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Purification and characterization of a growth factor from rat platelets for mature parenchymal hepatocytes in primary cultures

(hepatocyte growth factor/hepatotropic factor/liver regeneration)

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ABSTRACT **A growth factor (HGF) stimulating DNA synthesis of adult rat hepatocytes in primary culture was found in rat platelets. HGF was purified from rat platelets to homogeneity by a three-step procedure: stimulation of its release from platelets by thrombin, cation-exchanger fast protein liquid chromatography on a Mono S column, and heparin-Sepharose chromatography. HGF was clearly distinguishable from the platelet-derived growth factor (PDGF) by fast protein liquid chromatography. HGF was a heat- and acid-labile cationic protein that was inactivated by reduction with dithiothreitol. Its molecular mass was estimated to be 27 kDa by NaDodSO$_4$/PAGE and its amino acid composition was very different from that of PDGF. The purified HGF stimulated DNA synthesis in adult rat hepatocytes at 2 ng/ml and was maximally effective at 20 ng/ml; its effect was additive or synergistic with those of insulin and EGF, depending on their combinations. HGF did not stimulate DNA synthesis of Swiss 3T3 cells, while PDGF did not stimulate that of hepatocytes. Thus, HGF showed clearly different cell specificity from PDGF in its growth-promoting activities. These findings indicate that HGF is a growth factor in platelets for mature hepatocytes.**

Many attempts have been made to demonstrate a "hepatotropic factor" that acts as a trigger for liver regeneration after partial hepatectomy. However, no such humoral factor has been purified and characterized fully to date, because no simple *in vitro* system has been available for its identification and purification.

Recently, several groups including ours found that adult rat hepatocytes in primary culture, which retain many of their *in vivo* liver functions, can proliferate when cultured at low density in medium containing insulin and epidermal growth factor (EGF) (1–6). Thus, primary cultures of adult rat hepatocytes are a suitable *in vitro* system for use in studies on the humoral factor for hepatocyte growth. Previously, we reported partial purification of a hepatocyte growth factor (HGF) from the serum of hepatectomized rats and showed that this factor was a heat- and acid-labile protein that differed from known factors such as fibroblast growth factor, platelet-derived growth factor (PDGF), and insulin-like growth factors (7).

Recently, Russell and coworkers (8, 9) reported that rat platelets contain a growth factor for adult rat hepatocytes in primary culture and suggested that the factor in serum is derived from platelets. More recently, Paul and Piasecki (10) showed that a heat-labile growth factor for adult rat hepatocytes is released from rat platelets when they aggregate on thrombin treatment. However, none of these growth factors has been purified or characterized extensively.

In this paper, we report isolation of a hepatotropic factor, HGF, from rat platelets and its purification to a homogeneous state by a three-step procedure, involving cation-exchanger, fast protein liquid chromatography (FPLC), and heparin-Sepharose chromatography. The purified HGF was found to be a heat- and acid-labile cationic protein of 27 kDa that was active at ≈2 ng/ml.

MATERIALS AND METHODS

Materials. The materials used for isolation and culture of hepatocytes were as described (11). EGF was purified from the submaxillary glands of male adult mice by the method of Savage and Cohen (12). Mono S and heparin-Sepharose CL-6B columns were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Pure thrombin from bovine plasma was purchased from Sigma. [*methyl*-^{3}H]Thymidine (54.2 Ci/mmol; 1 Ci = 37 GBq) was from Radiochemical Centre. Swiss 3T3 cells were a gift from Y. Takai (School of Medicine, Kobe University). Stock cultures of Swiss 3T3 cells were grown as monolayers in 10-cm Corning dishes in Dulbecco's modified Eagle's medium with 10% calf serum.

