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Abstract

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CO-EXISTENCE OF INHIBITORY AND STIMURATORY FACTORS MODULATING CELL PROLIFERATION IN RAT LIVER CYTOPLASM

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Abstract. Factors that inhibit and stimulate cell proliferation were found to coexist in rat liver supernatant. The inhibitory and stimulatory factors were separated by ethanol fractionation. Both factors were sensitive to heat- and trypsin-treatment. The activity of the inhibitor was diminished by partial hepatectomy. The inhibitor from normal livers inhibited DNA and RNA synthesis in the L-cell system, but the same fraction from regenerating livers caused little or no inhibition of nucleic acid synthesis. The stimulatory factor from regenerating livers had a stronger effect on cell proliferation than that of normal livers. Furthermore, the inhibitor from normal livers depressed DNA synthesis *in vivo* in regenerating livers.

Key words : growth factors, growth stimulants, growth inhibitants, rat liver cytoplasm, cell proliferation

The triggering of hepatocytic proliferation has been studied by many investigators after partial hepatectomy. Humoral factors, such as serum factors (1–4), growth hormone (5, 6), portal factors (7–9), and neuronal factors (10) are considered more important than hepatic factors. In modulated humoral states after partial hepatectomy, however, hepatic factors other than cyclic nucleotides and protein phosphokinase might play a role. Liver extracts have been studied as inhibitors of cell proliferation (11–13), and other organ extracts have been found to act as stimulants, e. g., fibroblast growth factor (14), epidermal growth factor (15), and nerve growth factor (16), or as inhibitors, e. g., chalone (17). These previous reports indicate that organs contained either a stimulant or an inhibitor but not both. It appears reasonable to assume that both co-exist as cellular factors and respond to changes in humoral conditions induced by partial hepatectomy.

In this report we present evidence of the existence of both factors in the same rat liver supernatant and on some changes in their biological activities induced by partial hepatectomy. The role of the hepatic regulatory factors in the regeneration mechanism is discussed.

MATERIALS AND METHODS

Preparation of hepatic factors from rat liver. Wister rats (60–100 g) were partially hepatectomized by the method of Higgins and Anderson (18). Regenerating livers were harvested 24 h after the operation, and 40 to 60 regenerating livers were used in one sample batch; seven batches of samples were obtained. Normal and regenerating livers were separately homogenized in STE medium that contained 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, and 1 mM ethylenediamine tetraacetic acid. The post-mitochondrial supernatant was centrifuged at 104,000×g for 1 h, and the supernatant (S2) was gradually saturated with absolute ethanol to 45 and 70% in the presence of 0.5 mM 2-mercaptoethanol. After standing for 1 h, the ethanol saturated S2 was centrifuged stepwise at 10,000×g for 10 min in a 45% system (abbreviated N45 for normal liver; R45 for regenerating liver) and then in a 70% system (N70 and R70, respectively). All residues were re-suspended in dialysis medium that contained 1 mM Tris-HCl, pH 7.4 and 0.5 mM 2-mercaptoethanol, and lyophilized after extensive dialysis. All procedures were performed at 0°–4°. The samples were dissolved in phosphate buffered saline [PBS (−)] and sterilized just prior to use.

Assay of effect of hepatic factors on cell proliferation. The inhibitory and stimulatory effects of hepatic factors on L-929 mouse fibroblast (L-cell) proliferation were assayed for 48 h at 37° in a CO_2 gas incubator. The assay was started by replacing the resting medium (RM) that contained 2% tryptose phosphate broth (TPB, DIFCO) and 0.4% bovine serum in Eagle's minimum essential medium (MEM) with fresh resting medium and samples for stimulatory activity assay, or by replacing with fresh growing medium (GM) that contained RM plus 10% bovine serum and samples for inhibitory activity assay. The cell number was determined by a hemocytometer (Coulter counter) after trypsinization (19). Triplicate identical plastic dishes (7.55 cm^2) were used per assay point to obtain the mean cell number. Percent inhibition was calculated as: [Cell count in GM − Cell count in (GM + sample)] ÷ Cell count in GM × 100. Percent stimulation was calculated as: [Cell count in (RM + sample) − Cell count in RM] ÷ Cell count in RM × 100. The standard deviation of cell counts by the mechanical and trypsinization procedure did not exceed 10% of mean value.

