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HEPATOCYTES FROM THE LIVER OF MICE WITH EXPERIMENTAL POST-TOXIC CIRRHOSIS STIMULATE DNA SYNTHESIS IN HETEROKARYONS IN NUCLEI OF RESTING NIH 3T3 FIBROBLASTS

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Chronic inflammation and necrosis in the liver parenchyma in cirrhosis of varied etiology are accompanied by pathological morphological and functional changes caused by local or general proliferation of the hepatic connective tissue. Fibrosis of the liver not only prevents the course of regeneration in the parenchyma of the lobules, but also disturbs function of the vascular system of the liver, leading to portal hypertension and to further aggravation of the pathological process [2]. The question arises of the mechanisms of stimulation of proliferative activity of the fibroblasts of the connective-tissue basis of the liver in chronic inflammatory and degenerative processes, for knowledge of these mechanisms would enable ways of their specific blocking to be found.

Experiments with fusion of hepatocytes, obtained from the liver regenerating after partial hepatectomy, and also of cells obtained from embryonic liver (15-18-day embryos) with resting NIH 3T3 fibroblasts have shown considerable stimulation of DNA synthesis in heterokaryons in the fibroblast nuclei [3]. We also observed stimulation of DNA synthesis in mononuclear cells (monokaryons) both in fusion experiments and during combined culture of fibroblasts with these hepatocytes. These facts suggest that hepatocytes obtained from the actively proliferating liver form factors which can stimulate proliferation of cells of mesenchymal origin.

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It can be tentatively suggested that hepatocytes obtained from a liver with experimental post-toxic cirrhosis [6] also have the ability to stimulate fibroblast proliferation. To test this hypothesis, we carried out fusion of hepatocytes from the liver of mice exposed to chronic poisoning by the hepatotoxin CCl_4 with resting NIH 3T3 fibroblasts. We found that in heterokaryons obtained as a result of fusion of these hepatocytes with fibroblasts resting in a medium with low serum concentration active entry of the fibroblast nuclei into the S-period takes place after fusion of the cells in medium with a low serum concentration, i.e., in the absence of exogenous stimulation of their proliferation.

EXPERIMENTAL METHOD

Experiments were carried out on female BALB/c mice weighing initially 20-25 g. Pathological changes in the liver (experimental post-toxic cirrhosis) were induced by repeated inhalation of CCl_4 in a special chamber for 3.5-4 h twice a week, with CCl_4 in a concentration of 30 mg/liter in air [6]. After 10 to 12 inhalations the liver of the anesthetized animals was removed, and using nonrecirculating two-stage (nonenzymic and enzymic) perfusion, isolated hepatocytes were obtained by the method described previously [3]. The fraction of dead cells in the hepatocyte suspension was determined by means of a 0.5% solution of trypan blue in 0.85% NaCl at pH 7.2, and amounted to 10-12% for the suspension of "CCl_4-hepatocytes" and 4-6% for the suspension of intact adult hepatocytes. The hepatocytes thus obtained were kept until fusion in complete NCTC-135 medium, containing 0.2% fetal calf serum (FCS), 0.1% bovine serum albumin, 0.1% glucose, 0.05 μg/ml insulin ("Serva"), 50 mg/ml dexamethasone, 0.1 mM β-mercaptoethanol, 20 mM HEPES-buffer, and 100 μg/ml kanamycin sulfate. Heterocyte suspensions from two animals were pooled and used for fusion with resting NIH 3T3 fibroblasts. The resting fibroblasts were obtained by culturing the cells for 3 days in medium NCTC-135 containing 0.2% FCS and 0.2 mM glutamine. The index of ^{3}H-thymidine-labeled cells in this case was 6-9%. To improve adhesion of the hepatocytes to the coverslips in the fusion experiments, before the fibroblasts were seeded (10,000-15,000 cells/cm^2) the glass was treated with type I collagen, obtained from rat tails by the method in [4]. The collagen solution was sterilized by filtration and the coverslips were coated under sterile conditions and dried for 2 days at room temperature.