Primary Culture of Adult Rat Hepatocytes. Adult rat hepatocytes were isolated and cultured as monolayers as reported (11). Inocula of 2.5×10^5 cells were introduced into 2-cm-diameter wells of Linbro multiwell plastic dishes that had been coated with collagen from rat tail. The cells were cultured in 1 ml of Williams medium E supplemented with 5% calf serum/2 nM insulin/10 nM dexamethasone under 5% CO_2 and 30% O_2 in air at 37°C. After 4 hr, the medium was changed to serum-free Williams medium E with aprotinin at 0.1 μg/ml (bovine pancreatic trypsin inhibitor).

Assay of DNA Synthesis. The test substance or insulin (0.1 μM) and EGF (20 ng/ml) were added to cultures of rat hepatocytes 20 hr after plating. At 12 hr after addition of these substances, [^{3}H]thymidine (2.5 μCi/ml, 0.27 Ci/mmol) was added to the fresh medium and culture was continued for 24 hr. Incorporation of [^{3}H]thymidine into DNA was determined as described (1). One unit of HGF activity is defined as the amount of EGF required for half-maximal stimulation of DNA synthesis by adult rat hepatocytes, because HGF itself cannot be used as a standard since the maximal activity of the crude HGF preparation varies owing to variable contamination with growth inhibitor. The labeling index was assayed as described (2).

For assay of PDGF activity with Swiss 3T3 cells, the cells were inoculated into 2-cm wells of Linbro multiwell plastic dishes at 1.25×10^4 cells per well and cultured in Dulbecco's modified Eagle's medium supplemented with 1% calf serum for 4 or 5 days to subconfluency. After 4 or 5 days, test samples were added to the cultures and [^{3}H]thymidine (2.5 μCi/ml, 0.27 Ci/mmol) was added 20 hr later. After a further 4 hr, the cells were washed twice with phosphate-buffered saline (PBS), fixed with 10% trichloroacetic acid, and solubil-

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Abbreviations: HGF, hepatocyte growth factor; FPLC, fast protein liquid chromatography; EGF, epidermal growth factor; PDGF, platelet-derived growth factor.

ized in 0.1 M NaOH at 37°C. Radioactivity in the NaOH solution was measured in a scintillation counter.

Release of HGF from Rat Platelets. Adult rat blood was collected in a syringe containing 0.1 vol of 0.15 M NaCl and 77 mM EDTA (pH 7.4). The blood was centrifuged at 200 × *g* for 15 min, and the resulting supernatant was recentrifuged at 2500 × *g* for 15 min to precipitate platelets. The precipitate was washed twice with PBS by centrifugation, yielding a platelet preparation of >99% purity as judged by microscopy. The washed platelets obtained from 100 adult rats (200–400 g) were suspended at 1×10^{10} platelets per ml in PBS and incubated with pure thrombin (2 units/ml) for 15 min at 37°C to induce their aggregation. After 15 min, phenylmethylsulfonyl fluoride was added at a final concentration of 1 mM, and the suspension was centrifuged at 15,000 × *g* for 10 min. The supernatant was used as crude HGF.

Cation-Exchanger FPLC on a Mono S Column. The crude HGF preparation was applied to a Mono S column (1 × 10 cm) previously equilibrated with 50 mM Tris·HCl (pH 8.5) containing 0.15 M NaCl, 10 mM Hepes, and 2 mM $CaCl_2$. The column was washed with the same buffer and then HGF was eluted with a linear gradient of 0.15–1.0 M NaCl at a flow rate of 60 ml/hr. For measurement of the growth-stimulating activities of HGF and PDGF of fractions, 100-μl aliquots of all fractions were diluted with 4 vol of a solution of bovine serum albumin (2.5 mg/ml) in PBS and sterilized by filtration through a 0.22-μm filter (Millex-GV, Millipore).

