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MITOGENIC ACTIVITY IN PLATELET-POOR PLASMA FROM RATS WITH PERSISTENT LIVER NODULES OR LIVER CANCER

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Platelet-poor plasma (PPP) from F-344 rats with chemically-induced preneoplastic liver nodules or hepatocellular carcinoma stimulated S-phase DNA synthesis in monolayer cultures of normal rat hepatocytes. Similar mitogenic activity was detected in PPP 6 hrs to 1 week after partial hepatectomy (PH) or after necrotizing doses of CCl_4 or diethylnitrosamine (DENA). Very little activity was found in PPP from control rats. The mitogenic activity in PPP from animals with nodules was non-dialyzable (> 14 kd) and bound to a heparin-sepharose affinity column. None of the mitogenic PPPs competed with [^{125}I] epidermal growth factor (EGF) for binding sites on A431 cells or normal rat hepatocytes. These studies indicate that persistent proliferation of preneoplastic and neoplastic hepatocytes is associated with increased circulating levels of mitogenic hepatocyte growth factor.

A serious limitation in the early diagnosis of internal cancer is the difficulty in the identification of preneoplastic and precancerous cellular lesions in the intact individual. Since hepatocyte proliferation is an important continuing property of liver during the long period of progression to cancer (1) and since acute regenerative liver cell proliferation has been found to be associated with the appearance of growth stimulating activity or factors in the serum (2-7), serum preparations from rats with precancerous lesions or with cancer were examined for their ability to stimulate the proliferation of isolated hepatocytes in vitro. The results from such assays were highly variable. Since serum from normal or partially hepatectomized (PH) rats has been shown also to inhibit hepatocyte proliferation (8-10) and to contain TGF-β and other inhibitors (11-13) and since platelets are at

ABBREVIATIONS: PPP, platelet-poor plasma; PH, partial hepatectomy; DENA, diethylnitrosamine; EGF, epidermal growth factor; TGF, transforming growth factor; WE, Williams E medium; LI, labeling index; BSA, bovine serum albumin; PBS, phosphate buffered saline; TdR, thymidine.

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least one source for some inhibitors, the assay was modified by the use of platelet-poor plasma (PPP). Using such preparations, consistent growth stimulating activity was found in plasma from animals with precancerous liver nodules and with liver cancer as well as after PH or liver cell damage with two hepatotoxins, CCl_4 and DENA. These results together with some properties of the factor are the subject of this communication.

MATERIALS AND METHODS

Animals and Preparation of PPP. Animals used were male F-344 rats (Charles River, Kingston, NY) maintained on Purina chow diet (5001) and managed according to the guidelines of the Canadian Council for Animal Care. Hepatocyte nodules and cancers were generated as described (14) following initiation by DENA and a modified selection by 3 doses of 20 mg/kg 2-acetylaminofluorene and PH (15). Blood was collected from the abdominal aorta under ether anesthesia at 5 and 8 months and at 12 and 14 months after initiation. Three month old rats were also bled at 6,24 and 48 hours and 7 and 14 days after 2/3 PH (16), at 6,24 and 48 hours after 2 mg/kg CCl_4 given intragastrically, and at 5,8,10 and 15 days after 200 mg/kg DENA given intraperitoneally. Controls were normal age-matched rats. Blood was pooled from 2 to 10 animals at each time point. Serum was prepared from blood clotted overnight at 4°C, then separated by centrifugation in a Sorvall RT 6000B (4°C 20 min, at 3500 rpm). For the preparation of PPP, blood was collected into 10 ml syringes containing 1 ml acid citrate dextrose and centrifuged (4°C, 20 min, 3500 rpm, Sorvall RT 6000B) to yield platelet free supernatant. PPP samples were subsequently defibrinated by the addition of 0.1 M $CaCl_2$ and 0.04 M $MgCl_2$ (in PBS) at 37°C. The clot was removed by centrifugation (4°C, 25 min, 10000 rpm, Sorvall RC2B). PPP was then dialyzed for 24 hours against 3 changes of Ca/Mg-free PBS in 14 kd dialysis membranes and filtered (0.22 um) prior to assay.

