL-60 derived HGF immunoreactivity

After 48 hr of cultivating PMA-stimulated HL-60 cells, the media was removed from the cells and tested on the primary hepatocyte culture. The graded amount of media significantly increased DNA synthesis in the hepatocytes in a dose-dependent manner (data not shown). Fresh medium (RPMI 1640 containing 2.5% FCS and 1 ng/ml PMA) did not stimulate the DNA synthesis. Therefore, the hepatocyte growth-promoting activities did not appear to be due to factors in the media (serum or PMA) but rather to the addition of the factor derived from the HL-60 cell.

To confirm whether or not this factor was similar to human HGF, we analyzed the medium using the human HGF-specific ELISA system. As shown in fig. 1, the response for the conditioned medium was superimposed on a standard curve for human HGF.

The factor was secreted by PMA stimulation, but not by Con-A, PHA and LPS. The dose-response relationship between PMA concentration and HGF immunoreactivity generation is presented in fig. 2. A concentration of 1 ng/ml PMA was found to be optimal for generation of the factor. A significant increase in immunoreactivity was observed as early as 3 hr after addition of PMA , then progressively increased to 100-fold that (to approximately 10 ng/ml human HGF eq.) of the non-stimulated culture in 72 hr. Hepatocyte growth promoting activity was proportional to the increments in the immunoreactivity. We have not observed the generation of this factor in U937 (histocytic leukemia cell), THP-1 (monocytic leukemia cell) or CCRF-CEM (lymphoblastic leukemia cell)

Characterization of HL-60 HGF

Previously, we purified HGF from rat platelets via a three-step procedure, cation ion exchange FPLC, heparin affinity chromatography and C_4 reverse phase

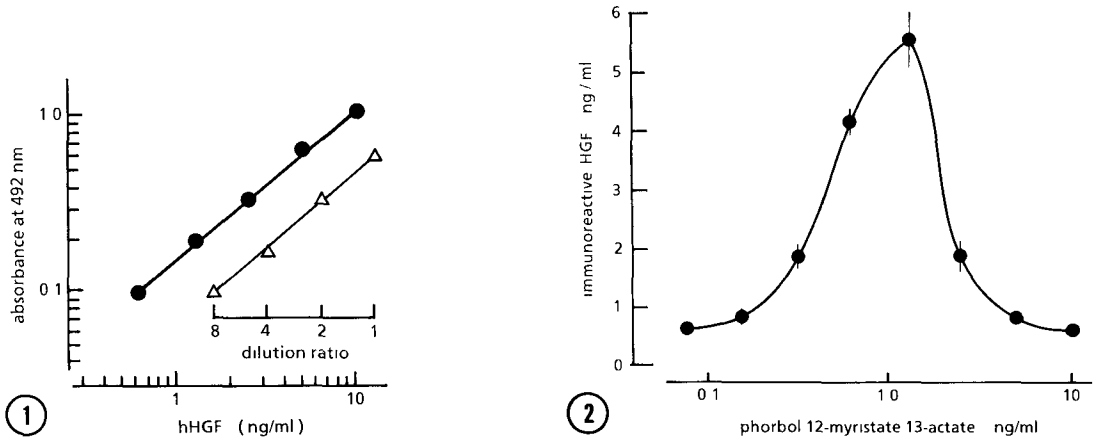

Fig. 1. Dose response curve of HL-60 conditioned medium in human HGF specific ELISA. HL-60 cells (1x10^6 cells/ml) were stimulated by PMA (1 ng/ml) for 72 hr. Then, culture supernatant was harvested and analyzed with the ELISA △—△. Human HGF for standard ●——● was purified from the plasma of patients with FHF.

Fig. 2. The effect of PMA on generation of the HGF immunoreactivity by HL-60 cells. HL-60 cells (1x10^{6}cells/ml) were stimulated by various concentrations of PMA for 48 hr. Assay of the HGF immunoreactivity in culture supernatant was performed by ELISA. The results are the mean of 4 experimants.

HPLC (1). The purification described here also consists of the same steps. The elution pattern of HL-60 derived HGF immunoreactivity in each case was completely compatible with hepatocyte growth promoting activity. The elution profiles also resembled that of rat platelet derived HGF (1) and human plasma-derived HGF (data not shown). The active peak obtained by C_4 reverse phase HPLC (fig. 3A) was subjected to SDS-PAGE. The final preparation revealed a broad band, migrating between molecular weights 78 and 90 kD (average 84 kD) with silver stain after SDS-PAGE under nonreduction conditions (fig. 3B). When this was reduced with 2-mercaptoethanol and subjected to SDS-PAGE, one major band with a molecular weight between 56 and 65 kD, two major bands with molecular weights of 32 and 34 kD, and a minor band with a molecular weight of 50 kD were detected (fig. 3B). A minor band of 50 kD might be a contaminant in the reducing reagent, because this band was also detected in the lane of molecular mass markers. These findings indicated that HL-60 HGF consists of the two chains linked together by disulfide, in manner previously reported in rat (1) and human HGF (2). In short, for the heavy chain (α-chain), there was a broad band between 56 and 65 kD. For the light chain (β-chain), there were two different size chains (β_1: 34 kD, β_2: 32 kD).

