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POWERS OF DIFFERENTIATION OF CLONAL STRAINS OF BONE MARROW FIBROBLASTS

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Diploid strains of bone marrow fibroblasts, obtained in cultures involving passage of progenies of stromal colony-forming cells (CFU-f) have been shown to possess marked osteogenic activity, which is exhibited on back transplantation into the donor [2-5, 8, 10].

The writers showed previously [7] that the number of osteogenic cells in the composition of the strains increases during passage.

In the investigation described below the powers of differentiation of monoclonal strains of bone marrow fibroblasts, derived from single CFU-f, were studied.

METHODS

Part of the wing of the ilium was resected from Californian rabbits aged 1.5-2.5 months under pentobarbital anesthesia. Cells contained in its medullary cavity were flushed out into culture medium, after which a suspension of disaggregated cells was obtained by repeated pipeting. The cell suspension was filtered through 4 layers of Kapron. Completeness of disaggregation was tested by introducing 10^5 cells into plastic flasks (area 25 cm^2) coated with polylysine. After 60 min flasks were fixed with alcohol and stained by Giemma's method. The bone marrow cells were cultured on HAM medium with 20% embryonic calf serum. To determine the efficiency of stromal colony formation (ECF-f) [4, 7] and to obtain colonies for passage, between 10^4 and $3 \cdot 10^4$ bone marrow cells were explanted into flasks with an area of 20-40 cm^2.

For passage of single colonies primary cultures were treated for 1-3 min with a 0.25% solution of trypsin, and during the next 10-15 min the wet flasks were kept at 37°C, after which the single colonies were removed with gelatin sponges. The cells were washed out of the sponges by pipeting and subjected to further culture. Repeated passages were carried out when the cultures reached confluence. X and Y chromosomes were identified [9] in dividing cells of the colonies in mixed cultures of male and female bone marrow and in cells of strains obtained by passage of colonies derived from mixed cultures.

Cells of the 2nd or 3rd passages, taken from the cultures, were transplanted allogeneically into two types of diffusion chambers: type A (volume of chamber 0.015 mm^3, height 0.1 mm [4], and type O (volume of chamber 0.15 cm^3, height 2 mm) [8].

The diffusion chambers were fixed on the 30th-90th day with alcohol-formol, calcium-formol, or 96° alcohol, and subjected to histologic treatment, including preliminary decalcification in 5% HNO_3. Series of paraffin sections were stained with hematoxylin and eosin, by Cossa' method, and by Gomori's method for alkaline phosphatase.

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TABLE 1. Transplantation of Strains of Cloned Bone Marrow Fibroblasts in Diffusion Chambers

Strain No.	Number of Cells in initial colony ($\times 10^4$)	Number of cells in culture ($\times 10^5$)	Number of cells per chamber ($\times 10^5$)	Result of transplantation
		Type A chamber		
1	0,125	1	1	B
2	0,25	1,4	1,4	B
3	0,5	2	2	B
4	1	2	2	NF
5	1,125	2	2	NF
6	0,625	2,5	2,5	F
7	2,25	3	3	F
8	0,5	3,5	3,5	NF
9	0,125	4	4	F
10	0,5	8	4	B
11	0,125	9	1	NF
12	0,25	10	1	F
13	0,625	2,0	1	B
13	» »	» »	» »	B
14	0,25	6	3	B
15	1,5	6	3	F
16	0,875	7	3	B
17	0,875	14	1	B
		Type C chamber		
18	0,5	1,7	1,7	NF
19	0,25	2	2	F
20	0,625	2	2	B, C
21	1	2,4	2,4	B, C
22	3	2,8	2,8	F
23	0,875	3	3	NF
24	0,125	3	3	F
25	0,125	3,5	3,5	B, C
26	0,75	4	4	B, C
27	0,125	4	4	NF
28	1,25	5	2,7	B
29	0,375	23	8	F
14	0,25	6	3	NF
15	1,5	6	3	F
16	0,875	7	3,5	NF
17	3,5	1	1	NF

Legend. B) Bone tissue in diffusion chamber, NF) no fibroblasts in diffusion chamber, F) growth of fibroblasts in diffusion chamber, C) cartilage tissue in diffusion chamber.

RESULTS

In the course of 60 min all the cells adhered to the surface of the polylysine-coated flasks, and no stromal aggregates were found among $5 \cdot 10^5$ filtered bone marrow cells. The concentration of these aggregates among the explanted cells was therefore under $2 \cdot 10^{-6}$. ECF-f in cultures from which the colonies were taken for further passage was $(13.7 \pm 1.8) \cdot 10^{-4}$. Hence it follows that fewer than 0.1% of the colonies could have been formed by stromal aggregates, i.e., that virtually all the colonies developed from initially disaggregated stromal cells.

Cells of 15 colonies in mixed cultures, into which a mixture of male and female bone marrow in the ratio of 1:1 was explanted, were subjected to karyologic analysis. In each colony from 3 to 10 metaphase plates were typed. In 8 colonies all the metaphase plates contained a Y chromosome, and 7 colonies contained two X chromosomes. Fibroblast cultures at the 1st or 2nd passage, obtained from single colonies, were pure strains of fibroblasts. Karyologic analysis of dividing cells in 10 such strains, obtained from colonies derived from mixed cultures, showed that all the metaphase plates in 4 of them contained a Y chromosome, and in 6 cultures there were 2 X chromosomes.

