STUDY OF FIBROBLAST-LIKE CELLS IN MONOLAYER CULTURES OF MOUSE EMBRYONIC LIVER

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Two categories of cells can be clearly distinguished in monolayer cultures of mouse embryonic liver: histiocytes and fibroblast-like cells. Unlike histiocytes from bone marrow cultures of adult mice, the histiocytes from 13-day cultures continue to proliferate actively (as shown by thymidine labeling). The fibroblast-like cells form colonies, the number of which rises as a linear function of the number of explanted cells; one colony-forming unit corresponds to approximately 10^5 explanted cells.

Embryonic liver from mice in the second period of intrauterine development is an organ of intensive hematopoiesis. Cloning of the cells of hematopoietic tissue in vivo [7] shows that ancestral cells which form hematopoietic foci in the spleen of irradiated mice [4, 8] are present in cell suspensions prepared from embryonic liver. Precursor cells capable, under monolayer conditions of cultivation, of giving rise to colonies of fibroblast-like cells [2, 3] are present in cell suspensions made from spleen and bone marrow of adult mice and guinea pigs. These cells retain some of their powers of differentiation on cultivation. For instance, fibroblasts from cultures of guinea pig bone marrow, in contrast to fibroblasts from cultures of the spleen, are osteogenic cells [1].

The object of this investigation was to study the formation of fibroblasts in monolayer cultures of mouse embryonic liver.

EXPERIMENTAL METHOD

The liver donors were 16-19-day mouse embryos of line CBA. The liver cells were suspended in medium No. 199 by pipeting or by injection through a syringe with needles of decreasing diameter, and filtered through Kapron. The resulting suspension was cultivated in 250-ml flasks. A medium of the following composition was used for cultivation: 80% medium No. 199, 20% bovine serum, inactivated by heating for 40 min at 56°C; to every 100 ml of this medium 400 mg glucose, 7 mg ascorbic acid, and 10,000 units each of penicillin and streptomycin were added.

The medium was changed the first time 48 h after transplantation, when the number of cells which did not settle was counted, and they were transferred to a fresh series of flasks; in these cultures the medium was also changed after 48 h and the number of settling cells counted. The medium was changed thereafter once or twice a week.

After 14 days the cultures were fixed with 96° alcohol and stained with Carazzi's hematoxylin. The number of colonies of fibroblast-like cells was counted under a binocular loupe. A colony was regarded as consisting of not less than 5 cells.

In parallel tests the same cell suspensions were cultivated in flasks with cover slips. Into each flask was poured 2 ml of a suspension containing $(2-3) \cdot 10^6$ cells/ml, and the medium was changed for the first

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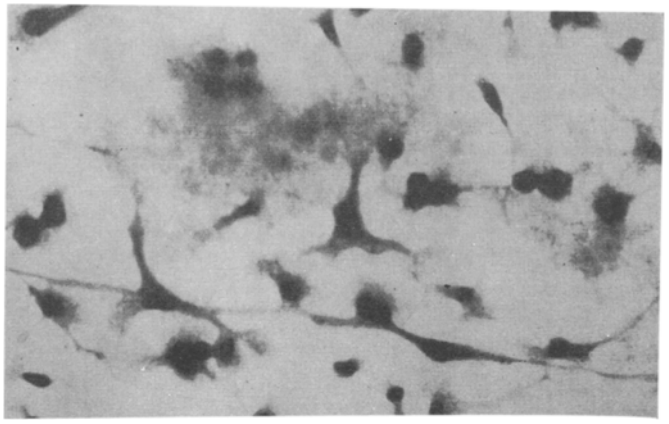

Fig. 1. Two-day old culture. Histiocytes, macrophages, and reticulum cells, 20×.

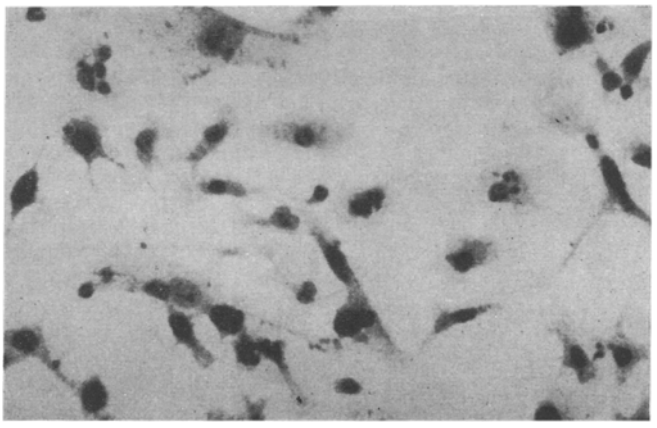

Fig. 2. Two-week old culture. Single fibroblast-like cells (top and bottom left) among histiocytes, 20×.

time 48 h after transplantation. The cover slips were fixed at various times of cultivation, up to the 30th day, stained with Carazzi's hematoxylin, and mounted in balsam.

On the 8th and 13th days of cultivation thymidine-H^3 (specific activity 1.2 mCi/ml) was added to some flasks in a concentration of 1 μCi/ml medium. After 24 h the cultures were washed, fixed with 96° alcohol, and autoradiographs prepared.

