NUMBER OF OSTEOGENIC PRECURSOR CELLS IN BONE MARROW AND THEIR MULTIPLICATION IN CULTURE

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UDC 612.419.014.2:612.6]:612.753.014.2]-085.23

KEY WORDS: precursor cells; bone marrow fibroblasts.

Stromal cells with high proliferative potential are found in cultures of bone marrow cells *in vitro*: They behave like clonogenic cells (CFU_f) and give rise to diploid strains of bone marrow fibroblasts [3, 4]. On reverse transplantation *in vivo* these strains form bone and the stroma of heterotopic medullary organs [3, 4, 6, 7]. Hence it follows that at least some CFU_f are osteogenic precursor cells and that osteogenic cells are preserved in cultures of bone marrow fibroblasts.

However, it remained unclear whether these cells multiply during culture.

The aim of this investigation was to study which fraction of CFU_f isolated from bone marrow possesses osteogenic properties and whether the number of osteogenic precursor cells increases during culture of bone marrow fibroblasts.

EXPERIMENTAL METHOD

Experiments were carried out on Californian rabbits aged 1.5-2.5 months. To obtain bone marrow, the right pelvic bone was resected under pentobarbital anesthesia. The contents of the medullary cavity were flushed out into culture medium and a suspension of isolated cells was prepared by repeated passage through a Pasteur pipette. The cell suspension was filtered through four layers of Kapron and explanted into monolayer cultures [3]. Culture was carried out in flasks, the bottom of which had an area of 20-162 cm^2, on Alpha medium in the MEM medium modification, with 20% embryonic calf or rabbit serum in the presence of extra feeder: allogeneic bone marrow cells irradiated in a dose of 6000 rads [2]. To determine the efficiency of fibroblast colony formation, explantation was carried out with an initial density of about 10^4 bone marrow cells/cm^2, which ensured the clonal nature of the colonies [3]. The efficiency of colony formation [3] was determined on the 10th-12th day for cells of each of the 15 rabbits used in the work. The rosette-formation test was carried out on some cultures with erythrocytes sensitized with FC and C_3 [1]. Cultures for passage were treated with trypsin, and the detached cells were counted and transferred to new culture vessels, without the addition of feeder. Cells also were removed for subsequent autologous transplantation in Ivalon sponges beneath the renal capsule [2] or for allogeneic transplantation intraperitoneally in diffusion chambers [3].

Transplants in sponges and diffusion chambers were analyzed histologically [2-4]. After fixation some of the chambers were dried and the dry weight of the bone tissue formed was determined.

EXPERIMENTAL RESULTS

On the 12th-14th day of culture colonies of fibroblasts were contaminated with macrophages, which also were distributed between the colonies. Later the macrophages disappeared and the space between the colonies was virtually cell-free. The efficiency of colony formation varied for different rabbits from 2.2 to 16.0 (mean 5.1 ± 1.0) per 10^4 explanted bone marrow cells.

Laboratory of Immunomorphology, N. F. Gamaleya Institute of Epidemiology and Microbiology, Academy of Medical Sciences of the USSR, Moscow. (Presented by Academician of the Academy of Medical Sciences of the USSR D. S. Sarkisov.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 98, No. 11, pp. 605-608, November, 1984. Original article submitted January 13, 1984.

 0007-4888/84/9811-1570$08.50

TABLE 1. Cultures of Bone Marrow Fibroblasts after First Passage

Number of bone marrow cells explanted initially	Number of cultures	Area of cultures, cm^2	Number of fibroblasts removed $\times 10^6$	Increase in number relative to initial CFU_f	Mean number of duplications of progeny of CFU_f
10^7	14	136	5,6±0,6	1768±75,2	10
$5 \cdot 10^6$	8	136	5,2±1,0	3587±619	11
$3 \cdot 10^6$	6	136	3,9±0,5	3941±830	11,5
10^6	14	66	2,6±0,8	7400±1956	12
$5 \cdot 10^5$	8	66	2,3±0,7	13997±3898	13,5
10^5	10	66	1,3±0,2	3400±9235	14,4
$5 \cdot 10^4$	5	22	1,4±0,2	49180±16334	15
10^4	1	22	1,8	360000	18