Assay of hepatic factors on synthesis of nucleic acid and protein in L-cells. The assay of nucleic acid and protein synthesis was carried out by the method of Rudland (20) and Schaefer (21) modified as follows: Induction of G_1-S phase transition of resting cells was performed by replacing RM with fresh GM, and labelling was performed with ^{3}H-thymidine (New England Nuclear, NEN, 0.5 Ci $mmol^{-1}$, 2.5 μCi ml^{-1}) for DNA synthesis, and ^{3}H-uridine (NEN, 0.6 Ci $mmol^{-1}$, 2 μCi ml^{-1}) for RNA synthesis, and ^{14}C-leucine (Radiochemical Centre, Amersham, 0.63 Ci $mmol^{-1}$, 0.25 μCi ml^{-1}) for protein synthesis. The assay was performed for 4 h from 16 to 20 h after medium change or 20 min for kinetic assay from the time indicated in the figures. Radioactivity on the glass filter (Whatman, 2.4 cm, GF/C) was counted in a toluene-based scintillator (22) using a scintillation counter, and radioactivity in the soluble fraction was counted in a scintillator containing Triton X-100 (22). The cell numbers used were 10^6 per plate (18.1 cm^2)

for DNA synthesis and 4×10^5 per plate for RNA synthesis. Four identical plates were used to obtain the mean value of each experimental point. Percent variation from the control value was calculated for inhibitory activity as: [c. p. m. in GM−c. p. m. in (GM+sample)]÷c. p. m. in GM×100. Percent variation from control for stimulatory activity was calculated as: [c. p. m. in (RM+sample) −c. p. m. in RM]÷c. p. m. in RM×100. The standard deviation did not exceed 15.4% of the mean value.

Protein concentration was determined by the method of Lowry *et al.* (23).

In vivo assay of in hibitory fraction on DNA synthesis in regenerating rat liver. The inhibitory fraction was dissolved in PBS (−) and sterilized, and 2.5−25 μg protein per g body weight was injected intravenously after partial hepatectomy (24). DNA synthesis *in vivo* was assayed for 1 h or 4h from 18h after hepatectomy by intravenous injection of ^{3}H-thymidine (25 μCi per rat). The radioactivity in the DNA fraction was counted in a scintillator containing Triton X-100 after DNA extraction (22).

RESULTS

Fig. 1 shows that the addition of the 45% ethanol saturation precipitate of normal liver (N45) inhibited proliferation of L-cells. On the other hand, the 70% ethanol fraction (N70) stimulated cell proliferation. These results sugest that normal hepatic cytoplasm contained an inhibitory factor and a stimulatory factor for cell proliferation. Table 1 shows that the 45%fraction from re-

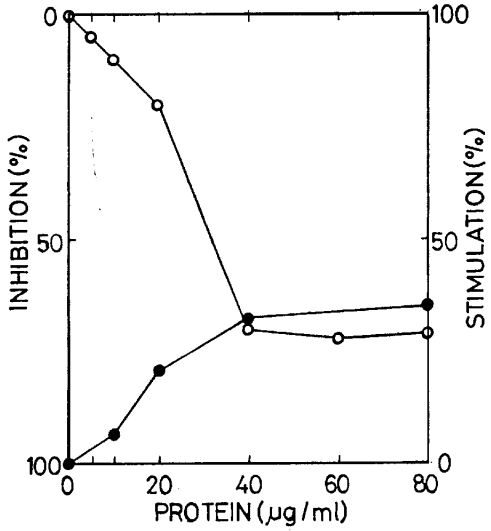

Fig. 1. Inhibitory and stimulatory effects of N45 and N70 on cell proliferation. N45 (○——○) was added to the growing medium (GM), N70 (●——●) was added to the resting medium (RM) at the amount indicated. The assay procedure is described in Materials and Methods.

