The results of the present investigation are evidence that the stromal tissue of the bone marrow microenvironment in mice undergoes significant structural changes with age. However, these changes can be revealed only under experimental conditions that simulate an extraordinary local regenerative demand.

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INHIBITION OF α-FETOPROTEIN SYNTHESIS IN ADULT MOUSE HEPATOCYTES BY DEXTRAN SULFATE IN VIVO AND IN VITRO

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UDC 616.36-008.939.6-053.13-02:615.384

KEY WORDS: α-fetoprotein biosynthesis; mouse hepatocytes; dextran sulfate.

Much evidence has recently been published that charged polymers are activators of cells of the immune system [4, 5]. The mechanism of action of polyelectrolytes on cells has not been finally elucidated, but even at this stage it can be asserted that it is largely connected with direct interaction of charged macromolecules with components of the plasma membrane [4, 5]. The use of polyelectrolytes as agents regulating various cell functions through the membrane may also be interesting when other cell types are to be acted upon.

The effect of polyelectrolytes on primary monolayer cultures of adult mouse hepatocytes was investigated. It was found previously that hepatocytes from the intact adult mouse liver in culture change their functional activity significantly and begin to synthesize the embryospecific protein α-fetoprotein (AFP) intensively [2, 3, 8]. Despite numerous attempts, it has not yet proved possible to regulate this process. One synthetic polyanion, dextran sulfate (DS), has been found to induce considerable morphological changes in hepatocytes in culture, when AFP synthesis is appreciably inhibited; DS also inhibits AFP synthesis in vivo in the mouse liver regenerating after CCl_4 poisoning.

EXPERIMENTAL METHOD

A suspension of hepatocytes was obtained from the liver of adult C57BL/6 mice after perfusion with solutions of EGTA and collagenase. The procedure used to isolate and culture the cells was fully described previously [2, 3]. On the 3rd-6th day of culture the hepatocytes

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Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 103, No. 4, pp. 470-473, April, 1987. Original article submitted April 2, 1986.

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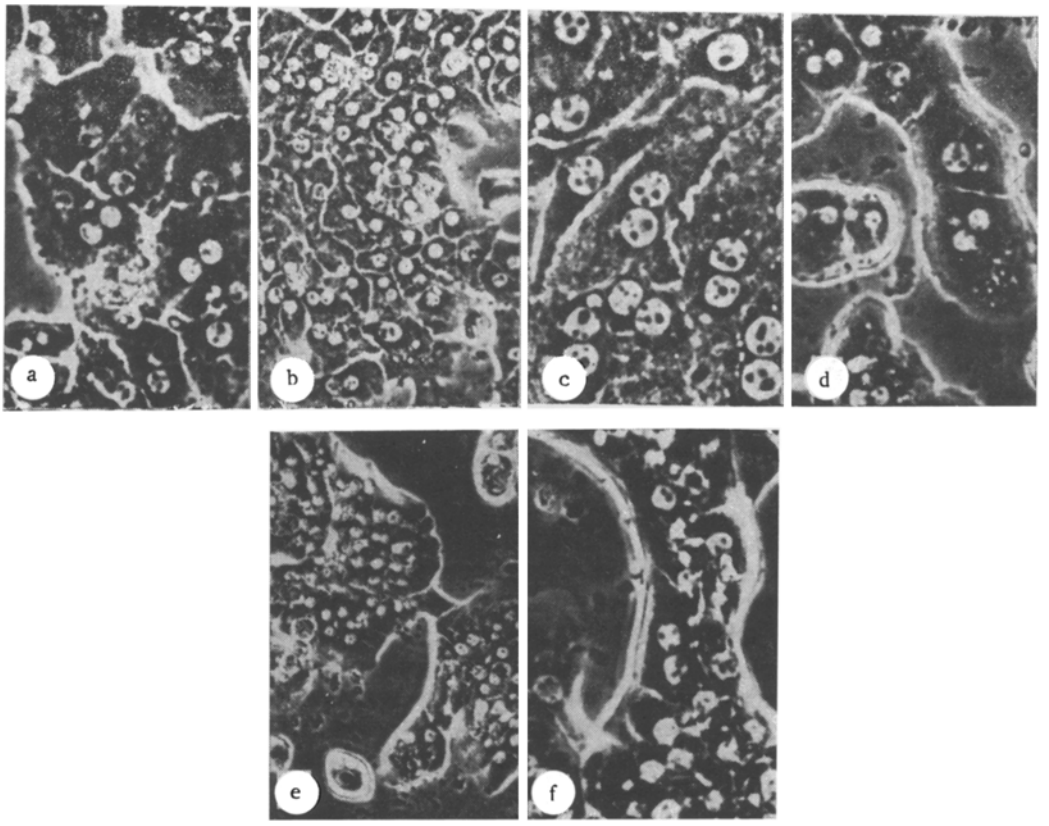

Fig. 1. Morphology of hepatocytes cultured in monolayer without DS (a-c) and with DS (d-f). a, d) First day of culture (250 ×); b, e) 2nd day of culture (120 ×); c, f) 3rd day of culture (magnification: c — 500 ×, f — 360 ×). Living culture, phase contrast.

were fixed with 4% formaldehyde solution in buffered physiological saline with 0.05% saponin for 20 min at room temperature. The cells were washed free from formaldehyde with buffered physiological saline containing saponin for not less than 4 h.