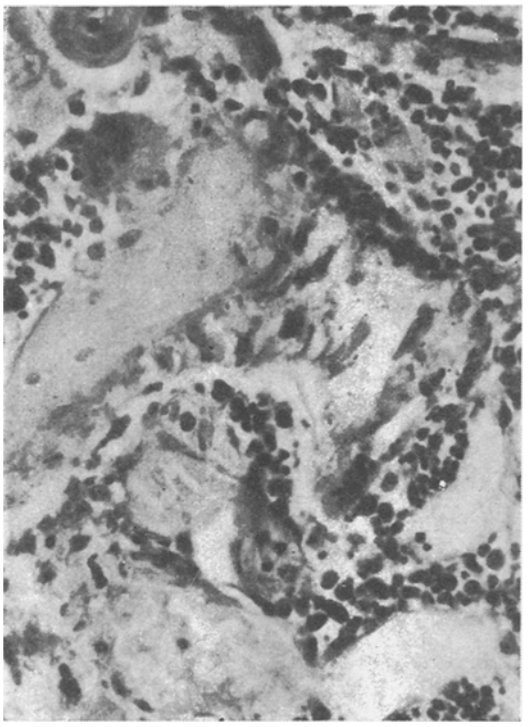

Fig. 1

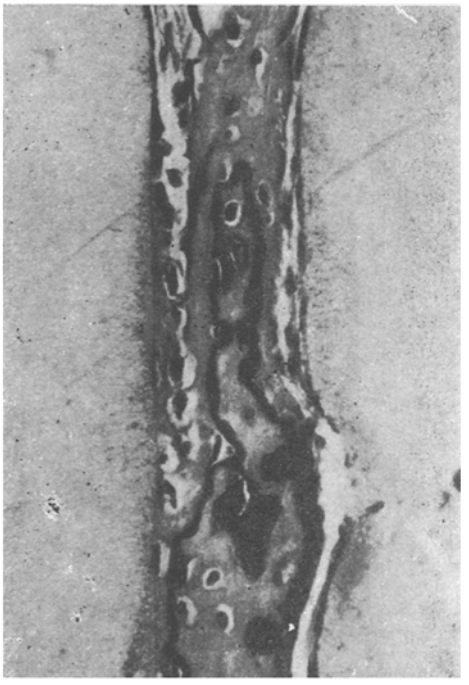

Fig. 2

Fig. 1. Reverse transplantation of fibroblasts at first passage of strain started from 150 CFU_f (76 days). Bone tissue present in transplant. Here and in Fig. 2, staining with hematoxylin and eosin.

Fig. 2. Transplant of 10^6 fibroblasts at 9th passage of strain No. 5 (83 days). Bone tissue filling diffusion chamber.

The colonies differed considerably in size and number of fibroblasts composing them. Large colonies (5-10% of the total) had a diameter of 8-10 mm, medium sized (20-25%) a diameter of 2-7 mm, and small (67-75%) a diameter of 0.2-0.4 mm. The large colonies contained $2.5 \cdot 10^4$-$10 \cdot 10^4$ fibroblasts, the small colonies at least 50 fibroblasts. The first passage was undertaken 16-20 days after primary explantation. Cultures after passage were virtually pure strains of fibroblasts. In the rosette-formation test fibroblasts in primary cultures and all cells of subcultures did not form rosettes, by contrast with macrophages in primary cultures, which did form rosettes.

Table 1 shows the number of fibroblasts removed from cultures at the first passage during formation of a confluent cell layer depending on the cell density (2nd-7th day of passage). The increase in the number of fibroblasts relative to the initial number of CFU_f was from 10^3 to $36 \cdot 10^4$; the number of growing cells, moreover, depended on the area of the culture flask and not on the number of CFU_f explanted. The magnitudes of the cell yield reflected the area of the culture available for colonization by fibroblasts.

TABLE 2. Repeated Passage of Bone Marrow Fibroblasts

Number of bone marrow cells explanted	Number of CFU_f	Passage					
		1st	3rd	5th	7th	8th	10th
10^7	15 000	4,8 / 0,3	17,4 / 1,3	110 / 7,3	2 800 / 186,6	13 000 / 866,6	
10^6	1 500	2,7 / 1,7	10,9 / 7,3	56,7 / 36,7	1600 / 1 000,0		
10^5	150	1,2 / 8,0	5,2 / 33,3	26,0 / 166,7	268 / 1,666,7	n.d.	4 000 / 26 666,7

Legend. Above the line — number of fibroblasts in passages $\times 10^6$; below the line — increase in number of fibroblasts relative to initial CFU_f $\times 10^3$. n.d.) Not determined.

TABLE 3. Reverse Transplantation of Bone Marrow Fibroblasts after Repeated Passages

Strain No.	Number of CFU_f explanted initially	Number of passages	Number of fibroblasts in last passage	Number of fibroblasts per chamber	Chambers with bone / total number of chambers
1	3000	4	$6{,}7 \cdot 10^7$	$2{,}4 \cdot 10^6$	1/1
2	300	3	$1{,}6 \cdot 10^7$	$1{,}2 \cdot 10^6$	1/1
3	150	4	$6{,}4 \cdot 10^7$	$3{,}2 \cdot 10^6$	1/1
4	30	3	$7{,}2 \cdot 10^6$	$6 \cdot 10^5$	1/1
5	150	9	$1{,}5 \cdot 10^9$	$3 \cdot 10^5$	3/3
				$1 \cdot 10^5$	3/3

During repeated passages intensitive multiplication of the bone marrow fibroblasts continued (Table 2). In the course of 10 passages the mean number of cell duplications reached 26. Fibroblasts from cultures at the first passage (Table 1) were transplanted in sponges beneath the renal capsule. The cultures arose from different numbers of initially explanted CFU_f, namely: Eight cultures were started on average from 5000 CFU_f, five cultures from 2500 CFU_f, four cultures from 1500 CFU_f, eight cultures from 500 CFU_f, two cultures from 250 CFU_f, four cultures from 150 CFU_f, five cultures from 50 CFU_f, and four cultures from 25 CFU_f. All cells of each of the 40 cultures were introduced into one sponge for transplantation. After 74-114 days bone tissue was found to be formed in all 40 transplants, from which it follows that at least one of every 25 medullary CFU_f is an osteogenic precursor (Fig. 1).