Monolayer Hepatocyte Assay. The preparation of hepatocyte monolayers has been described previously (17). Viable hepatocytes (200,000 per dish) from normal male F-344 rats were cultured at 37°C in air/CO_2 (95:5) on dishes coated with collagen (60 µg per 35 mm dish) (Collagen Corp, Palo Alto, CA) in modified WE medium supplemented with fetal bovine serum (10% v:v), insulin (20 U/l) L-glutamine (2 mM), HEPES (10 mM), penicillin (100 U/ml) and streptomycin (100 ug/ml). After 3 hours in culture, attached cells were washed and further incubated in serum-free modified WE, supplemented with L-proline (2 mM), sodium-pyruvate (10 mM) and 5 uCi/dish [^{3}H]TdR (New England Nuclear, Boston MA). PPP was added from 2.5 to 20% (v:v). Positive and negative controls contained similar concentrations of BSA (5 mg/ml in PBS) with or without 20 ng/ml mouse EGF (Collaborative Research Inc., Bedford, MA) or synthetic human TGF-alpha (Biotope Inc., Bellevue, WA). Porcine platelet TGF-beta (0.5 ng/ml) (R and D Systems, Inc. Minneapolis,MN) was added to some dishes at this time. After 48 hours in culture, dishes were washed, fixed in 10% buffered formalin, and processed for autoradiography. The labeling index (LI) was determined on a minimum of 400 hepatocytes per dish from randomly chosen fields. Aliquots of nodule PPP were tested for sensitivity to heat treatment (20 min at 56°C and 70°C and 2 min at 100°C) or to several enzyme treatments: trypsin (10 µg/ml and 1 µg/ml for 2 hrs followed by Soybean trypsin inhibitor - 80 µg/ml), RNase A (125 µg/ml for 2 hrs)

and DNase (10 µg/ml for 2 hrs). Aliquots were then kept on ice until added to hepatocyte cultures.

Competitive Binding Assays. An EGF radioreceptor assay kit (Biomedical Technologies Inc., Stoughton, MA) was used to assay the concentration of EGF receptor ligands in PPP. The amount of EGF activity was determined by the degree of inhibition of [^{125}I]EGF binding to purified A431 receptors. Competitive binding of [^{125}I]EGF to rat hepatocytes by PPP was determined in monolayer cultures after 3 hours saturation binding at 4°C with [^{125}I]EGF (New England Nuclear, Boston, MA; 80,000 cpm/ng EGF; 2.5 ng/250,000 hepatocytes). Cells were washed 4 times, scraped from culture dishes and radioactivity determined by liquid scintillation counting. Final results were expressed as ng EGF bound per mg protein (18).

Heparin-Sepharose Chromatography. Nodule PPP (1.5 ml) was applied to a column of heparin-sepharose CL-6B (Pharmacia Canada Ltd., Dorval, Quebec) (1.6 x 2.0 cm, 4 ml bed volume) equilibrated with 10 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl. After extensive washing, the bound proteins were eluted with Tris buffer containing 2.0 M NaCl. Fractions were dialyzed separately against 1/10 PBS for 24 hrs and lyophilized. Each sample was reconstituted with 2.5 ml of culture medium and filtered (0.22 µm) just prior to assay for mitogenic activity in the hepatocyte monolayer system.

RESULTS

Hepatocytes cultured for 48 hours in serum or PPP-free modified WE with L-proline, pyruvate and [^{3}H]TdR routinely had a baseline LI of 6 to 12%. EGF at a concentration of 20 ng/ml increased the 48 hr LI to 55 to 85%.

