# OSTEOGENIC PROPERTIES OF ADHESIVE CELLS FROM MOUSE BONE MARROW DEXTER CULTURES

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In bone marrow cultures prepared by Dexter's method, long-term maintenance of hematopoiesis is effected by a supporting layer of adhesive cells [5], which plays the role of stromal microenvironment in vitro. As a rule the cell types of the adhesive layer are identified on the basis of morphological features or of histochemical and antigenic markers [3, 4]. In the investigation described below the presence of stromal fibroblasts in the adhesive layer of Dexter cultures of mouse bone marrow was determined by means of a function test (capacity for osteogenesis).

## EXPERIMENTAL METHOD

C57BL and (CBA × C57BL)$F_1$ mice weighing 18-22 g were used. Bone marrow was explanted by Dexter's method [5, 6] into some of a series of plastic flasks (area of bottom 25 $cm^2$), by flushing out the entire contents of one femur. Suspensions of disaggregated bone marrow cells or stromal tissue were explanted into the other flasks.

Trypsinized Suspension. The bone marrow was pipeted in MEM(α) medium and centrifuged for 10 min at 900 rpm; the residue was resuspended in 0.25% trypsin solution and placed on a magnetic mixer (30 min at room temperature). The resulting suspension was filtered through four layers of Kapron, the trypsin was inactivated by the addition of serum, the sample was centrifuged, and the residue was resuspended in fresh medium.

Mechanical Suspension. The bone marrow was pipeted in medium, mixed on the magnetic mixer, and the suspension was allowed to stand for 5 min. The sedimenting cell aggregates constituted an unbroken stromal residue, almost free from hematopoietic cells. The unsedimented suspension was filtered and centrifuged and the residue resuspended in fresh medium. The culture medium consisted of 75% MEM(α) medium, 25% fetal calf serum, glutamine (50 mg/100 ml), penicillin and streptomycin (6000 U/100 ml of each), and hydrocortisone ($10^{-7}$ M). The cells were cultured at 33°C. Secondary transplantation of the bone marrow cells was not carried out. Every week half of the culture medium was replaced by fresh; the medium withdrawn was used to count floating cells and to prepare films. After various time intervals the cultures were used for heterotopic transplantation. Cells of the adhesive layer were removed with 0.25% trypsin solution and broken up by pipeting. Different quantities of suspended cells (and in some cases, equal amounts of the unbroken residue, within the confines of each experiment) were introduced into gelatin sponges weighing 0.6 mg, which were transplanted under pentobarbital anesthesia one at a time beneath the renal capsule of syngeneic or semiallogeneic recipients [1]. After 5 months the transplants were removed, fixed with 96% ethanol, and decalcified with 5% nitric acid, after which serial paraffin sections were prepared. The morphology of the transplants was assessed in sections stained with hematoxylin and eosin and the number of hematopoietic cells was counted. The volume of the transplants was determined by the equation:

$$V = \frac{\pi h}{6}(h^2 + 3r^2),$$

where h is the height and r the radius of the base of the transplant. The mean number of cells in 10 fields of vision under a 100× objective was counted; the number of cells in the transplant was determined by the equation:

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TABLE 1. Changes in Number of Floating Cells in Bone Marrow Cultures with Period of Culture (number of living cells per culture × $10^5$)

| No. of culture | Strain of donor mouse | Method of culture | Period of culture × $10^5$, weeks | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
| 1 | C57Bl | Trypsinized suspension, 1.2 × $10^7$ | 21 | 0,7 | 13 | 3 | 5,5 | 10 | 4,5 | 2,5 | 2 | 2 | 1,4 |
| 2 | C57Bl | Dexter's method | — | 1,7 | 3,5 | 30 | 18 | 27 | 6 | 4,6 | 13 | 23 | 4,4 |
| 3 | C57Bl | Stromal residue from seven femora | 12 | 6,3 | 15 | 7 | 8 | 5 | — | 6 | 2 | | |
| 4 | $F_1$ | Dexter's method | — | 4,5 | 7 | 3 | 0,7 | 2 | 2,5 | 0,7 | | | |
| 5 | $F_1$ | » » | — | 0,3 | 9 | 31 | 29 | 32 | 2,5 | 0,3 | | | |