Heparin-Sepharose CL-6B Chromatography. A column of heparin-Sepharose CL-6B (1 × 2.5 cm, 2-ml bed volume) was equilibrated with 10 mM Tris·HCl, pH 7.5/0.3 M NaCl. Active fractions from Mono S FPLC were pooled, adjusted to pH 7.5 with 1 M HCl, and diluted with 3 vol of distilled water. The diluted samples were applied to the column. The column was washed with 10 column vol of equilibration buffer, and then material was eluted with 30 ml of a 0.3–2.0 M NaCl gradient at a flow rate of 20 ml/hr. Fractions of 1 ml were collected and their absorbance at 215 nm and HGF activity were measured. Before assay of HGF activity, 50-μl aliquots were diluted with 20 vol of bovine serum albumin solution and sterilized as described above.

Treatments of Purified HGF. For measurement of the heat stability of HGF, the purified preparation (3 μg/ml) was heated for 20 min at 70°C or in boiling water for 1.5 min and cooled. The acid stability of the factor was examined by incubating it with 1 M acetic acid for 5 hr at 20°C and then neutralizing the solution with 6 M NaOH. Then, the sample was supplemented with bovine serum albumin (2.5 mg/ml). For examination of the effect of a reducing agent, HGF was incubated with 50 mM dithiothreitol for 1 hr at 20°C. Then, bovine serum albumin was added and the mixture was dialyzed against PBS overnight at 4°C. The susceptibility of HGF to protease digestion was examined by incubating HGF (3 μg/ml) with trypsin (10 μg/ml) for 2 hr at 37°C and then stopping the digestion by adding 1 mM phenylmethylsulfonyl fluoride. These samples were diluted with 10 vol of bovine serum albumin (2.5 mg/ml) and sterilized by filtration through a Millex-GV filter.

Reverse-Phase FPLC of Purified HGF. A sample of 200 μl of purified HGF was injected into a ProPRC HR 5/5 column (C_8) equilibrated with 0.1% (vol/vol) trifluoroacetic acid and 10% acetonitrile. Protein was eluted with a linear gradient of 10–50% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 0.5 ml/min. Fractions of 1 ml were collected and their absorbance at 215 nm was measured.

NaDodSO$_4$/PAGE. NaDodSO$_4$/PAGE of purified HGF was performed with 12.5% acrylamide as described by Laemmli (13). After electrophoresis, the gel was fixed in 40% methanol/10% acetic acid for 90 min, washed with 10% ethanol/5% acetic acid, and stained with silver (14).

Amino Acid Analysis. Purified HGF eluted from the heparin-Sepharose column was desalted by reverse-phase FPLC. The protein was hydrolyzed in 6 M HCl in evacuated sealed tubes for 24, 48, and 72 hrs at 105°C, and the hydrolyzed samples were examined in an amino acid analyzer (Hitachi 835).

RESULTS

Purification of HGF from Rat Platelets. Crude HGF was obtained from fresh platelets of 100 rats by treating them with thrombin. The HGF released from platelets stimulated DNA synthesis in primary cultured hepatocytes at a dose of 20 μg/ml and was maximally effective at 100 μg/ml, as shown in Fig. 1. The crude HGF lost activity completely on heating at 70°C for 20 min or on incubation with 1 M acetic acid overnight at 4°C (data not shown).

The crude preparation was subjected to cation-exchanger FPLC on a Mono S column. Much protein with no detectable HGF activity was not adsorbed and was washed out from the column. Then, on increasing the NaCl concentration, HGF was eluted as a very small peak of protein with 0.68 M NaCl (Fig. 2). This step of purification was very effective, increasing the purity ≈200-fold over that in the crude preparation (Table 1). Moreover, in this step, HGF was separated almost completely from PDGF, as judged by measuring ability to stimulate DNA synthesis in Swiss 3T3 cells; PDGF was eluted with 0.6 M NaCl just before HGF. These findings indicate that both HGF and PDGF are strongly basic proteins, but that they are chromatographically distinguishable. The fraction in which PDGF was the most active for 3T3 cells did not stimulate DNA synthesis of hepatocytes at all. Active fractions (nos. 26–31) were pooled and diluted with 2 vol of distilled water to decrease the NaCl concentration of the eluate to ≈0.3 M. In the final step of purification, the diluted eluate was applied to a heparin-Sepharose column (Fig. 3). All the HGF activity was adsorbed, but most of the protein was not retained on the column. On gradient elution, a large peak of activity was eluted with 1 M NaCl. After elution of HGF, other adsorbed proteins with little activity were eluted with 1.4 M NaCl.