TABLE 1. INHIBITORY AND STIMULATORY EFFECTS OF HEPATIC FRACTIONS ON PROLIFERATION OF L-CELLS

Systems	Cell count at 48 h incubation	% Variation from control
Growing control (GM)	4.68×10^5	100
GM+N45 (40 μg/ml)	0.86×10^5	18.5
GM+R45 (40 μg/ml)	5.58×10^5	119
Resting control (RM)	1.16×10^5	100
RM+N70 (40 μg/ml)	1.66×10^5	143
RM+R70 (40 μg/ml)	2.28×10^5	197

The experimental conditions are described in Materials and Methods. The abbreviated codes are described in the text.

generating liver (R45) did not have an inhibitory effect on cell proliferation and that the 70% fraction from regenerating liver (R70) strongly stimulated cell growth. This low inhibitory activity of R45 comparing with N45 was observed in five of seven batches of R45, and the data shown in Table 1 is from one of the typical cases.

TABLE 2. INHIBITORY AND STIMULATORY EFFECTS OF HEPATIC FRACTIONS ON DNA AND RNA SYNTHESIS

Systems	DNA synthesis (%)	RNA synthesis (%)
Growing control (GM)	100[a]	100[b]
GM+N45 (100 μg/ml)	32	41
GM+R45 (100 μg/ml)	108	95
Resting control (RM)	100[c]	100[d]
RM+N70 (100 μg/ml)	159	184
RM+R70 (100 μg/ml)	179	219

The assay systems and abbreviated codes are described in Materials and Methods.
The values indicate percent variation from control, assuming that the control is 100%.
The standard deviations did not exceed 15.4% of the mean values.
a Measured mean value was 5.92×10^4 c. p. m. per mg protein.
b Measured mean value was 10.7×10^4 c. p. m. per mg protein.
c Measured mean value was 1.12×10^4 c. p. m. per mg protein.
d Measured mean value was 3.53×10^4 c. p. m. per mg protein.

Table 2 shows the co-existence of inhibitory and stimulatory factors on nucleic acid synthesis in normal hepatic cytoplasm. N45 inhibited DNA and RNA synthesis in S-phase and N70 stimulated nucleic acid synthesis in S-phase. Table 2 also shows that R45 did not inhibit nucleic acid synthesis and that R70 stimulated nucleic acid synthesis in S-phase

Kinetic assays of the inhibitor (N45) on synthesis of DNA, RNA, and protein were performed in the S-phase system of L-cells. DNA synthesis was most strongly inhibited; RNA synthesis was slightly inhibited; and protein synthesis was not suppressed (Fig. 2). These results suggest that inhibition of proliferation and inhibition of nucleic acid synthesis are direct effects of N45.