The cells were fused in filed penicillin flasks in which the round coverslips with the resting fibroblasts were placed. A suspension of hepatocytes (2 ml, about 100,000 cells per flask) was layered above them, and the cells were sedimented by centrifugation at 1000g for 5 min on a hematologic centrifuge with rotor of special design. After centrifugation the flasks were placed for 30 min in a CO_2-incubator with 6% CO_2. The supernatant was then decanted and 0.5 ml of a 40% solution of polyethylene-glycol (PEG; 6000, from "Merck") was poured into each flask and centrifugation continued for 1 min. The PEG solution was diluted and the cells washed off with pure medium NCTC-135. After fusion the cells were transferred into medium NCTC-135 containing 0.2% or 10% FCS, 0.5 mM glutamine, 0.1 mM β-mercaptoethanol, 20 mM HEPES-buffer, 100 μg/ml kanamycin sulfate, and $3.7 \cdot 10^4$ Bq/ml (1720 TBq/mole) ^{3}H-thymidine. The cells were incubated in this medium for 16-22 h before fixation. Nuclei labeled with ^{3}H-thymidine were detected by autoradiography, using type K2 nuclear emulsion ("Ilford"). After development of the autoradiographs they were stained by the Giemsa method and examined under the microscope.

The mitogenic activity of medium conditioned by "CCl_4-hepatocytes" and also of the blood serum obtained from the experimental animals was determined and compared with activity of other conditioned media and serum by measuring stimulation of DNA synthesis in NIH 3T3 fibroblasts, resting or stimulated for 10 h with 10% FCS. For this purpose cells cultured in 24-well planchets ("Linbro") ($5 \cdot 10^4$ cells per well) were transferred into the corresponding medium, or a particular serum (the data were given in the caption to Fig. 2) and ^{3}H-thymidine (3.7 kBq/well, 187 GBq/liter) were added to serum-free medium. After 16 h the cells were washed with Hanks' solution and removed with the aid of 2.5% trypsin ("Flow Laboratories"). The cells were then harvested on GF/C filters ("Whatman"), washed with 0.85% NaCl solution and 7.5% TCA, and fixed with 96% ethanol. The dried filters were examined in a liquid scintillation β-counter. Five wells were tested at each point.

Condition media were obtained by culturing hepatocytes (100,000 cells per well in 2 ml medium) in complete NCTC-135 medium with 2% FCS for 2 days.

EXPERIMENTAL RESULTS

The liver of mice receiving 10-12 inhalations of CCl_4 differed morphologically from the intact liver. It was paler in color and firmer in consistency. Under a binocular loupe its surface had a finely nodular structure, with nodules measuring

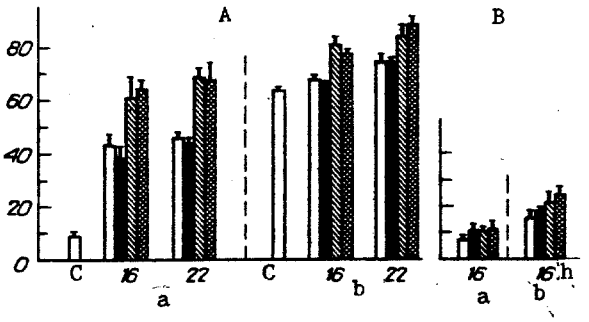

Fig. 1. Incorporation of ^{3}H-thymidine into nuclei of resting fibroblasts after their fusion with hepatocytes obtained from liver damaged by CCl_4 (A) and from intact adult liver (B) in medium with 0.2% (a) or 10% (b) serum. Cells were cultured before fixation for 16 or 22 h. In the control, fibroblasts were cultured without hepatocytes. Unshaded columns — monokaryons; black — homodikaryons; obliquely shaded — heterodikaryons; cross-hatched — heterotrikaryons. C) Control; 16, 22) 4 and 10 p.m. Ordinate, number of labeled nuclei (in %).