The dose–response curves of fractions at the three steps of purification are shown in Fig. 1. The final preparation obtained from heparin-Sepharose was effective at a concentration as low as 2 ng/ml, and it was maximally effective at 20 ng/ml. Results of a typical purification are summarized in Table 1. The overall recovery of HGF activity was ≈40%

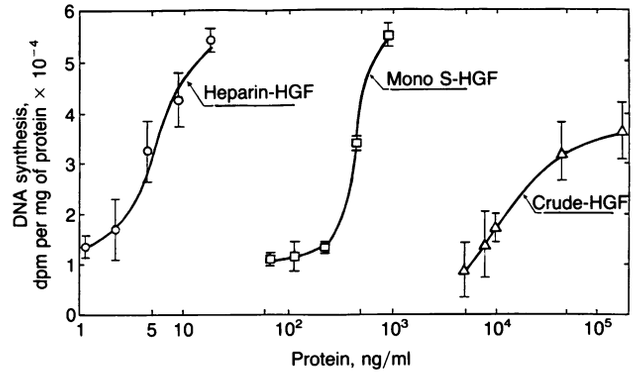

FIG. 1. Dose-dependent effects of HGF at three purification steps on DNA synthesis by adult rat hepatocytes. HGF samples obtained at the three steps were diluted with bovine serum albumin (2.5 mg/ml) and sterilized, and their HGF activity at the indicated concentrations was assayed. Protein was measured by the method of Lowry *et al.* (15). Values are means ± SD for four dishes in two separate experiments.

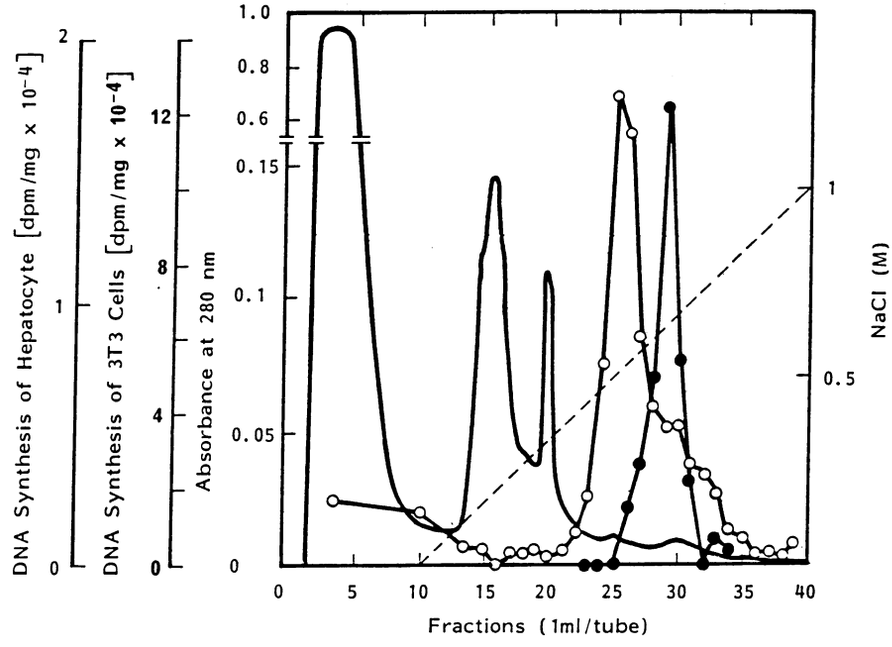

FIG. 2. Cation-exchanger FPLC on a Mono S column of crude HGF released from platelets. Crude HGF obtained from platelets of 100 rats was applied to a Mono S column and eluted by a gradient (30 ml at total volume) of 0.15–1.0 M NaCl as described. Fractions of 1 ml were collected and absorbance at 280 nm (—), conductivity (----), PDGF activity (○), and HGF activity (●) were measured.