Various natural and artificial polyelectrolytes were added to the cultures: DS (50-100 μg/ml, from Pharmacia, Sweden), DEAE-dextran (1-10 μg/ml, Pharmacia), polylysine with mol. wt. of 110 kilodaltons (1-10 μg/ml, from Sigma, USA), heparin (50-200 μg/ml, Sigma), chondroitin sulfate (100 μg/ml, from Koch-Light, England), and carrageenan (50-100 μg/ml, from Sigma). The polyelectrolytes were added either immediately after adhesion of the hepatocytes to the substrate and washing to remove nonadeherent cells, or at different times during culture.

Regeneration of the liver was induced in AKR mice by the action of CCl_4 vapor [1]. Injections of DS were given 3-5 h after inhalation of CCl_4, and subsequently repeated after 24 and 48 h. DS was injected intraperitoneally in physiological saline in doses of 50 , 25, and 5 μg/g. The mice were killed on the 2nd and 3rd days of regeneration. The blood AFP level was determined by semiquantitative gel immunodiffusion, using a standard test system [6].

The localization of AFP in the hepatocytes cultured was studied by the indirect immunoperoxidase method. Permeability of the cells for antibodies was ensured by the presence of 0.05% saponin in the fixing solution and all subsequent fluids. AFP was detected in the regenerating mouse liver in paraffin sections prepared by the method described previously, using the same immunoperoxidase technique. The localization of AFP was determined with monospecific affinity-purified rabbit antibodies to mouse AFP and porcine antibodies against rabbit IgG, conjugated with horseradish peroxidase (Dako, Denmark). Peroxidase activity was detected with diaminobenzidine tetrahydrochloride.

EXPERIMENTAL RESULTS

As was described previously [2, 3], isolated hepatocytes became adherent to the substrate (gelatinized plastic) in the course of 1-2 h in the presence of serum, after which they began to spread out. The spreading process took 12-16 h, and as a result, if the density of the

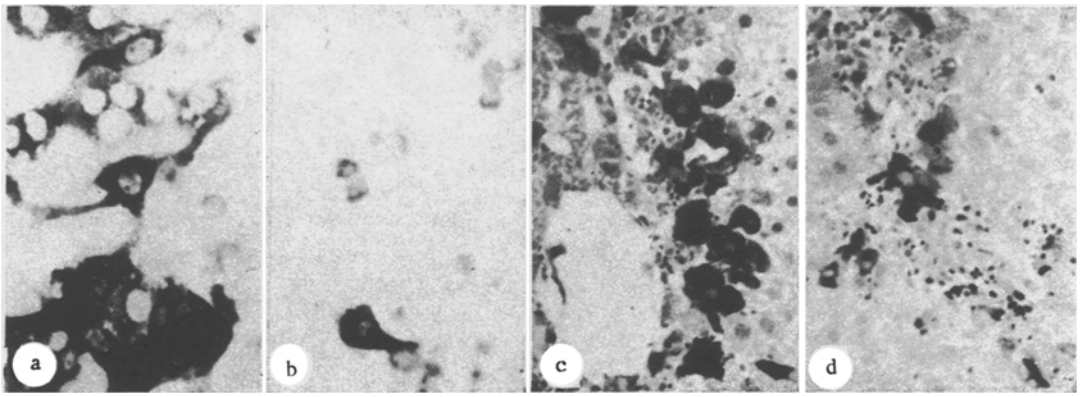

Fig. 2. Detection of AFP in hepatocyte cultures and sections of regenerating mouse liver by the indirect immunoperoxidase method. a, b) AFP in hepatocytes on 4th day of culture; a) control cultures, b) culture in presence of DS (300 ×); c, d) AFP in regenerating mouse liver on 3rd day after CCl_4 poisoning; c) control liver; d) mouse liver after three injections of DS (150 ×).