Colonies of bone marrow fibroblasts from rabbits are thus cell clones and each is formed from a single CFU-f, which is a stromal clonogenic cell, whereas cultures obtained by passage of single colonies are monoclonal strains of bone marrow fibroblasts. To obtain monoclonal

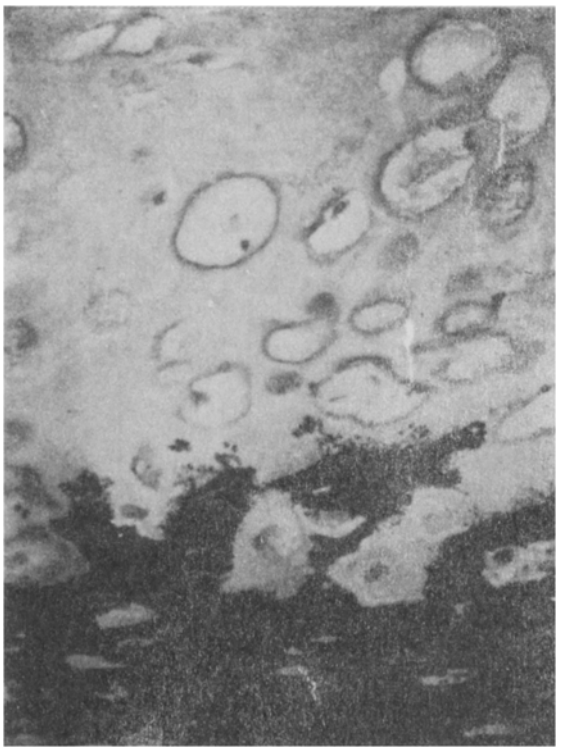

Fig. 1. Bone and cartilage tissue in type O diffusion chamber into which the cells of cloned strains were transplanted after the second passage (not more than $4 \cdot 10^5$, time 60 days). Below, mineralized bone tissue; above, uncalcified cartilage tissue. Cossa's reaction. 400 ×.

strains in order to transplant them *in vivo*, 37 colonies were subcultured from primary cultures. In the first passage growth was obtained in 44 cultures, in the second in 42 of 44 cultures, and in the third in 12 of 12 cultures. Data on the numbers of fibroblasts in the original colonies and in the cloned strains are given in Table 1. Only large colonies, in which the progenies of the original CFU-f completed 10-15 cell doublings, were subcultured. These colonies accounted for about 10% of all colonies growing in the primary cultures of bone marrow cells. In the course of 3 passages the total number of cell doublings in the monoclonal strains reached 22. Results of determination of the osteogenic potential of 29 monoclonal strains also are given in Table 1. Of the 29 strains 14 (48%) possessed osteogenic potentials, as was shown by bone tissue formation in the chambers. The morphology of the bone developing in the chambers was typical: the bone consisted of calcified ground substance with immured osteocytes, it contained osteoblasts, and it had the characteristic trabecular structure of bone. In chambers of the O type, besides bone tissue, cartilage tissue also was formed (in 4 of 5 chambers). It consisted of noncalcified homogeneous ground substance with cartilage cells immured in it.

The reaction for alkaline phosphatase was positive in the bone tissue but negative in cartilage (Figs. 1 and 2). It will be noted that cartilate tissue developed in chambers of the O type, whereas bone tissue developed in chambers of both the A and the O type.

On the basis of the results described in this paper and also of those obtained previously [7] it can be concluded that CFU-f possess high proliferative potential, although in bone marrow *in situ* it is outside the mitotic cycle [1]. At the same time, CFU-f were common precursors for bone and cartilage tissue. CFU-f can therefore be considered to be osteogenic stem cells [2]. The results described above were obtained during testing of the osteogenic properties of only large colonies of bone marrow fibroblasts. They are evidence that the concentration of osteogenic stem cells (OSC) in bone marrow is not less than 10^{-4}. In fact, the OSC concentration may prove to be significantly greater, when the osteogenic powers of fibroblasts of small colonies are tested.

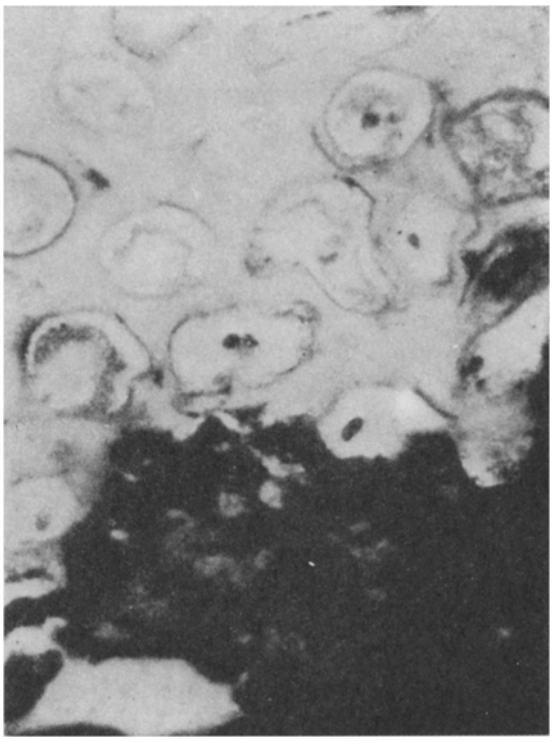

Fig. 2. The same object as Fig. 1. Gomori's reaction for alkaline phosphatase: bone tissue (below) is phosphatase-positive; cartilage tissue (above) is phosphatase-negative. 400 ×.

Differentiation of CFU-f progenies in the direction of cartilage or bone, as follows from the results described above, depends on epigenomal factors. The action of these factors, the nature of which is not yet explained, is evidently different for transplantation into diffusion chambers of A and O types, which differ from one another in depth. This difference evidently produces different conditions of oxygenation and tension inside the chambers, and as many authorities consider [6], this has a selective effect on osteogenesis and chondrogenesis.

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