Table 1 compared the amino acid compositions of purified HL-60 HGF, purified HGF from the plasma of patients with FHF and pre-pro HGF calculated from cDNA sequence reported by Miyazawa et al. (4). The amino acid

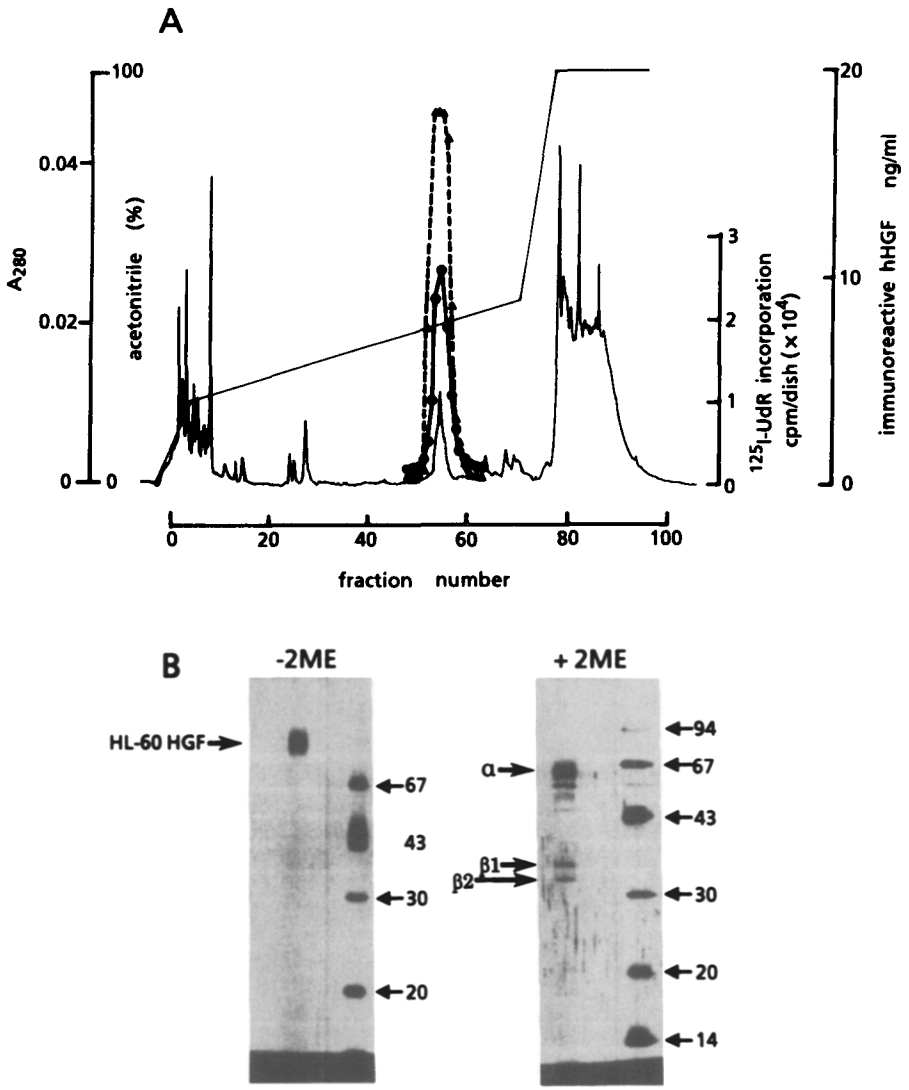

Fig. 3. (A) Reverse phase HPLC of HL-60 derived HGF. The active fraction from a heparin Sepharose chromatography was injected into a Hi-pore Rp 304 column and eluted with a gradient 0-90% acetonitrile in 0.1% trifluoroacetic acid. Fractions of 1ml were collected, promptly neutralized with 0.1% $NaHCO_3$ solution. Then, HGF bioactivity ●——● and immunoreactivity ▲- - - -▲ were assayed after dilution and sterilization. Solid line indicates the absorbance at 280 nm. Dotted line indicate the acetonitrile concentration.

(B) SDSPAGE of the purified HL-60 derived HGF. The purified HL-60 derived HGF with or without treatment of 2-mercaptoethanol were subjected to SDSPAGE as described by Laemmi (14). After electrophoresis, the gel was fixed and stained with silver (15). The molecular mass (in kD) markers used were as follows; glycogen phosphorylase, 94; BSA, 67; ovalbumin, 43; carbonic anhydrase, 30; soybean trypsin inhibitor, 20; lactalbumin, 14.

compositions were all in close agreement with each other. However, slight differences were apparent in the amino acid composition of HL-60 HGF. For example, HL-60 HGF contained larger amounts of Lys and Ala than others.