with ≈12,400-fold increase in specific activity over that in the crude preparation released from platelets. About 1.5 μg of pure HGF was obtained from the platelets of 100 adult rats. The purified HGF had a specific activity of 384,000 units per mg of protein. By repeating the purification procedure 20 times, we obtained ≈30 μg of pure HGF from 2000 adult rats.

Characterization of HGF Purified from Rat Platelets. The homogeneity of HGF obtained from a heparin-Sepharose column was analyzed by $NaDodSO_4$/PAGE and reverse-phase FPLC. HGF migrated as a single band in a position corresponding to 27 kDa, as shown in Fig. 4. Analysis by reverse-phase FPLC also showed that it was highly pure, giving a single sharp peak in 38% acetonitrile (data not shown). Table 2 shows the amino acid composition of the purified HGF, compared with that of PDGF. The amino acid composition of HGF was very different from that of PDGF. Like PDGF, HGF had a high content of basic amino acids, but it differed greatly from PDGF in its contents of alanine, valine, methionine, and tyrosine.

Table 3 shows the effects of various treatments of the purified HGF on its hepatocyte growth activity. The activity was lost completely on heating at 70°C for 20 min or in boiling water for 1.5 min. It was inactivated by digestion with trypsin and was labile on treatment with 1 M acetic acid. More than 90% of the activity was lost on reduction of HGF with dithiothreitol. Table 4 shows some biological properties of HGF. HGF alone markedly stimulated DNA synthesis of adult rat hepatocytes and its potency was stronger than that of EGF. The effect of HGF was additive or synergistic with those of insulin and EGF, depending on their combinations. After treatment with three factors, >80% of the cells entered the S phase, judging from their labeling index. Increases of DNA synthesis after various additions correlated well with those of the labeling index. Purified HGF did not stimulate DNA synthesis in Swiss 3T3 cells, and, conversely, PDGF did not enhance DNA synthesis in hepatocytes.

DISCUSSION

In addition to PDGF, platelets are known to contain several mitogens, such as platelet basic protein (17), connective tissue activating peptide III (18), and transforming growth factor β (19). All these growth factors are heat- and acid-stable proteins and are mitogenic for cells derived from mesenchymal tissues. On the contrary, HGF purified from rat platelets in this work stimulated DNA synthesis in mature rat hepatocyte in primary culture, but not in fibroblasts such as 3T3 cells. HGF is a heat- and acid-labile protein, with a molecular mass of 27 kDa. Thus, besides differing in cell specificity and chemical properties, HGF is different from known platelet-derived factors in heat and acid stability and molecular size. Moreover, various known growth factors such as fibroblast growth factor, insulin-like growth factors I and II, transferrin, and thrombin did not stimulate DNA synthesis in cultured rat hepatocytes (7). These findings indicate that HGF is a different PDGF for mature hepatocytes.

HGF has marked affinity for heparin. We first noticed that HGF from rat serum has strong affinity to heparin-Sepharose CL-6B during a survey of good ligands for affinity chromatography of HGF (7). This affinity for heparin greatly facilitated purification of HGF and, consequently, HGF could be

Table 1. Purification of HGF from rat platelets

Purification step	Total activity, units	Specific activity, units per mg of protein × 10^{-3}	Purification, -fold	Recovery, %
Crude HGF	4649	0.031	1	100
Mono S FPLC	2941	5.882	188	63
Heparin-Sepharose	1823	384.6	12,408	39

Material released from the platelets of 100 rats was used as starting material. HGF activity was measured at three or four different concentrations, and the activity was determined from the linear portion of the dose–response curve. One unit of activity is defined as the quantity of EGF required for half-maximal stimulation of DNA synthesis of adult rat hepatocytes in primary culture.

