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A correlation between liver plasma membrane-associated stimulatory activity (PMASA) and experimental cirrhosis formation

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In the course of experimental CCl_4-induced cirrhosis, an increase of the membrane-associated factor stimulating 3T3 cells' proliferation in vitro was observed. Gel filtration showed an approximate molecular mass of 150 kDa. Extraction of growth stimulatory activity by liver perfusion in situ demonstrated a peripheral plasma membrane protein localization. The activity increased with an increasing number of CCl_4 treatments, reaching a maximum at the tenth intoxication, faster than the proliferation of connective tissues. Cessation of treatment caused a decrease in activity to the level of untreated liver, although the amount of fibroblast-like cells remained large, which is evidence in favour of an hepatocyte origin of the factor.

Liver cirrhosis; Membrane protein

1. INTRODUCTION

Plasma membrane-associated activity stimulating fibroblast proliferation in vitro has been found on liver cells [1]. Excessive proliferation of connective tissue cells is the main morphological sign of both human and experimental cirrhosis of different etiology [2–5]. This process results in alterations in the liver structure. However, the mechanism of the induction of connective tissue cell proliferation has yet been unknown. We suppose that a correlation exists between experimental cirrhosis formation and PMASA levels.

2. MATERIALS AND METHODS

2.1. *Experimental animals*

Adult male A/He mice weighing 25–30 g were used. The experimental cirrhosis was induced [6] by CCl_4 inhalations at 30 mg/l air twice weekly for 4 h during 15 weeks.

2.2. *Cell culture*

Murine 3T3 (Swiss) cells were grown in 75 ml flasks (Costar, USA) in MEM with 0.1 mg/ml glutamine and 10% calf serum. For growth assays the cells were removed with a trypsin-EDTA solution (Flow, USA) and plated on MEM, containing 10% calf serum, on 12-well plates (Flow, USA) at a density of 5×10^4 to 1×10^5 cells in 1 ml per well.

2.3. *Plasma membrane preparations*

At the various times of CCl_4 treatment mice were sacrificed by cervical dislocation. The isolated liver was immediately washed in a cool 0.25 M sucrose solution, connective tissues were removed by pressing the liver through a metal sieve. Three volumes of sucrose solution were added to the pulverized liver tissue and homogenized with 4 passes of a teflon Potter–Elvehjem homogenizer at 1000–1200 rpm. Enriched plasma membrane fractions were isolated as described for rat livers [7] with the exception of the liver perfusion. The membranes were stored at −40°C until use.

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2.4. *Extraction of PMASA*

Step-by-step extraction of stimulatory activity regulating fibroblast proliferation was basically performed as described by Lieberman [1]. High-salt extracts containing PMASA were obtained by membrane incubation in 0.5 M NaCl, 50 mM Tris-HCl, pH 7.4 at 4°C for 1 h. After centrifugation at 78000 × *g* for 1 h, the supernatant was dialyzed against 50 mM Tris-HCl, pH 7.4, and concentrated to 1/2 of initial volume in Ficoll. The protein content was determined by Lowry [8]. The PMASA preparations were added to medium to achieve a final concentration of 1.5–2.0 μg/ml.

2.5. *Extraction of PMASA by perfusion*

Mice were sedated with 100 mg/kg hexabarbital (Serva, Germany) intraperitoneally. Catheters were inserted in the vena cava inferior above the renal vein, and in the portal vein and were secured with the sutures. The diaphragm was incised and the vena cava inferior was ligated supra-hepatically. In such a way the liver was perfused in situ through the portal vein [9] with a cool isotonic sucrose solution at 2 ml/min rate until it was clear of blood. The perfusate was then sequentially changed with 0.14 M NaCl and 0.5 M NaCl in 50 mM Tris-HCl buffer, pH 7.4. 4 ml of each solution were circulated in a closed perfusion system for 5 min. The solutions were collected, dialyzed against the initial buffer, and assayed for protein and growth stimulatory activity. Cellular integrity during perfusion with a hypertonic NaCl solution was assessed by the activity of lactate dehydrogenase (LDH-R Reagent, SCE Labsystems, Finland).

2.6. *Gel filtration*

High-salt extract (35 μg protein) was dialyzed against 50 mM Tris-HCl buffer, pH 7.4 and was applied to a 1 × 5 cm Sephadex G-150 column equilibrated with this solution. 0.5 ml fractions were collected and assayed for the stimulatory activity in DNA synthesis as described below. For the determination of the molecular mass, Blue

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dextran (2000000, Reanal, Hungary), IGG (160000, Sigma, USA), BSA (67000, Sigma, USA) and trypsin (23000, Spofa, CSSR) were used as markers.

2.7. PMASA assay

PMASA was assayed in extracts from the hepatocytes of the intact liver and from the liver after 1, 2, 5, 14, 23, 26 and 30 treatments with CCl_4. The assays were repeated on animals 6 weeks after the 30th treatment as well. 3T3 cells plated at a density of 1×10^5 cells/well were incubated for 24 h in MEM containing 10% calf serum. Cell growth was retarded by incubation in medium containing 1% plasma for 24 h. Then PMASA was added to the cells for 12 h. Cells incubated for 24 h in 1% plasma alone and those restimulated in MEM plus 10% serum were used as controls. All the experiments and controls were repeated 4–6 times. Mitogenic activity of the extracts was assayed by [^{3}H]thymidine incorporation into DNA of 3T3 cells. [^{3}H]Thymidine (50 mCi/mmol) was added in a concentration of 1 μCi/ml medium. After a 24 h incubation cells were removed, precipitated with 10% TCA and trapped on nitrocellulose filters (1.5 μm pore size, SYNPOR, CSSR). The control cells (cultivated in 1% plasma or 10% serum) were treated identically.

