OSTEOGENIC POTENTIAL OF LONG-TERM DIPLOID CULTURES OF BONE MARROW CELLS

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At the 17th subculture (after cultivation for 3.5 months), a diploid strain of cells obtained from the bone marrow of an adult rabbit, with cell differentiation characteristic of fibroblasts, exhibited osteogenic potential when retransplanted in vivo.

Regular changes in cell composition take place in monolayer bone marrow cultures. In the late periods, instead of a heterogeneous cell population, a morphologically homogeneous culture of fibroblasts is formed. This has been shown to develop from clone colonies arising from a very small number of precursor cells present in the original suspension of bone marrow cells. Fibroblasts from primary bone marrow cultures possess ostegoenic properties when retransplanted in vivo [6]. At the same time, they can be subcultured for long periods during which they remain diploid.

The object of this investigation was to determine whether these cells retain their osteogenic potential during prolonged cultivation with frequent subculture.

EXPERIMENTAL METHOD

Bone marrow of an adult male rabbit was flushed out of the femur and resuspended in medium No. 199. The marrow was cultivated in medium No. 199 (89.5%) with bovine (10%) and rabbit (0.5%) serum in 250-ml flat-bottomed flasks. The medium was changed every 2-4 days. Cells for subculture were taken from the glass after brief treatment of the cultures with trypsin. The first subculture was taken on the 20th day and each subsequent subculture every 5-7 days.

After subculture, some cells were placed in Leighton's tubes with cover slips and fixed on the 2nd-7th day. The specimens were stained with hematoxylin, azure II-eosin, azan, and silver for reticulin fibers, and also by Feulgen's method, by methylgreen-pyronine, Sudan B and III, the PAS method, for acid and alkaline phosphatase by Gomori's method, and for esterase, NAD- and NADP-diaphorase, and succinate dehydrogenase.

At the 20th subculture, L. D. Safronova and Yu. S. Demin carried out a cytogenetic analysis of 210 metaphase plates obtained by Moorhead's method [1]. All cells with a diploid number of chromosomes were assessed for euploidy (the presence or absence of 4 large chromosomes of pairs I and II, telocentrics of pairs XIX-XXII, and a Y-chromosome). Twelve euploid and three aneuploid plates were karyotyped by Nichols' method [2]. Analysis showed that the number of diploid cells was 75.2%. The aneuploid cells (24.8%) consisted mainly of hypodiploid cells, and they were evidently produced by loss of chromosomes during preparation of the specimen. The frequency of hyperdiploid cells (0.95%) did not exceed the level of spontaneous mutation, nor did the number of tetraploids (5.2%) exceed the limits accepted for diploid cultures. The number of aberrations – chromatid and mixed deletions (about 1%) – agreed with the level of

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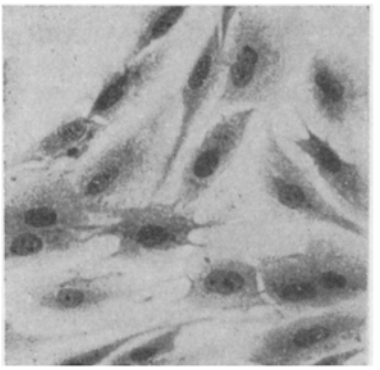

Fig. 1.

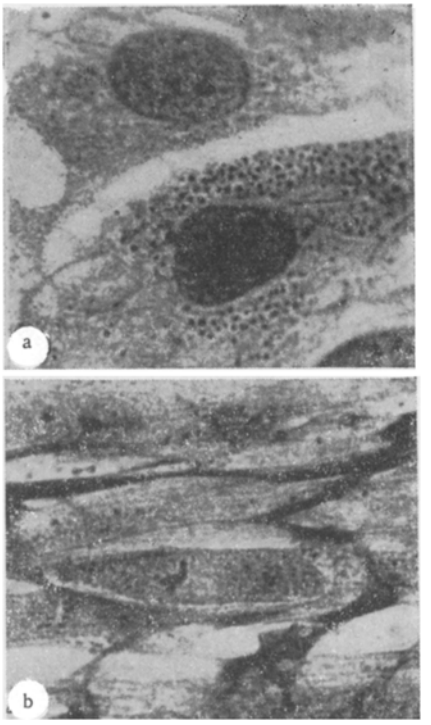

Fig. 2.

Fig. 1. Diploid culture at 11th subculture of bone marrow cells (azure II-eosin, 20×).

Fig. 2. Azan-positive granules (a) and fibers (b) in cells (azan, 90×).