TABLE 2. Results of Transplantation of Adhesive Cells from Cultures beneath Renal Capsule

| No. of culture | Strain of donor mouse | Time in culture, days | Number of cells removed ×$10^5$ | Number of cells transplanted into sponge × $10^5$ | Strain of recipient | Number of cells in transplant × $10^6$ | Presence of bone |
|---|---|---|---|---|---|---|---|
| 1 | C57Bl | 77 (in primary) + 27 (in passage) | 30 | 4 | C57Bl | 5,3 | — |
| | | | | 4 | $F_1$ | 0,7 | — |
| | | | | 3,3 | C57Bl | 4,5 | — |
| | | | | 3,3 | C57Bl | 7,0 | — |
| | | | | 3,3 | C57Bl | 3,2 | + |
| 2 | C57Bl | 82 | 18 | 2 | C57Bl | 1,6 | — |
| 3 | C57Bl | 68 | 36 + residue | 4 | $F_1$ | 20,3 | + |
| | | | | 12 | $F_1$ | 33,3 | + |
| | | | | residue | C57Bl | 1,1 | + |
| | | | | » | C57Bl | 14,6 | + |
| 4 | $F_1$ | 61 | 15 + residue | 5 | $F_1$ | 12,4 | + |
| | | | | 5 | $F_1$ | 19,0 | + |
| | | | | residue | $F_1$ | 15,6 | + |
| | | | | » | $F_1$ | 16,6 | + |

Legend. No. of cultures corresponds to No. of cultures in Table 1.

$$\text{number of cells} = \frac{\text{volume of transplant x mean number of cells in field of vision.}}{\text{area of field of vision (9500 } \mu^2\text{)} \times 10 \text{ (diameter of cells)}}$$

The result thus obtained agreed closely with the result of directed counting (cells flushed out of half of the transplant).

## EXPERIMENTAL RESULTS

Morphology and Cell Composition of the Culture. Four methods of explantation of the bone marrow cells were used: Dexter's method, mechanical suspension, trypsinized suspension, and stromal residue. The dynamics of the change in number of floating cells in these cultures (except mechanical suspension cultures, in which prolonged hematopoiesis was not observed) is shown in Table 1. In most cultures the number of floating cells at first fell compared with the initial value [(1-2) × $10^7$], but later it rose again, and remained for some time at a level above 1 × $10^6$, after which it was maintained for a long time at a lower level. Macrophages, megakaryocytes, and neutrophilic granulocytes, including young forms, were identified in films prepared from cultures at different times of culture. Toward the end of the 2nd week a continuous layer of adhesive cells formed on the bottom of the flasks; foci of young hematopoiesis developed on this layer and appeared as zones of distinctly outlined polygonal or round cells, in close contact with one another. Later (3-6 weeks) these regions grew in width and occupied a significant part of the adhesive layer.

Morphology and Cell Composition of Transplants. Cells taken from primary cultures (Nos. 2, 3, and 4) or from cultures obtained by passage of the primary culture (No. 1) were subjected to heterotopic transplantation. Altogether 23 sponges were grafted, and in 14 cases transplants were obtained; nine animals died.

The transplants had a characteristic morphology in sections. A bone marrow organ lay next to the renal tissue: hematopoietic cells of all branches were present in abundance, together with sinusoids and individual vessels. Reticular cells could be distinguished among the hematopoietic cells. Externally the transplant was surrounded by a connective-tissue capsule. In nine of 14 transplants bone was found (Table 2), most frequently with the appearance of large,

elongated trabeculae, lying in the capsule or more centrally. Less frequently bone was present only in small fragments. The bone had a lamellar structure, contained osteocytes, and appeared fully viable.

During formation of the transplant the gelatin sponges were absorbed; they could no longer be found.

The results are clearly insufficent to allow final conclusions to be drawn on whether in this system the volume of the transplant depends on the number of transplanted cells, whereas the presence of bone depends on the volume of the transplant and the parameters of culture. All that can be stated is that if the total number of cells of the transplant is determined (i.e., in real terms the size of the stromal base formed) as their peak value, a value significantly in excess of the initial number of explanted cells will be obtained for most cultures ($9 \times 10^7$ for culture No. 4, $21 \times 10^7$ for culture No. 3).

On the whole, cultures comparable for duration of hematopoiesis with Dexter cultures can be obtained by explantation of suspensions of trypsinized bone marrow cells or stromal tissue of bone marrow, which has not disaggregated due to the gentle mechanical treatment. At the site of transplantation of cells of the adhesive layer of all three types of cultures a bone marrow organ formed, and in most cases it contained bone. In heterotopic bone marrow transplants stromal tissue is formed by the donor's cells [7], and osteogenesis is one path of differentiation of medullary fibroblasts [2]. Consequently, during culture for 2 months or more stromal bone marrow fibroblasts are components of the adhesive layer of the cultures described and they participate in the maintenance of hematopoiesis in vitro, retaining their capacity for osteogenic differentiation and for transfer of the medullary microenvironment.

## LITERATURE CITED


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