The investigation showed that castration, like free ovarian transplantation after preliminary castration, leads to a decrease in the serum zinc concentration, and reduction of zinc excretion with the urine. The significant differences in the serum estradiol concentration in the animals of these groups do not lead to any significant differences in zinc metabolism, probably due to the lower blood testosterone level than in the control.

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EFFECT OF CONDITIONED MEDIUM OF NEONATAL RAT HEPATOCYTES ON MIXED CULTURE OF KUPFFER CELLS AND FIBROBLAST-LIKE LIVER CELLS

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Kupffer cells (KC) of the liver account for about one-tenth of the cell mass of the liver [9]. The KC are small and occupy about 2% of the volume of the liver [5]. The importance of KC in the body is great. They are unique "cleaners," they play an important role in purification of the circulating fluid from defective and foreign compounds and particles, including aging erythrocytes, liposomes, microorganisms and endotoxins, they remove cholesterol esters [10], and they specifically generate glycoproteins from the blood and utilize them [7]. KC form prostaglandins and thromboxane, and accordingly they play an important role in the regulation of glycogenolysis and of the hemodynamics in the liver [11], and regulate the blood flow through the hepatic sinusoids, working like sphincters [6].

During work with a culture of neonatal rat hepatocytes [2] we paid particular attention to the fact that during culture of these cells for more than 2 weeks, fibroblast-like cells actively proliferate, and small independent colonies of KC and endothelial cells also begin to appear, although the latter usually cannot be cultured without growth factors. The morphological picture observed suggests that during mixed culture the role of growth and differentiation factors is played by cell junctions or by soluble compounds present in the culture medium. We studied the effect of conditioned medium of a neonatal rat hepatocyte culture on a mixed culture of KC and fibroblast-like rat liver cells during culture for 12 days.

EXPERIMENTAL METHOD

The method of obtaining the neonatal rat hepatocyte culture was previously described in detail [2].

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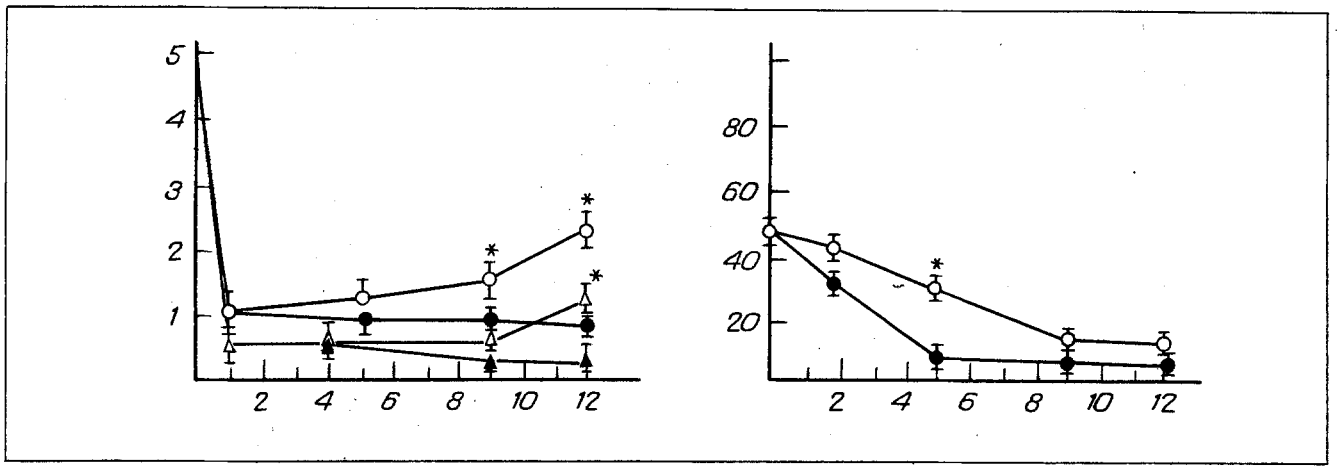

Fig. 1 Fig. 2

Fig. 1. Changes in number of cells in mixed cultures of fibroblast-like and Kupffer cells during culture in Eagle's medium with addition of 10% fetal calf serum, and with the use of conditioned medium of neonatal rat hepatocytes. Abscissa, duration of culture (in days); ordinate, number of cells ($\times 10^{-3}$). Filled circles and triangles denote control cultures of neonatal and mature rats respectively, empty circles and triangles denote culture of the same cells in the presence of conditioned medium of neonatal rat hepatocytes.