3. RESULTS

High-salt extracts from liver cells of CCl_4-treated animals were tested for the growth stimulatory function in vitro after various periods of CCl_4 treatment. DNA labelling in 3T3 cells was much greater than in control cells even after the first inhalation. PMASA increased during the experiment and achieved a maximum in the extracts derived from the liver cells after 10 inhalations. Six weeks after cessation of the inhalations, the level of PMASA fell to that of the untreated animals (Fig. 1), though morphological analysis showed that the content

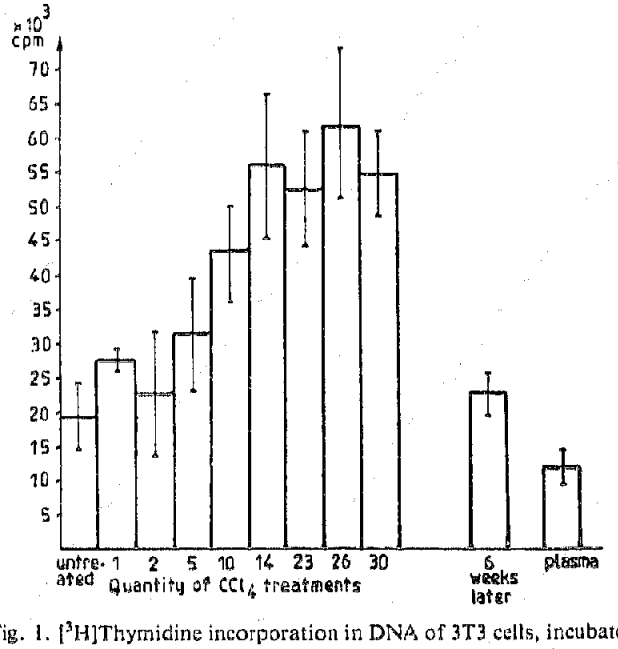

Fig. 1. [^{3}H]Thymidine incorporation in DNA of 3T3 cells, incubated in 1% plasma, after addition of PMASA from normal livers and from the livers after 1, 2, 5, 10, 14, 23, 26 and 30 CCl_4 treatments, and 6 weeks after cessation of inhalations. Cells incubated in 1% plasma without addition of active substance were used as controls. $n = 6$.

Table I

Mitogenic activity of the two fractions obtained by gel filtration on G-150 Sephadex

	150 kDa fraction (cpm)	40 kDa fraction (cpm)
Intact animals	200 ± 115	582 ± 74
14 CCl_4 treatments	1642 ± 78	876 ± 106

Cells incubated in 1% plasma without addition of active substance were used as controls. The values after subtraction of controls are shown. $n = 4$.

in connective tissue cells remained much higher than in the controls [10].

Salt extracts from livers of the untreated animals and from the animals after 14 treatments were subjected to gel filtration on G-150 Sephadex. Fractions were assayed for mitogenic activity. Two fractions were shown to have marked mitogenic activity eluting at approximate molecular masses of 150 kDa and 40 kDa. The high molecular mass fraction exhibited an 8.2-fold increase in stimulatory activity after the 14th inhalation compared with the untreated animals, while the 40 kDa fraction showed only a 1.5-fold increase (Table I).

The fact that mitogenic activity was also eluted in the solutions with which the liver had been perfused in situ may prove that the growth stimulatory activity is localized on the external surface of the membrane. The highest activity was extracted with a 0.5 M NaCl solution, similar to the PMASA extraction from membranes (Table II).

Lactate dehydrogenase activity was not detectable in the perfusates suggesting that cells remained undamaged during the perfusion.

4. DISCUSSION

These results suggest that PMASA may regulate proliferation of connective tissues during the development of experimental cirrhosis. During repeated exposure to CCl_4, an increase in stimulatory activity is followed by growth of connective tissue, as determined histologically. Enhanced activity of the factor having an apparent molecular mass of 150 kDa (PMASA), which is similar to that previously observed in intact livers [1], was

Table II

Mitogenic activity of the solutions, with which the livers of mice were perfused after mice had been treated with CCl_4 14 times

	0.14 M NaCl	0.5 M NaCl
cpm	588 ± 104	1665 ± 128

Cells incubated in 1% plasma without addition of active material were used as controls. The values after subtraction of controls are shown. $n = 6$.

shown. PMASA is supposed to be a peripheral plasma membrane protein which is localized on the external surface. During the course of CCl_4 exposure PMASA accumulation precedes connective tissue forming. Cessation of treatment leads to a decrease in PMASA levels to that of untreated liver, though the amount of connective tissue remains large.

Thus, our data show that repeated disruptions of liver parenchyma lead to a dramatic increase in PMASA levels. The fact that these changes precede morphological signs of cirrhosis allows speculations about the regulatory role of PMASA in experimental cirrhosis.

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