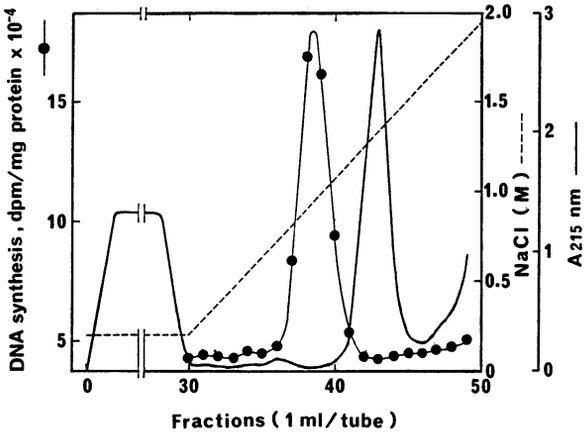

FIG. 3. Purification by heparin-Sepharose CL-6B chromatography of partially purified HGF. The HGF preparation eluted from the Mono S column (13.5 ml) was applied to a heparin-Sepharose CL-6B column and eluted by 30 ml of a gradient of 0.3–2.0 M NaCl as described. Fractions of 1 ml were collected and their absorbance at 215 nm (—), conductivity (---), and HGF activity (●) were measured.

purified to homogeneity in high yield by only three steps, one being heparin-Sepharose chromatography. Since Shing *et al.* (20) first reported that heparin affinity chromatography was very effective for the purification of a capillary endothelial cell growth factor from chondrosarcoma, this procedure has been used in purification of fibroblast growth factor from bovine brain and pituitary glands (21), endothelial cell growth factor from bovine brain (22), and cartilage-derived growth factor from bovine scapular cartilage (23). Platelets contain many proteins and peptides that bind to heparin and are released by thrombin or platelet-aggregating agents, such as platelet factor 4, PDGF, platelet basic protein, and β-thromboglobulin. These heparin affinity proteins are stored in the α granules of human platelets (24). The biological significance of the affinities of many mitogens in platelets including HGF for heparin is unknown.

Previously, we partially purified and characterized a growth factor for adult rat hepatocytes in primary culture from serum of rats (7). This factor was found in the serum of rats after partial hepatectomy and was named HGF or hepatotropin. The HGF activity of this factor in rat serum increased time dependently after partial hepatectomy, reaching a maximum of ≈5-fold the initial level 24 hr after the operation. This serum-derived HGF was a heat- and acid-labile acidic protein of ≈150 kDa. This serum-derived HGF also had strong affinity for heparin. Thus, the HGF derived from rat serum and that released from platelets have very

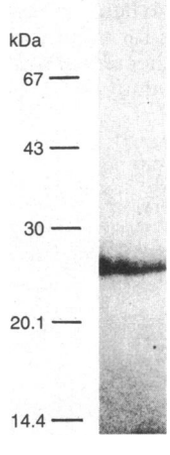

FIG. 4. NaDodSO$_4$/PAGE of purified HGF. Samples (20 μl, 0.1 μg) were subjected to NaDodSO$_4$/PAGE as described. After electrophoresis, the gel was fixed and stained with silver. The molecular size markers used were as follows: bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; soy bean trypsin inhibitor, 20.1 kDa; lactalbumin, 14.4 kDa.