Normal PPP (at concentrations of 1 to 20%) caused only a slight increase in LI over baseline (12 to 22%) (Fig. 1, Table 1). However,

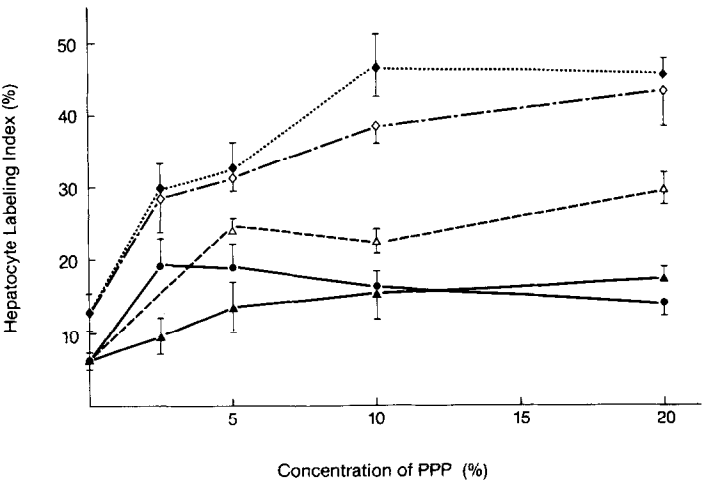

Fig. 1. Stimulation of DNA synthesis in rat monolayer hepatocytes, by PPP from rats with hepatic nodules at 5 mo (Δ---Δ) or 8 mo (◇—◇), with liver cancer (◆-----◆) or from 12 month old control rats (▲------▲) or by whole serum from nodules at 8 mo (●----●). Blood was pooled from 2 to 10 animals. Values are mean ± S.D. of at least 3 assays.

Table I

Effects of 20% PPP from rats at various times after treatment with CCl_4 or DENA on DNA synthesis of monolayer hepatocytes

		Labeling Index (%)
PPP	0-BSA control	10.1 ± 2.0
	Normal[a]	20.1 ± 2.3
CCl_4[b]	6 hours	28.3 ± 6.0
	24 hours	65.2 ± 4.4[c,d]
	48 hours	56.2 ± 0.3[d]
	7 days	20.9 ± 2.4
DENA[e]	5 days	43.6 ± 1.0[d]
	8 days	42.5 ± 4.6[d]
	10 days	29.0 ± 4.5[d]

[a] age-matched control animals, treated with saline.
[b] dosage 2 ml/kg.
[c] comparable LI from whole serum was 16.1 ± 3.4.
[d] Significantly greater than control, $p < .001$.
[e] dosage 200 mg/kg.

PPP from rats with liver nodules (at 5 or 8 months) or liver cancers (at 12 or 14 months) had much more mitogenic activity in EGF-free hepatocyte cultures resulting in a dose-dependent increase in LI to a maximum of 33 to 47% at 20% PPP (Fig. 1). These values were highly significant compared to control ($p < .001$, Students T test, unpaired variables).

PPP from PH rats also stimulated hepatocyte DNA synthesis for up to 1 week. Highly significant activity ($p < .001$) was detectable from 6 to 48 hours after PH (46 to 48% LI). High levels of mitogenic activity were also observed in PPP from animals treated with CCl_4 or DENA (Table 1). A maximum LI of 65 ± 4% was found in PPP 24 hours after CCl_4 treatment. PPP from DENA-treated rats had mitogenic activity for at least 5 to 10 days after treatment.

Mitogenic activity was not detectable in whole serum from nodule-bearing (Fig. 1), PH rats, or CCl_4-treated rats (Table 1) suggesting that mitogenic activity in PPP was inhibited in serum. PPP-induced DNA synthesis was reduced by about 80% in the presence of 0.5 ng/ml TGF-beta (Table 2). The mitogenic activity in nodule PPP was lost after heating to 70°C or 100°C, but only slightly decreased in samples heated to 56°C. Treatment with DNase, RNase or trypsin had little effect on whole plasma; however the activity of heparin-bound PPP was sensitive to trypsin.