Fig. 2. Change in relative proportion of KC in population during combined culture with fibroblast-like cells of neonatal rat liver. Abscissa, duration of culture (in days); ordinate, fraction of KC in population (in %), determined on fixed preparations. Filled circles – control cultures of neonatal rats, empty circles – culture of the same cells in presence of conditioned medium of neonatal rat hepatocytes; *) significant ($p < 0.01$) differences compared with control.

To obtain a mixed culture of KC and fibroblast-like neonatal rat liver cells, the supernatant collected after sedimentation of the hepatocytes was centrifuged at 2000*g* for 5 min. The residues were collected, resuspended, and the cells were seeded on glass in Carrel's flasks or Petri dishes at the rate of 5×10^5 cells/ml in Eagle's medium with the addition of 10% fetal calf serum ("Sigma") and 200 U/ml of penicillin. The medium was changed on the day after seeding of the cells. When conditioned medium was used, conditioned hepatocyte medium was added to the original growth medium in the ratio of 2:1. Later the medium was changed twice a week.

Conditioned medium was collected on the 2nd-4th days of culture of the neonatal rat hepatocytes [2]: 2 ml of medium was collected with cells in culture. The medium was collected 48 h after a routine change, and centrifuged at 5000 rpm for 10 min. The supernatant was separated and kept at 4°C for up to 6 weeks without loss of activity.

Neonatal rat hepatocytes were isolated by two-stage enzymic treatment of the liver in situ [1]. For enzymic dissociation of the tissue 0.5 mg/ml collagenase in medium 199 was used. The hepatocytes were cultured under the same conditions as the hepatocytes of the newborn rats. Mixed cultures of KC and fibroblast-like cells were obtained in the same way as the corresponding neonatal rat cultures.

The morphological tests were carried out on preparations stained with hematoxylin and eosin. For quantitative evaluation of the morphological changes, at least 1000 cells were counted in each preparation (objective 40, ocular 10). The mean value was determined for four or five preparations taken from different experiments. The total number of cells in the population was estimated on native preparations stained with trypan blue (0.6%), after removal from the glass with 0.25% trypsin solution. The numerical results were subjected to statistical analysis by Student's *t* test.

EXPERIMENTAL RESULTS

By seeding neonatal rat cells collected after sedimentation of the hepatocytes on glass without a collagen support, a mixed culture of KC and fibroblast-like cells was obtained. The concentration of the cells during seeding must not fall below 5×10^5 cells/ml, for a high proportion of the cells, including endothelial cells and blood cells, was washed off the

TABLE 1. Mitotic Activity and Changes in Number of Nuclei in KC and Fibroblast-like Cells of Neonatal Rat Liver during Combined Culture (M ± m)

Parameter	Time of culture, days			
	2	5	9	12
Mitoses, %	0	0	0	0
	Eagle's medium with addition of 10% fetal calf serum			
Number of nucleoli				
in fibroblasts	2,27±0,09	2,15±0,06	2,04±0,05	1,46±0,03*
in KC	1,13±0,07	1,12±0,07	1,17±0,06	1,15±0,04
	Addition of conditioned hepatocyte medium			
Mitoses, %	0	0,11±0,05	0,61±0,07*	0,97±0,11*
Number of nucleoli				
in fibroblasts	2,17±0,07	2,35±0,07	2,84±0,09*	2,75±0,09*
in KC	1,2±0,06	1,27±0,06	1,39±0,09	1,85±0,09*

Legend. *) Differences from original data significant.

glass at the first change of medium. In the original population 24 h after seeding 40-45% consisted of KC and the remainder of fibroblast-like liver cells.