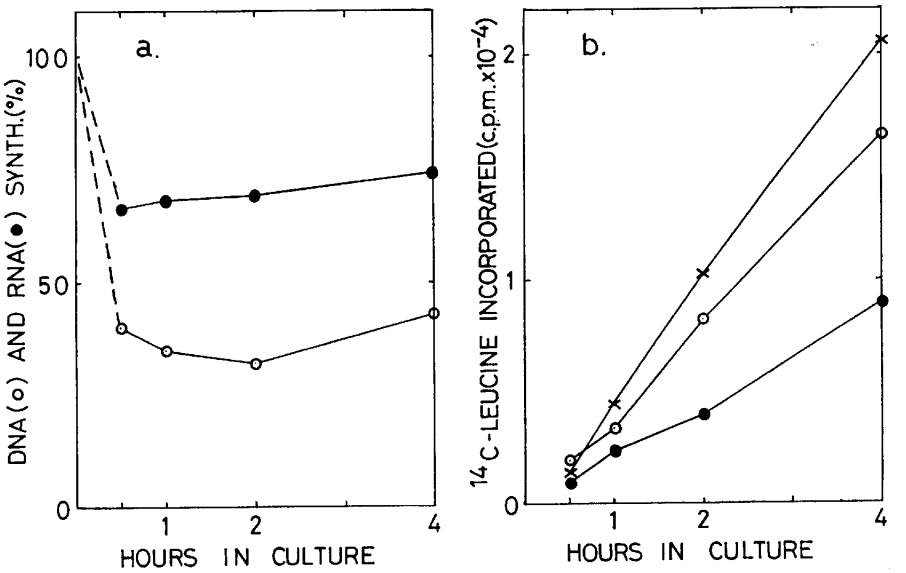

Fig. 2. a. Kinetic assay of N45 inhibitory effect on DNA (○——○) and RNA (●——●) synthesis. The inhibitor (N45) was added (100 μg/ml) at 0 h indicating that was 16 h after the addition of 10% bovine serum. L-cells were incubated with N45 for 0.5, 1, 2, and 4 h as indicated. The pulse labelling with ^{3}H-thymidine or ^{3}H-uridine was performed for 20 min at the time indicated. DNA synthesis of inhibitor-free control culture was 3.25×10^{4} c.p.m. per plate and RNA synthesis of control culture was 8.60×10^{3} c.p.m. per plate. Data are expressed as percent of control.

b. Effect of N45 on protein synthesis assayed kinetically by the same assay procedure. Data are expressed as c.p.m. of ^{14}C-leucine incorporated per plate. ●——●; Radioactivity in resting cell system, ○——○; growing control system, ×——×; experimental system (GM+N45).

The inhibitor in N45 also inhibited DNA synthesis *in vivo* (Table 3). Intravenous N45 injection just after hepatectomy inhibited DNA synthesis about 50% compared with the control. This indicates that the inhibitor is one of the native regulatory factors in the regenerating mechanism. Heat and trypsin treatment destroyed the inhibitory and stimulatory activities of these preparations (not shown).

TABLE 3. INHIBITORY EFFECT OF N45 ON DNA SYNTHESIS *in vivo* AFTER PARTIAL HEPATECTOMY

Experiment	Operation system	No. of rats	DNA synthesis[a]	% Variation from control[b]
I	Sham	6	517±150	—
	Hepatectomy (Hept.)	6	3040±932	100
	Hept.+N45 (2.5 μg/g body weight)	6	1980±722	58.0
	Hept.+N45 (25 μg/g body weight)	4	2200±772	66.0
II	Sham	3	442±119	—
	Hept.	3	21800±1200	100
	Hept.+N45 (24 μg/g body weight)	3	10400±2740	47.0

The assay system and radioactivity measurements are described in Materials and Methods. Rats weighed from 80-100 g. Abbreviations are the same as in Table 1.

a DNA synthesis indicate ^{3}H-thymidine incorporated in c. p. m. /mg protein ± standard deviation (S. D.).

b In Experiment I, the assay was performed for 1 h *in vivo* and in Experiment II for 4 h.

DISCUSSION

In liver regeneration after partial hepatectomy, humoral factors are of prime importance in triggering hepatocyte proliferation (1-10). Next in importance are changes in concentrations of cellular cyclic nucleotides and the activity of regulatory enzymes (25-27). Thirdly, stimulation of protein synthesis, RNA synthesis and a series of changes in enzyme activities follow to induce G_1-S phase transition.

In this investigation hepatic regulatory factors were shown to respond to changes in humoral factors induced by partial hepatectomy. The co-existence of an inhibitor and a stimulant modulating cell proliferation in the cytoplasm was demonstrated. This macromolecular inhibitor and stimulant may be included among other hepatic regulators of regeneration. The weakening of R45 inhibitory activity in regenerating liver is consistent with hepatocytic proliferation, and the data in the *in vivo* system suggest that the inhibitor is a native cytoplasmic regulator.

The physico-chemical properties and enzymatic activities of both factors and their roles in the regenerating mechanism have still to be investigated. The reason for the decreased R45 inhibitory activity not being observed in all batches is still unclear. Purification of the hepatic regulators is relevant for further analysis of the regulatory mechanism of hepatic regeneration.

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