Table 2

Effects of various treatments on mitogenic activity of nodule PPP in hepatocyte cultures

Treatment[a]	Hepatocyte labeling index
No PPP	8.0 ± 1.2
+ Soybean trypsin inhibitor	10.3 ± 2.2
+ RNase	9.7 ± 0.1
+ DNase	12.3 ± 0.9
+ TGF-beta	2.3 ± 0.2
10% nodule PPP	27.9 ± 2.0
+ heat 56°C	21.4 ± 0.2
+ heat 70°C	9.8 ± 0.8
+ heat 100°C	10.9 ± 2.5
+ trypsin 1 µg/ml	20.8 ± 0.8
+ trypsin 10 µg/ml	18.8 ± 0.5
+ RNase	20.5 ± 4.5
+ DNase	26.4 ± 2.7
+ TGF-beta	4.6 ± 1.8
Heparin bound PPP	24.0 ± 4.1
+ trypsin 10 µg/ml	3.1 ± 0.4

[a]Concentrations and exposure times were as described in materials and methods. Values are means ± S.D. for duplicate dishes.

In primary cultures of rat hepatocytes, PPP caused little or no decrease in [^{125}I]EGF bound to hepatocytes. In an EGF radioreceptor assay, using purified EGF receptors from A431 cells, we detected only low levels of EGF activity (< 2 ng/ml) in mitogenic PPP samples. Equivalent concentrations of EGF or TGF-alpha added to normal PPP did not induce significant levels of DNA synthesis in rat hepatocyte monolayers.

Passage of nodule PPP in Tris-NaCl buffer through a heparin sepharose CL-6B column resulted in a loss of mitogenic activity. The unbound proteins were assayed separately and did not stimulate hepatocyte DNA synthesis. However, elution of the bound protein with 2.0 M NaCl resulted in a small protein peak which had quite significant mitogenic activity. A maximum LI of about 30% was observed in hepatocytes exposed to these fractions (Fig. 2).

DISCUSSION

The removal of platelet-derived inhibitors has allowed us to readily detect circulating hepatocyte mitogenic activity in rats with neoplastic and regenerative liver proliferation.

The mitogenic activity in plasma of rats with nodules is a non-dialyzable (>14 kd), heat-sensitive heparin-binding substance. The

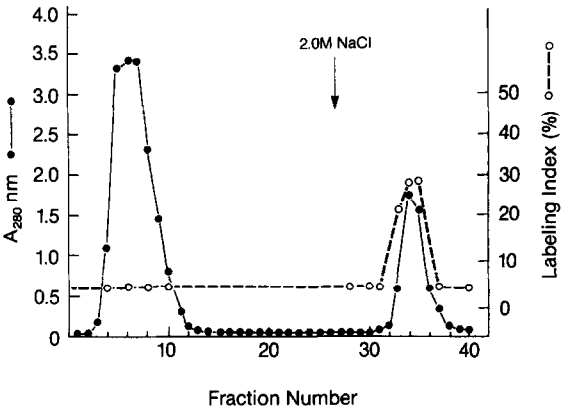

Fig. 2. Stimulation of DNA synthesis by nodule PPP fractionated by heparin-sepharose chromatography. ●—● $A_{280\ nm}$; ○---○ labeling index (%).

active heparin-bound factor is trypsin-sensitive, although this sensitivity is not observed in whole plasma, possibly due to the presence of trypsin inhibitors. Since EGF activity was less than 2 ng/ml in all PPPs tested and since binding to EGF receptors on hepatocytes or purified from A431 cells was not detected, it appears unlikely that PPP activity is due to EGF or other EGF receptor ligands such as TGF-alpha. Also, persistently proliferating nodule and carcinoma hepatocytes have reduced EGF receptors (19) and decreased EGF responsiveness (20), suggesting that EGF in plasma is not the important mitogen.

The presence of hepatocyte mitogenic activity in PPP of rats with liver nodules or carcinomas does suggest that persistent hepatocyte proliferation in preneoplastic and neoplastic lesions may be associated with a humoral growth stimulus much like those associated with post PH (21,22) or post-necrotic (23) regeneration. Hepatocyte proliferation in preneoplastic nodules is well regulated, increasing in response to PH, but returning to a higher than normal rate after the regenerative stimulus subsides (24). Persistent proliferation in these populations could be due to their greater sensitivity to humoral hepatopoietins. However the origins and regulatory signals for secretion of humoral liver growth factors are unknown. The presence of these humoral growth factors at preneoplastic stages, many months before the appearance of cancer, may be useful as an early diagnostic marker for developing neoplasia.

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