suspension was high enough (0.4-0.6 million living cells per Petri dish 4 cm in diameter), a monolayer of well-spread polygonal hepatocytes was formed after 20-24 h in culture. This monolayer persisted until 48-72 h of culture. After that time the hepatocytes, especially those at the periphery of the islets and large sheets of cells, spread out even more and became spindle-shaped, and sometimes fibroblast-like. Some hepatocytes began to divide. In some cases irregularly shaped cells formed a kind of network above the layer of hepatocytes. The hepatocyte monolayer is thus not a stable formation, and by the 3rd day of culture it commences its irreversible evolution (Fig. 1a-c). On the addition of DS immediately after adhesion of the hepatocytes, within a few hours a clear difference from the control was observed. With DS in a concentration of 50-100 μg/ml, spreading of the hepatocytes was significantly inhibited. The cells often formed characteristic bands, rather reminiscent of hepatic trabeculae. Inhibition of spreading was observed throughout the time when DS was present in the culture (Fig. 1d-f) and it was still preserved even after incubation of the cells for 24 h to remove DS. Addition of DS to the culture after 24 h, and not during the first few hours of culture, led to equally effective inhibition of spreading after 48 h of culture, just as when DS was present in the culture during the first few hours of its existence. Addition of DS on the 2nd-4th day of culture to hepatocytes which had already spread out not only prevented further morphological evolution of the cultures, but also returned the cells to a less widely spread state.

Longer exposure of the hepatocytes to collagenase than in the present experiment (we isolated the hepatocytes after perfusion with 25 ml of 0.02% collagenase and subsequent incubation of the isolated liver in the same collagenase solution for 10 min) significantly weakened the action of DS on hepatocyte morphology.

During the action of other polyelectrolytes it was found that polylysine and DEAE-dextran were highly toxic for hepatocytes and did not affect their spreading out. Heparin and chondroitin sulfate were not toxic for hepatocytes but likewise had no effect on their spreading out. Finally, the action of carrageenan was exactly like the action of DS.

As has previously been stated [2, 3, 8], AFP synthesis takes place in cultures of adult mouse hepatocytes on the 3rd day of culture. After 4 days AFP can usually be detected immunohistochemically in 20-30% of hepatocytes (Fig. 2a). On the 5th-6th day of culture the proportion of hepatocytes producing AFP may be even higher. AFP production is sharply inhibited in the presence of DS. The number of AFP-producing cells was reduced by 6-8 times compared with the control. In some cases only solitary AFP-producing cells were found in the presence of DS, and sometimes their total absence was observed (Fig. 2b). If DS was added, not during the first few hours of culture, but after 24, 48, or 72 h, marked inhibition of AFP synthesis was observed by the 4th day. If DS was added only on the 4th day (when active AFP synthesis was already in progress) no significant inhibition of AFP synthesis was found by the 5th day, although transition of the hepatocytes into a less widely spread state and the formation of protein-like structures took place as before.

Of all the remaining polyelectrolytes investigated only carrageenan inhibited AFP synthesis, while the rest did not affect this process.

Having obtained definite inhibition of AFP synthesis with the aid of DS in vitro, it was decided to study the effect of DS on AFP synthesis in vivo during regeneration of the liver. During regeneration of the liver after CCl_4 poisoning, the greatest rise of the blood AFP level and the peak of the number of AFP-containing cells in the liver are observed on the 3rd-4th day [7]. In the AKR mice used for these experiments, on the 3rd day of regeneration after CCl_4 poisoning about 50-100 μg/ml of AFP was found in the blood. Daily injection of 50 μg/g of DS lowered the AFP level below 10 μg/ml on the 3rd day. Reducing the dose of DS led to weaker inhibition of AFP production. Immunohistochemical detection of AFP in the regenerating liver showed that DS reduces the number of AFP-containing cells about by one order of magnitude (Fig. 2c, d).

DS is thus a powerful inhibitor of AFP re-expression in adult hepatocytes both in vitro and in vivo. The mechanism of this phenomenon is not yet completely clear. We consider it most probable that it is connected with the ability of DS to stabilize protein complexes of the extracellular matrix [9]. DS simulates the action of heparin-sulfate-proteoglycan in this process. This is to some extent clear from the similarity of action of DS and carrageenan. Both these polysaccharides are high-molecular-weight compounds, which are strongly sulfated, and from this point of view they are similar to heparin-sulfate-proteoglycan. The natural polyanions heparin and chondroitin sulfate are less strongly sulfated than DS, and of course they have a much smaller molecular weight.

The possibility cannot be ruled out that similarity of DS with heparin-sulfate-proteoglycan is important also for the other functional effect of DS and, in particular, for its ability to act as a polyclonal activator of lymphocytes. It may be that DS in this case simulates the action of natural cellular modulators.

The authors are grateful to V. S. Poltoranina for providing monospecific antibodies to AFP, and also to R. M. Khaitov, G. I. Abelev, and R. I. Ataullakhanov for continuous advice and valuable help in the course of the work. The investigation was partially subsidized by the immunological division of WHO (World Health Organization).

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