Table 1. Results of amino acid analysis for purified HL-60 derived HGF and purified human HGF from the plasma of patients with FHF

	HL-60 HGF	Purified native hHGF	Prepro hHGF calculated
Asp	69	86	83
Thr	44	39	42
Ser	55	42	40
Glu	70	71	63
Gly	67	62	58
Ala	45	25	24
Val	40	31	35
Met	12	14	16
Ile	38	33	42
Leu	45	41	50
Tyr	30	29	33
Phe*	18	18	18
Lys	75	47	47
His	22	22	26
Arg	50	43	43
M.W	76783	68501	70757

Amino acid analysis was performed by fluorescence detection using a Hitachi model 7300 Amino Acid Analizer. Amino acid composition of human pre-pro HGF was calculated from cDNA sequence. Phenylalanine with asterisk was assumed to be 18.

Human HGF mRNA in HL-60 cell

We examined the HGF mRNA in HL-60 cell stimulated by PMA for 12 hr. Hybridization of poly (A)+ RNA from stimulated HL-60 cell with the human HGF cDNA probe detected a major 6 kb mRNA (fig. 4), which was consistent with previous reported data in rat liver (5) and human placenta (4). Nonstimulated cells contained a very low level of this mRNA. In contrast, the level of β-actin mRNA was not significantly changed by PMA stimulation.

DISCUSSION

In the present study, we demonstrated that PMA-stimulated HL-60 cell produced immunoreactive HGF. This immunoreactivity seems to be bioactive, because it increased along with the hepatocyte growth promoting activity. To ascertain the characterization of the immunoreactive HGF, we purified the factor

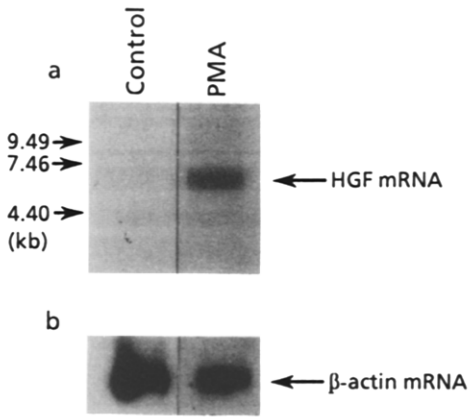

Fig. 4. Northern hybridization analysis of poly(A)$^+$RNA from HL-60 cells with or without stimulation with PMA. Ten μg of poly(A)$^+$RNA was applied in each lane. Two hybridizations were carried out independently on the same membrane using human HGF cDNA fragment (a) and β-actin one (b) as probe. The positions of RNA markers (BRL) are shown on the left.

from supernatants of HL-60 cells cultured with PMA. We had previously purified HGF from rat platelets via a three-step procedure (1). Human HGF from the plasma of patients with FHF was also isolated by the same procedure (data not shown). The behavior of the HL-60 HGF in each step resembled that of human plasma HGF. This indicated that the chemical properties of the HL-60 HGF were identical to human HGF.

Electrophoretic analysis of the purified HL-60 HGF suggested that the factor had a wide ranging molecular mass, 78-90 kD (average; 84 kD), and consisted of an α-chain (MW. average; 60 kD) and β-chains. There were two species of β-chain; β_1 had a molecular weight of 34 kD, β_2 a molecular weight of 32 kD. The components were in accord with rat platelet derived HGF and human plasma HGF. In the amino acid composition, the purified HL-60 HGF was the same as purified human HGF and pre-pro HGF calculated from human HGF cDNA sequence, except for a minor discrepancy with Lys and Ala. From these results, it seems that the molecular structure of HL-60 HGF closely resembles human plasma HGF.

We analyzed HL-60 HGF mRNA in PMA stimulated cells by northern blot hybridization using a fragment of the human HGF cDNA. One band corresponding to 6 kb was detected in stimulated HL-60 cells, but not in normal cells. The size of the mRNA was similar to human HGF mRNA as previously reported in human tissues (4). Thus, PMA induced not only HL-60 HGF secretion but also HGF mRNA transcription.

It is well known that HL-60 cells are differentiated into macrophages/ monocytes in the presence of phorbol esters (16)(17)(18), 1,25-dihydroxy vitamin D (18)(19) and others, and into granulocytes in the present of DMSO (20) and

others (21). The addition of DMSO to the HL-60 culture dose not stimulate production of the factor (data not shown). It was seen that HGF production was not correlated with the differentiation of HL-60 into macrophages/monocytes, because, in the optimal dose (1 ng/ml) of PMA for HGF production, the cell rarely adhered to the substrate of the culture dish. The cellular effects of PMA are mediated by the activation of the calcium and phospholipid-dependent protein kinase C (22). Stimulation of HL-60 HGF production by PMA might be caused through the activation of the protein kinase C.

In conclusion, HL-60 cell was an attractive model for studies of HGF producing mechanisms, the manner of secretion and the nature of the signals.

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