The results of experiments to determine the osteogenic potential of repeatedly subcultured bone marrow fibroblasts are given in Table 3. In these experiments a definite fraction of the cell progeny, grown from different numbers of initially explanted CFU_f, was transplanted in diffusion chambers. The osteogenic potential of the strains was preserved during passage, and the number of osteogenic precursors increased. This will be clear from the example of strain No. 5, which at the 9th passage consisted of $15 \cdot 10^8$ fibroblasts (the mean number of cell duplication during the period of passage was 24). For bone to be formed it was sufficient to transplant 10^5 cells of this strain into one diffusion chamber (Fig. 2). Hence it follows that the 9th passage of this strain contained at least 15,000 osteogenic units. This is 100 times more than the number of CFU_f from which the strain was started and, consequently, the greatest possible initial number of osteogenic cells in the strain, even if every CFU_f on primary explantation was an osteogenic precursor.

The dry weight of bone filling the diffusion chamber 1.5 months after transplantation of 10^5 cells at the 9th passage was 2 mg. This is equivalent to 30 g or the total number of 15,000 osteogenic units. The strain was obtained as a result of explantation of 10^3 cells contained in the medullary cavity of the pelvic bone. Consequently, the osteogenic potential of the progeny of medullary CFU_f of one such bone was sufficient to form at least 30 kg of bone tissue.

The writers showed previously that single colonies, consisting of clones of fibroblasts from primary cultures of bone marrow cells, form monoclonal bone organs on reverse transplantation [5]. The initial CFU_f for these clones are consequently osteogenic precursors with high morphogenic potential.

The results described above show that the proliferative potential of CFU_f is extremely great and that the progeny of CFU_f preserve the properties of osteogenic precursors during cell multiplication. During culture CFU_f give rise to cell populations which can form much more bone tissue than if present in the body. On the basis of all these features osteogenic medullary CFU_f must be regarded as self-maintaining, i.e., as osteogenic precursor stem cells. Osteogenic stem CFU_f account for not less than 4% of all clonogenic bone marrow stromal cells.

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EARLY CHANGES IN CELL AND LYSOSOMAL MEMBRANE PERMEABILITY IN THE RAT TESTIS AFTER LOCAL HEATING OF THE SCROTUM

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UDC 612.617.014.462.1-06:612.591

KEY WORDS: spermatogenesis; cell membranes; lysosomes.

The mechanism of the severe disturbance of spermatogenesis caused by the action of heat has not yet been explained. It has been shown that a suspension of spermatogenic epithelial cells, exposed to a high temperature (37°C), secretes more protein into the incubation medium than if incubated at 32°C. Such a suspension of spermatogenic cells, if incubated at 37°C, exhibits increased ability to be stained by trypan blue [5]. These observations show that the action of heat on a suspension of spermatogenic cells causes destabilization of the cell membranes. It must also be noted that lysosomes isolated from testes of sexually mature rats and incubated at 37°C secrete more hydrolytic enzymes than lysosomes obtained from liver cells [6]. This fact is evidence of the thermolability of the lysosomal membranes of testicular cells of adult animals. Increased acid phosphatase (AP) activity has been shown to be demonstrable histochemically in the abdominal testes of rats 24 h after operation [3], further evidence of destabilization of the lysosomal membranes. A biochemical study of AP activity in homogenates of abdominal testes revealed a decrease in activity of the enzyme, parallel to the degree of loss of sex cells. Free cathepsin D activity increased under these circumstances, irrespective of the time elapsing after the operation of artificial cryptorchidism [3]. An increase in AP and leucine-aminopeptidase activity was found in a histochemical study of the testes of rats whose scrotum was immersed in water at a temperature of 42°C for 30 min [4].

The aim of the present investigation was to investigate the disturbance of permeability of cell and lysosomal membranes in the rat testis after short-term heating of the scrotum at 41°C followed by incubation of fragments of the testis at 33°C.

EXPERIMENTAL METHOD

The scrotum of rats weighing 220-270 g was subjected to local heating by immersion of the lower part of the body in water at 41°C for 30 min. During heating the animals were kept in

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(Presented by Academician of the Academy of Medical Sciences of the USSR A. P. Avtsyn.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 98, No. 11, pp. 608-609, November, 1984. Original article submitted November 27, 1983.