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Histological Study of Rabbit Marrow Fibroblasts after Long-Term Culture in Vitro and in Vivo

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Abstract

The aging phenomena of the fibroblasts after long-term culture in vitro were investigated. The cells derived from young rabbit marrow have been grown in Eagle's modified MEM (Nissui) with 10% fetal bovine serum (Gibco Lab.). Continuous transfer and new outgrowth from a succession of about $1.9\times10^5\pm1.8\times10^4$ (mean±S.E.) cells have continued for a period of 1108 days. There are firbous tissues but no osseous tissue formed by the 47th passed cells in diffusion chambers in vivo.

Introduction

Many tissue culture studies on the phenomenon of aging have been reported. HAYFLICK and MOORHEAD (1961) proposed a hypothesis that many normal cells could not continue to grow indefinitely in tissue cultures. The present paper reports the continuous growth of culture of rabbit marrow cells for 3 years on plastics.

Materials and Methods

In vitro cultures. A white rabbit, 1540 g in weight, was killed by cervical dislocation, and the marrow from the femoral midshaft was flushed into serum-free medium (MEM, Nissui) with kanamycin 6 mg/100ml medium, 7%(g/100 ml) sodium bicarbonate 1.71 g/100 ml medium and 2.9%(g/100 ml) L-glutamin 1 ml/100 ml medium. A single cell suspension was created by drawing the marrow cells into a syringe several times through needles of decreasing sizes. Tissue culture flask (25 cm², Falcon) containing 5 ml modified MEM as described above supplemented with 10%(g/100 ml) fetal bovine serum (Gibco Lab.) was inoculated with 1.2×10^7 marrow cells. Cultures were fed with complete change of medium twice a week. After 30 days the confluent layer of fibroblasts could be harvested by treatment with a solution of 0.5%(g/100 ml) trypsin which was filtered three times by 0.45 μm membrane filters, in the serum free medium at pH 7.6. The culture was closely observed and its progress recorded two to three times weekly. When the culture reached confluent, it was transferred. New outgrowth from a succession of 1.9×10^5

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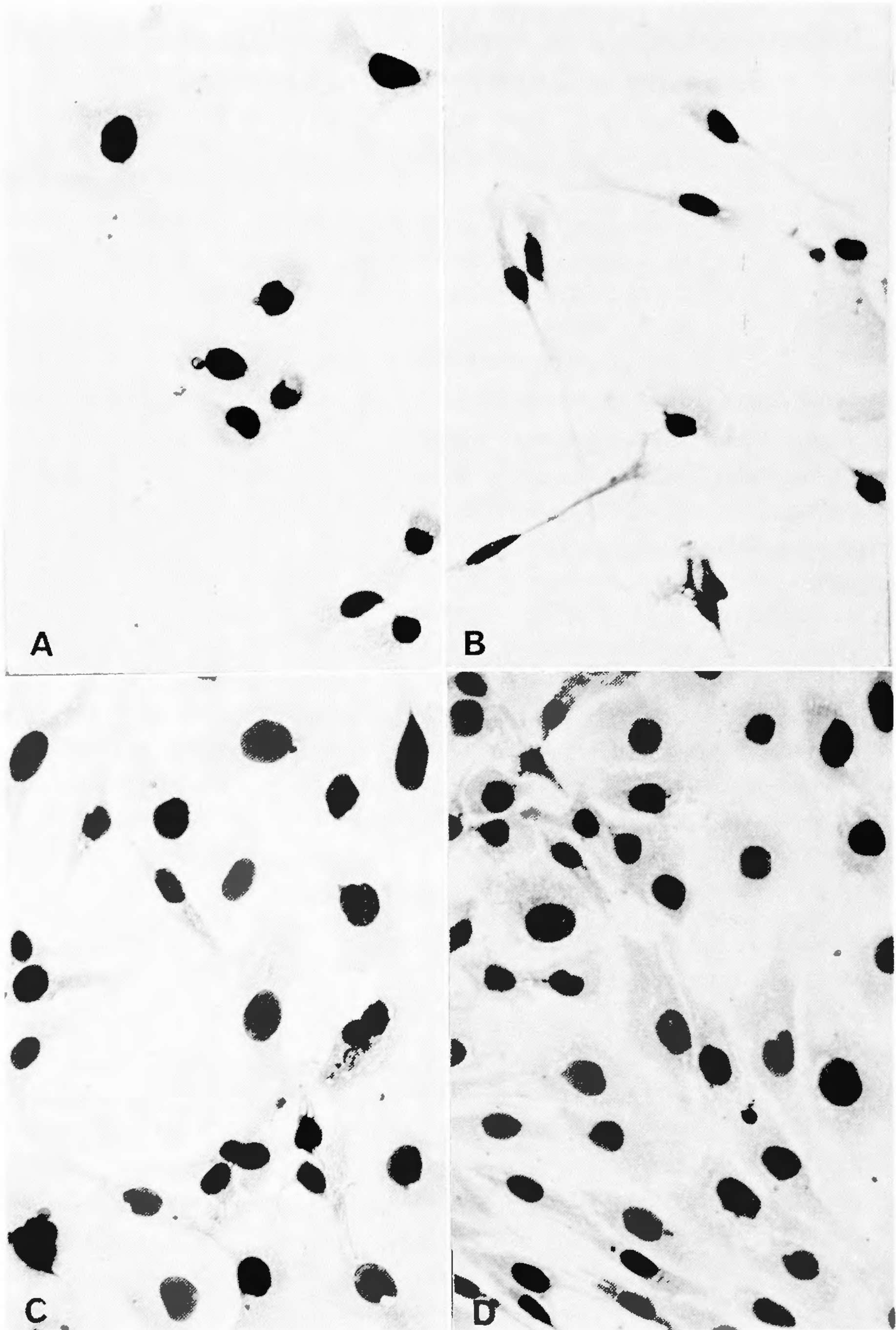