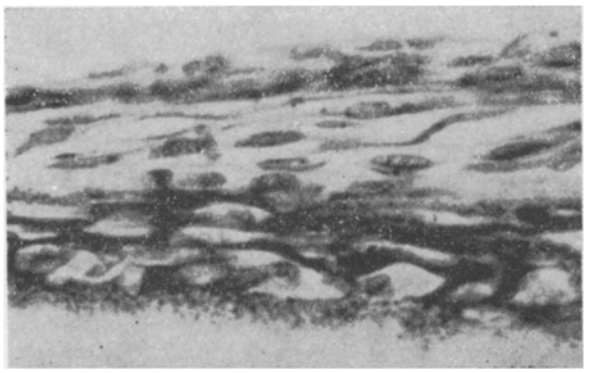

Fig. 3. Bone in diffusion chamber (section, PAS-hematoxylin, 40×).

spontaneous chromosomal mutations for many mammals. Hence, the culture at the 20th subculture used in the experiments can be taken as diploid.

Cells of the 17th subculture (3.5 months of cultivation) were transplanted into rabbits in 5 diffusion chambers made from NA filters with a pore size of 0.45 μ. The method of making the chambers, filling them with cells, and implanting them intraperitoneally was described previously [4]. The number of cells placed in each chamber was 10^6. On the 28th day the chambers were fixed with alcohol-formol, decalcified, and cut into series of sections which were stained with hematoxylin and by the PAS method.

EXPERIMENTAL RESULTS

The primary culture (before the 19th day) passed through the usual phases of changes in its composition. Initially most cells were leukocytes, followed by histiocytes, and these were replaced by fibroblast-like cells. After the 1st subculture, the cultures became homogeneous. They consisted of delicate fibroblast-like cells joined together with syncytia and possessing marked phagocytic activity. Among the large cells, arranged in layers and possessing from three to five processes, there were groups of smaller, elongated cells (Fig. 1). The nucleus, oval or round in shape and deficient in chromatin, contained from one to five large, pyroninophilic nucleoli.

The cytoplasm was poor in ribonucleoproteins, although many cells appeared basophilic when stained with azure II-eosin. On the 1st day after subculture, a few very tiny inclusions of neutral and bound fat

were observed in the cytoplasm. Their number increased by the 5th day, the inclusions were larger, and they were located near the periphery of the cytoplasm. The cells were in a state of fatty degeneration and few in number. On the 2nd day of growth, many tiny PAS-positive granules appeared in individual cells, while others contained a small quantity of glycogen, which accumulated by the 3rd-4th days and subsequently disappeared almost completely. At later periods, the fibers and small inclusions remained PAS-positive, and were also demonstrable by staining with azan (Fig. 2a). In some cases delicate, thin argyrophilic fibers and bundles of collagen fibers were impregnated in the cultures, and their number increased sharply after 10-15 days of growth (Fig.2b).

High acid phosphatase and esterase activity was found in the cells of the cultures, but no alkaline phosphatase activity could be detected. In most cells activity of oxidation-reduction enzymes was high, decreasing gradually after the 7th day of cultivation.

On the 28th day after transplantation into allogenic recipients, the cells of the 17th subculture showed marked osteogenic properties. All five chambers were filled with mature bone tissue (Fig. 3). In some cases the bone occupied the whole lumen of the chamber between the filters, while in others the bone fragments lay among layers of connective-tissue cells. On its outer side the bone was covered with a layer of osteoblasts. The ground substance of the bone tissue, in which osteocytes were immured, contained large quantities of polysaccharides and had the typical structure of well developed woven bone. The bone was so intensively calcified that the filters, when taken from the rings, did not bend until they were decalcified.

The culture of rabbit bone marrow cells described above maintained its diploid properties throughout 20 subcultures. In the monolayer culture, differentiation of its cells was characteristic of fibroblasts. This could be judged from the morphological and histochemical properties of the cells and the formation of fibers. Meanwhile, cells of this culture at the 17th subculture, when retransplanted in vivo under diffusion chamber conditions showed well marked osteogenic potential. Similar properties are possessed by freshly isolated bone marrow cells and primary cultures obtained from them [3, 5, 6]. Consequently, osteogenic elements are preserved in bone marrow cells undergoing prolonged subculture in vitro, although they do not exhibit their osteogenic function while in culture. It is not yet known whether all these cells which appear very similar morphologically and histochemically in a culture have identical osteogenic potential or whether this is possessed by only some cells in the population. Under all conditions, osteogenic potential can be maintained for a long time in cultures obtained from rabbit bone marrow cells.

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