Table 2. Amino acid composition of HGF

Amino acid	HGF, residues per mol of protein	PDGF, residues per mol of protein
Asx	29	24
Thr	15	19
Ser	15	20
Glx	25	36
Gly	26	17
Ala	9	19
Cys	ND	18
Val	11	27
Met	5	1
Ile	12	13
Leu	15	16
Tyr	11	3
Phe	7	8
His	7	6
Lys	20	21
Arg	14	23
Pro	ND	20
Trp	ND	ND
Total	(221)	291

ND, not determined. PDGF data are from ref. 16.

similar properties, except for their size. This difference in their molecular mass can be explained by supposing that since the platelet-derived HGF is a strongly basic protein it readily binds to some acidic proteins in plasma after its release from platelets. Thus, serum HGF could be derived from rat platelets. Very recently, we demonstrated that human platelets also contained HGF, which could be purified by similar procedures to those described in this paper (unpublished data). Moreover, HGF activity was increased in sera of patients with fulminant hepatic failure (25). From these findings we concluded that HGF from platelets is the hepatotropic factor for liver regeneration that has long been suspected to exist.

Previously, we showed that the growth and differentiated functions of adult rat hepatocytes were reciprocally regulated by a cell-surface modulator via cell–cell contact (26–28). We also suggested that by cell–cell contact *in vivo*, this cell-surface modulator maintains mature hepatocytes in the G_0 state, in which liver-specific functions are expressed maximally and cell proliferation is strongly inhibited. When cell–cell contact in the liver is disturbed by injury, such as viral hepatitis, partial hepatectomy, or treatment with a hepatotoxin, suppression of growth by the cell-surface modulator would be lost and surviving hepatocytes in the G_0 phase pass to the G_1 phase, in which increased HGF in the plasma stimulates proliferation. When the hepatocytes have divided once or twice, cell contact becomes tight again and

Table 3. Effects of various treatments on the activity of purified HGF

Treatment	DNA synthesis, dpm per mg of protein × 10^{-4}
No HGF	0.77 ± 0.49
HGF	
Untreated	5.52 ± 0.81
70°C, 20 min	1.52 ± 0.14
1 M acetic acid, 20°C, 5 hr	1.60 ± 0.72
50 mM dithiothreitol, 20°C, 1 hr	0.27 ± 0.15
Trypsin, 37°C, 2 hr	0.49 ± 0.15

Purified HGF was treated as described and then samples of 10 ng/ml were used for assay of residual activity. Values are means ± SD for triplicate samples.

Table 4. Effects of HGF, insulin, and EGF on DNA synthesis and the labeling index of adult rat hepatocytes in primary culture

Hormones	DNA synthesis, dpm per mg of protein × 10^{-4}	Labeling index, %
None	0.77 ± 0.22	2.99 ± 0.86
Insulin (0.1 μM)	2.01 ± 0.64	8.89 ± 1.50
EGF (20 ng/ml)	5.58 ± 0.45	19.1 ± 3.53
Insulin + EGF	14.58 ± 2.78	35.1 ± 5.62
HGF (10 ng/ml)	9.08 ± 3.00	27.9 ± 2.65
Insulin + HGF	13.20 ± 2.25	33.8 ± 4.46
EGF + HGF	21.18 ± 2.19	51.0 ± 6.22
Insulin + EGF + HGF	39.99 ± 4.16	81.6 ± 7.60

Values are means ± SD for triplicate samples.

through cell-surface modulator-mediated regulation, the cells return to the G_0 phase, ceasing cell division and expressing liver-specific functions again. Moreover, the findings that both PDGF for cells of mesenchymal origin and HGF for epithelial cells are stored in platelets and are released from the platelets by a platelet-aggregating stimulus suggest the biological significance of these factors in the mechanism of wound healing due to the coordinated growth of epithelial and mesenchymal cells. Recently, we found that rat platelets contained two kinds of inhibitors of DNA synthesis of hepatocytes in primary culture (29), and we identified one of them as the transforming growth factor β (30). Thus, these stimulators and inhibitors of hepatocyte growth may be secreted sequentially in liver tissue and exert fine positive and negative controls on hepatocyte proliferation. Therefore, further studies on the structure and biological characters of HGF should provide information on the mechanisms of wound-healing, liver regeneration, and hepatocarcinogenesis.

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