Fig. 1. Cells from culture of 65th cells (total survival time, 1108 days). May Giemsa stain, ×280. Note the cultures are producing new outgrowth of the cells.
A) Cells from a 3 day culture B) Cells from a 4 day culture C) Cells from a 5 day culture D) Cells from a 6 day culture are almost confluent.

$\pm 1.8\times10^4$ (mean$\pm$S.E.) cells in the flasks has continued. Trypsinization described above was performed at intervals of 2 to 3 weeks.

In vivo cultures. Diffusion chambers (Millipore Ltd.) made from lucite rings bounded by 0.45 μm membrane filters (approximate volume 130 μl) were filled through a hole in the plastic ring with 3.0×10^6 47th passed cultured fibroblasts from marrow stromal tissue. The hole was sealed by gluing in a small plastic plug. The chambers were implanted intraperitoneally in adult rabbits and harvested after 3 to 7 weeks. In the test for malignant growth, the 47th passed cells were inoculated in the rabbit abdominal muscles and were examined after 3 months.

Light microscopy. In in vitro cultures of the marrow fibroblasts, the cells were fixed in 95% ethanol for 20 min. and stained with May Giemsa. Diffusion chambers were transferred directly from the host rabbit into 10% formalin. Sections were stained with HE, PAS and AZAN.

Results

In vitro cultures. In these cultures there was a decrease in cell numbers over the first 5 days. From the 7th day tiny colonies of small triangularly-shaped cells and large irregular flattened cells were visible. In the flasks most of the hemopoietic cells inoculated died within the first week of culture. By the 15th day fibroblast colonies were appeared. By the 30th day a confluent layer of fibroblasts covered the bottom of the flasks. Continuous growth was achieved. A photograph of cells from a 3 day culture of 65th passed cells (total survival time, 1108 days) is shown in Fig.

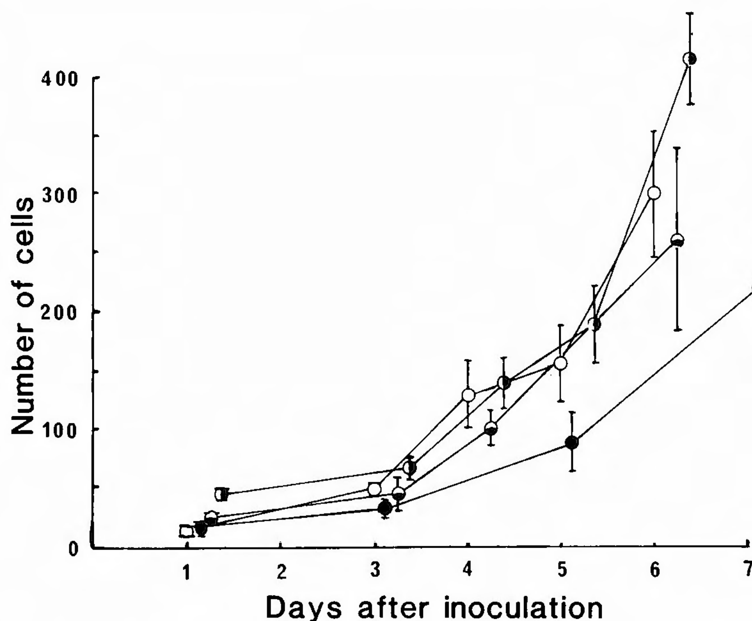

Fig. 2. Numbers of cells per 2.4 mm² of the bottom of flasks are shown as mean$\pm$S.D., (n=10). ○—○, 41st passed cells, total survival time 737 days; ●—●, 47th passed cells, total survival time 833 days; ◒- - -◒, 53rd passed cells, total survival time 927 days; ◑—◑, 65th passed cells, total survival time 1108 days. Note a slowing of the rate of production of the 47th passed cells. But in the cells of the 53rd and 65th passage, the rate recovered.