EXPERIMENTAL RESULTS

Starting from the second day of cultivation the explants consisted mainly of cells similar in their morphology to the reticulum cells of the bone marrow, histiocytes, or macrophages (Fig. 1). Many cells had characteristic long, branching processes. Some larger round cells with highly vacuolated "frothy" cytoplasm, but without processes and frequently binuclear, were seen. Cells of all these types had a darkly stained, round nucleus. Cells with this morphology will subsequently be described conventionally as histiocytes.

In the course of cultivation the number of histiocytes decreased, but they continued to proliferate, as shown by the results of autoradiography: in 8- and 13-day cultures, 15-20% of the histiocytes were labeled per day of incubation with thymidine-H^3. A histiocyte population was present in the culture after cultivation for 30 days.

From the early periods of fixation (2nd day) the explants contained cells extremely similar in their morphology to the fibroblasts described by Maximov [6] during cultivation of circulating guinea pig blood

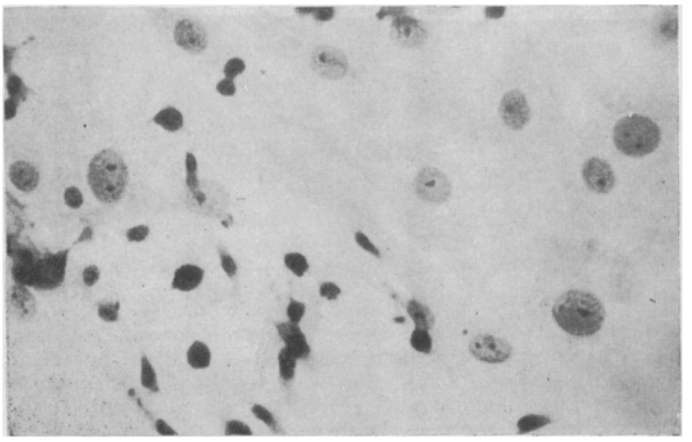

Fig. 3. Twelve-day culture. Area of a colony of fibroblast-like cells, 20×. Bottom left—group of histiocytes.

TABLE 1. Determination of Colony-forming Ability of Embryonic Liver Cells in Monolayer Cultures

Expt. No.	Flask No.	No. of explanted cells ($\times 10^7$)	No. of settling cells ($\times 10^7$)	No. of colonies in flask	Number of CFU/10^5 cells	
					settling	explanted
1	1	13.2	5.6	1260	2.2	0.9
	2	13.2	3.6	1167	3.2	0.9
	3*	29.2	29.2	4000	1.4	1.4
2	1	3.6	1.5	382	2.5	1.1
	2	3.6	0.8	114	1.4	0.3
	3	3.6	1.8	212	1.2	0.6

*Volume of flask 1 liter; cells not settling during 48 h in three 250-ml flasks, each containing $13.2 \cdot 10^7$ bone marrow cells, cultivated in it.

in a plasma clot, and by Chailakhyan et al. [2] during cultivation of guinea pig bone marrow and spleen in monolayer cultures. These fibroblast-like cells were much less strongly stained with hematoxylin than the histiocytes. They had a large cytoplasm, giving off processes and separated into layers around its wall, and a characteristic large, oval nucleus, with several nucleoli (Fig. 2). Among these cells were many binuclear and multinuclear cells; some had as many as ten nuclei. The proportion of multinuclear cells in the population rose appreciably with an increase in the cultivation time; for instance, in 7-day cultures 62.5% of the fibroblasts had one nucleus and 7% had three nuclei or more. By the 30th day, only 13% of all the fibroblasts were mononuclear and 61% were multinuclear.

The fibroblasts described above were arranged in diffuse colonies (Fig. 3). On the 4th day the colonies contained on the average 4-10 cells. By the 14th day they were of considerable size and could be seen with the unaided eye. Some colonies had a very compact structure and the cells composing them were packed closely together; smaller fibroblasts were present in the center, sometimes arranged in several layers; cells at the periphery of the focus were of the usual size. Many small, round histiocytes, without processes and with a round or lenticular nucleus, were frequently seen in these colonies. Other colonies were looser in structure and consisted of large fibroblast-like cells.

In the 8- and 13-day cultures 37% of fibroblast-like cells were labeled after incubation for 24 h with thymidine-H^3. The index of labeled cells in individual colonies was roughly the same. In the central part of the compact colonies fewer labeled cells were usually present than at the periphery; in the other colonies the distribution of labeled cells was uniform.

The results of calculation of the number of colony-forming units (CFU) in these cultures are given in Table 1.

These results show that the cells and the morphology of the colonies possess similar characteristics to the foci of fibroblast-like cells described in cultures of bone marrow and spleen from adult guinea pigs [2], and the number of foci also is a linear function of the number of explanted cells. The results obtained by the workers cited in experiments using a chromosome label, and supporting the clonal character of the colonies, suggest that foci of fibroblast-like cells in embryonic liver cultures are in fact clones.

In contrast to cultures of the bone marrow of adult guinea pigs, in which the histiocytes degenerate toward the 8th-10th day, and cultures of the bone marrow of adult mice [5], in which proliferation of histiocytes virtually ceases by the 3rd day, the histiocytes in embryonic liver cultures continue to proliferate actively for a long time, as is shown by the high index of cells labeled with thymidine-H^3 in 2-week cultures.

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