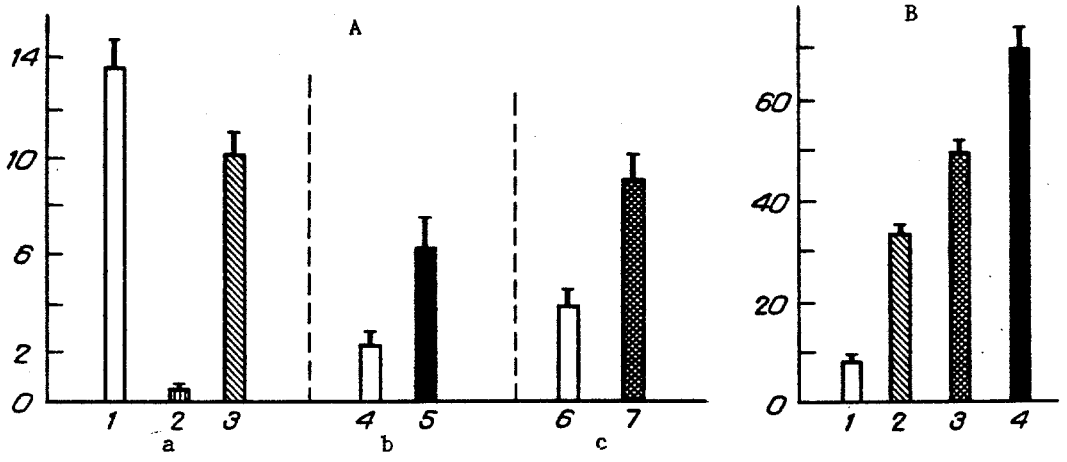

Fig. 2. Dependence of proliferative activity of fibroblasts on conditions of culture. A: a) Resting fibroblasts in medium with 10% serum (1); intact fibroblasts in medium with 10% serum (2); combined culture of resting fibroblasts and intact hepatocytes in medium with 10% serum (3); b) resting fibroblasts in medium with 2% serum, conditioned by intact hepatocytes (4); resting fibroblasts in medium with 2% serum conditioned by "CCl_4-hepatocytes" (5); c) stimulated fibroblasts in medium with 2% serum, conditioned by intact hepatocytes (6); stimulated fibroblasts in medium with 0.2% serum, conditioned by resting fibroblasts (7). Ordinate, incorporation of label (in cpm·10^{-4}). B) Resting fibroblasts in medium containing 0.2% (1) and 5% (2) fetal calf serum, 5% intact mouse serum (3), and 3% serum from mice receiving 10 inhalations of CCl_4 (4). Ordinate, incorporation of label (in cpm·10^{-3}). In all experiments the cells were cultured for 16 h.

3-5 mm, separated by fibrous septa 1 mm thick. During perfusion, this liver exhibited higher hydraulic resistance than the intact liver. The data in Fig. 1A show that the number of labeled fibroblast nuclei in heterodi- and heterotrikaryons in medium with 0.2% serum was greater than in mono- and homodikaryons, and significantly higher than in the control, when

fibroblasts were cultured in the absence of hepatocytes. In medium with 10% serum the index of labeled nuclei in the heterokaryons also was higher than in mono- and homodikaryons.

Conversely, during fusion with intact hepatocytes (Fig. 1B) entry of nuclei of the resting fibroblasts into the S-period was not stimulated in medium with 0.2% serum, and significant inhibition of proliferative activity was observed in medium with 10% serum, as also was observed during combined culture of intact hepatocytes and resting fibroblasts in medium with 10% serum (Fig. 2A, a). Furthermore, medium conditioned by normal hepatocytes possessed activity inhibiting proliferation of the stimulated fibroblasts (Fig. 2A, b). These results suggest that "CCl_4-hepatocytes" also introduce into heterokaryons a factor stimulating DNA synthesis in resting fibroblast nuclei. This factor is evidently secreted into the culture medium also, for stimulation of DNA synthesis also took place in mono- and homodikaryons.

Since heterokaryons are formed both on fusion of a fibroblast with two hepatocytes and from binuclear hepatocytes, it must be expected, taking into account the possible dose effect, that stimulation ought to be stronger in them than in heterokaryons. However, this was not so. It was found previously that the plasma membranes of hepatocytes from intact liver contain both stimulating factors and factors inhibiting proliferation of Swiss 3T3 fibroblasts [5]. The proliferation stimulating factor evidently is one of the surface proteins, for it is easily removed by mild treatment of the membranes by various methods. The data indicating topologic and temporal noncoincidence of the zones of damage and of subsequent fibrosis in cirrhosis led Belyaev and co-workers [1] to postulate that increased proliferation of stromal fibroblasts is linked with the appearance of inducers of fibrogenesis in the liver. The authors cited demonstrated that the proliferation-inducing activity of a factor extracted from hepatocyte plasma membranes increases in the course of development of experimental post-toxic cirrhosis of the liver in mice. It can be tentatively suggested that the action of this factor is aimed at stimulating proliferative activity of the hepatocytes themselves. However, even in heterokaryons, ^{3}H-thymidine was incorporated by only 0.7-7% of hepatocyte nuclei, and only solitary free-lying hepatocytes were labeled.

To study the ability of "CCl_4-hepatocytes" to secrete a mitogenic factor into the medium, we prepared a medium conditioned by them. The data in Fig. 2A, b show that this medium possessed significantly higher mitogenic activity than medium conditioned by intact hepatocytes.

Finally, blood serum from mice exposed to chronic CCl_4 poisoning also was found to have increased mitogenic activity, as is clear from the data given in Fig. 2B.

The study thus revealed the ability of hepatocytes from the liver of mice exposed to chronic CCl_4 poisoning to form a factor possessing the ability to stimulate proliferation activity of cells of mesenchymal origin and to secrete it into the medium (blood).

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