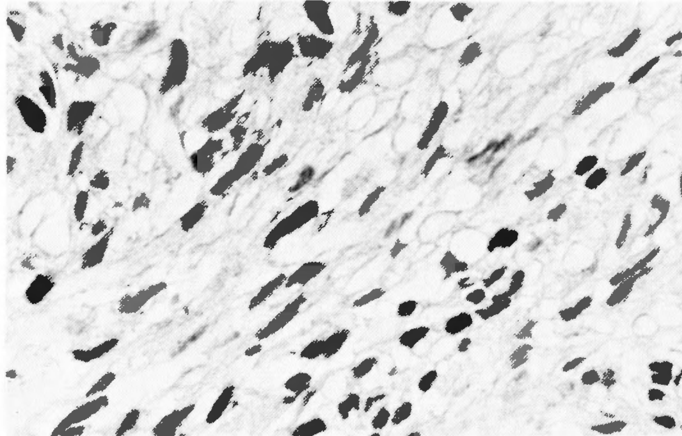

Fig. 3. A part of vertical section through the tissue formed in the diffusion chamber inoculated with cultured marrow fibroblasts and implanted intraperitoneally in a rabbit. The tissue is mainly fibroblastic and not osseous. HE stain. ×220.

1A. They were attached to plastic spread and proliferated. The number of these cells was 68.0±8.5 (mean±S.D.) per 2.4 mm² of the bottom surfaces of flasks. Fig. 1B shows cells from a 4 day culture of the same series, the number of these cells was 133.6±20.9 per the same area described above. Fig. 1C shows cells from a 5 day culture, the number of cells was 188.4±34.2 per the same area described above. Fig. 1D shows cells from a 6 day culture, the number of cells was 417.3±37.6 per the same area described above. The cultures are still alive and producing new cells. Fig. 2 gives the number of cells per 2.4 mm² of the bottom surfaces of flasks after inoculation. There was a slowing of the rate of production of new outgrowth on transfer of the 47th passed cells (total survival time, 833 days). But in the cells of the 53rd and 65th passages, the rate of production could get back to the former rate. It is difficult to estimate exactly how much growth or multiplication of cells occurred at each transplantation.

In vivo cultures. Fibroblasts harvested from in vitro culture of marrow cells, gave rise to fibrous tissue in diffusion chambers implanted into the peritoneal cavity for 3 to 7 weeks. Differentiation of the osteogenic and cartilaginous tissues in the diffusion chambers was not recognized. Fig. 3 shows fibroblastic tissue in the diffusion chamber contained in the 47th passed cultured cells from the marrow and implanted intraperitoneally in an adult rabbit for 6 weeks. In the test for malignant growth, no tumor growth was obtained within 3 months of inoculation of the cultured cells in the adult rabbit abdominal muscles.

Discussion

The basic difficulty in *in vitro* studies on cell growth in relation to the aging of cultured cells is both procedural and theoretical. HAYFLICK (1965) stated that there is a limit to the number of cell divisions which normal cells may undergo. The accepted method for determining the total number of cell generations is to separate the cells at each transfer and to count them. According

to GEY et al. (1974), chicken embryo cells maintained in this way have been carried from 14 to 38 generations and the cultures have continued to grow for a maximum of 4 months. They also reported that continuous growth for a period of 44 months was achieved only on a collagen substrate. Our previous report (HATTORI and ASHTON 1979) stated that well development of rabbit bone marrow cell cultures was observed on type I collagen.

The present study showed the continuous growth of culture of rabbit marrow cells for 3 years on plastic. GEY et al. (1974) performed the routine transfer of the original explant instead of trypsinized cells. They obtained new outgrowth from a succession of 2×2 mm fragments. HAYFLICK's (1965) method of subcultivation was a trypsinization. A solution of 0.4% trypsin was added to each culture for 1 min. All except 1 or 2 ml of the solution was then decanted and the bottle culture allowed to stand at room temperature for about 30 min. But in our experiments a solution of 0.5% was used. Under the microscopical observation cells were harvested. The cultures were trypsinized for about several minutes. New outgrowth from a succession of $1.9\times10^5\pm1.8\times10^4$ (mean$\pm$S.E.) cells in the flasks has continued. And potential clones of cells have survived through many trypsinizations.

The decrease in rate of growth which occurred after 833 days in culture has recovered. Our previous report (ASHTON et al. 1980) showed formation of the bone and cartilage by first passed marrow stromal cells in diffusion chambers in vivo. In the present study, however, only a fibrous tissue developed from the 47th passed cells in diffusion chambers.

Acknowledgments

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和文抄録

骨髄由来長期継代培養 fibroblast の in vitro および in vivo での検討

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Fibroblast の試験管内老化現象が検討された．幼若家兎の大腿骨骨髄から得られた細胞を，MEM 10%牛胎児血清を加えた培地で，継代培養を続けた．継代65代，1108日後でも細胞はなお正常な発育を続けた．47代の細胞を diffusion chamber に封入し家兎腹腔内に留置したが，骨組織は認められず線維組織を主体とするものであった．