In the course of culture for 1-2 days the fibroblasts spread out in a layer and numerous nucleoli appeared in their nuclei. The KC adhered to the glass but remained round, and their lamella was under strong tension.

By the 4th-5th days of culture the number of cells still remained unchanged (Fig. 1), and in fixed preparations KC accounted for not more than 10% of the population (Fig. 2). The fibroblast-like cells became rounded in shape and gradually began to separate from the glass. After 2 weeks of culture, single cells remained. The same picture was observed during culture of KC and fibroblast cells of mature rats, although in this case not more than 10-20% of the cells were fibroblasts. The results of these experiments were confirmed repeatedly with testing of the medium for sterility.

Death of the cells during combined culture was accompanied by a significant decrease in activity of the nucleolar apparatus of the fibroblasts (Table 1). No changes were found in the number of nucleoli in KC.

The use of conditioned medium of neonatal rat hepatocytes considerably prolonged the lifespan of the mixed culture, stimulated mitotic activity of the cells, and maintained their functional state, as could be judged by activity of the nucleolar apparatus (Table 1). Against the background of the conditioned medium, KC spread out more successfully on the glass, and constituted a large fraction of the population (Fig. 2). As regards the character of adhesion, and morphology of the nucleus and lysosomal apparatus, they resembled those described in the literature [6, 11, 12].

By the 12th-15th days of culture a state of a confluent monolayer was achieved due to active reproduction of fibroblasts, the increase in population being about 30%. KC were single or in small groups (up to six or seven cells), the round cells were about 6 μm in diameter, and the spread-out cells 10-12 μm. In some cases KC which had spread considerably could have two to four nuclei, and in such cases the cells were up to 20 μm in diameter. A decrease in the fraction of KC in the population (Fig. 2) could perhaps take place through an active increase in the number of fibroblasts. Some degree of proliferative activity may probably have appeared in KC, because KC with two to four nuclei could be seen during culture for 12 days.

The use of conditioned medium of neonatal rat hepatocytes during mixed cultures of cells from mature rats led to similar results (Fig. 1), but the response to the procedure was delayed by several days compared with neonatal rat cells.

When KC of mature rats were obtained their fraction of the population was large – about 90%. By using the limiting dilutions method, a few cultures of KC could be obtained without contamination by fibroblast-like cells. Exposure of these cell cultures to conditioned medium did not cause the appearance of signs of proliferation, although the KC under these conditions were more spread out and their diameter on average was 1.5 times greater than in the control cultures; consequently, an increase in the total number of cells and the appearance of mitotic activity were observed only in cases when KC were cultured together with hepatic fibroblasts and in the presence of conditioned hepatocyte medium. Increased proliferative activity of the hepatic fibroblasts preceded by a short time the increase in activity of the nucleolar apparatus of KC (Table 1) and an increase in the number of nuclei in them. Similar results were obtained on cultures from both newborn and mature rats.

Hepatic fibroblast-like cells are capable of repeated division in the presence of a small number of hepatocytes [2, 3, 4]. The proliferative activity of hepatic fibroblasts may probably be controlled by growth factor, secreted into the culture medium by the hepatocytes. During combined culture with KC their proliferative activity declines, probably because of the inhibitory action of KC. According to data in the literature [8], KC under certain conditions can synthesize a factor of protein nature, which corresponds in its immunologic, cytotoxic, and chemical properties to tumor necrosis factor, and secreted into the external medium. It has been suggested that this factor may also control normal cell proliferation.

Regulation of growth of liver cells is a highly coordinated and complex process, controlled by various exogenous regulatory factors and by a system of interaction between its cells. The results show that growth factors of conditioned medium of neonatal rat hepatocytes stimulate the mitotic and functional activity of hepatic fibroblasts. Activation of hepatic fibroblasts is probably an essential condition for stimulation of the nucleolar apparatus of KC and for the appearance of multinuclear KC.

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