LITERATURE CITED

1. P. D. Gorizontov (editor), Homeostasis [in Russian], Moscow (1976).
2. K. P. Zak, in: Progress on Hormones and the Mechanism of Their Action [in Russian], Kiev (1977), p. 323.
3. Yu. I. Zimin and R. M. Khaitov, Byull. Éksp. Biol. Med., No. 12, 68 (1975).
4. G. Bianco, R. Patrick, and V. Nussenzweig, J. Exp. Med., 132, 702 (1970).
5. H. N. Claman, New Engl. J. Med., 287, 338 (1972).
6. J. J. Cohen, J. Immunol., 108, 841 (1972).
7. A. S. Fauci, Fed. Proc., 33, 750 (1974).
8. V. M. Rusu and M. D. Cooper, J. Immunol., 115, 1370 (1975).
9. A. B. Wilson and R. R. A. Coombs, Int. Arch. Allerg, 44, 544 (1973).
10. D. T. Y. Yu, P. T. Clements, H. E. Paulus, et al., J. Clin. Invest., 53, 565 (1974).

EFFECT OF TRYPSINIZATION OF BONE MARROW ON EFFICIENCY OF FIBROBLAST COLONY FORMATION IN MONOLAYER CULTURES

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KEY WORDS: bone marrow; stroma; typsin; culture; cloning.

Stromal mechanocytes are responsible for the transfer of the hematopoietic microenvironment during heterotopic bone marrow transplantation [4]. These cells, together with hematopoietic cells, are readily liberated from tissue structures during preparation of cell suspensions by the ordinary mechanical method, such as by passing fragments of bone marrow through a needle from a syringe. On explantation of bone marrow cells into monolayer cultures stromal precursor cells give rise to colonies, which are clones of fibroblasts [3]. Cells forming fibroblast colonies (CFFC) are present in a suspension of bone marrow cells in a very low concentration — not more than one CFFC/10^4 hematopoietic cells [2]. However, in bone marrow sections and films stromal cells — reticular cells of all types — account for up to 3% of the total number of nucleated cells [5], i.e., fewer than 1% of stromal cells exhibit clonogenic properties. It can be tentatively suggested that the remaining stromal cells either have no clonogenic properties or, since they are firmly bound in bone marrow structures, they are deprived of their colony-forming ability because of partial injury in the process of suspension of the tissue.

The possibility of increasing the number of clonogenic stromal cells in a bone marrow suspension by preliminary treatment of fragments of hematopoietic tissue with trypsin was studied in the investigation described below.

EXPERIMENTAL METHOD

The bone marrow donors were guinea pigs weighing 180-350 g and (CBA × C57BL)F_1 mice weighing 18-20 g. Bone marrow from the femoral or tibial diaphysis was flushed out as fragments into 0.25% trypsin solution, in which it was incubated for 60-90 min at room temperature, with periodic gentle shaking. During incubation some of the cells were washed from the fragments into the trypsin, and for that reason every 20-30 min the cell suspension was drawn off and transferred to centrifuge tubes, with the addition of 10% embryonic bovine serum. The remaining fragments of bone marrow were covered with a fresh portion of trypsin solution. At the end of trypsinization the bone marrow cells were sedimented by centrifugation, resuspended by forcing through a needle from a syringe into medium No. 199, filtered

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TABLE 1. Colony-Forming Ability of Bone Marrow Cells after Trypsinization of Fragments of Hematopoietic Tissue

Source of bone marrow cells	No. of cells in bone, $\times 10^7$	No. of CFFC per 10^5 cells	No. of CFFC in organ, $\times 10^3$
Femur*	7,2/5,6	1.2(45,0	0,7/25,2
Same	9,9/7,8	10.7/72.0	5,3/56,2
"	8,0/9,5	5.2/50,0	4.2/47,5
"	10,0/10,0	2,1/31,8	2,1/31,8
Tibia*	5,0/3,8	0.5/9,7	0,2/3.7
Flat part of pelvic bone*	2,8/2,4	5,0/20,5	1,4/4.9
Femur*	5,6/6,0 †	2.2/19,7 †	1,2/11,8 ‡
Femur and tibia†	2,6/2,2	9,3/43,0	— —

Legend. Numerator — without trypsinization, denominator — with trypsinization. *) Trypsin solution + EDTA was used, ‡) donor of bone marrow was a guinea pig, ‡) donors of bone marrow were two mice.

through two to four layers of Kapron, and explanted in doses of $3 \cdot 10^5$-$10 \cdot 10^5$ cells into 100-ml flasks with culture medium consisting of media No. 199 and RPMI 1640 with 20% embryonic bovine serum. Homologous bone marrow cells, irradiated in a dose of 5000 R, were added in doses of $2 \cdot 10^7$ cells as feeder. The cultures were incubated at 37°C for 10-12 days, after which they were fixed in 96° ethanol, stained by the Romanovsky–Giemsa method, and the number of colonies of fibroblasts containing not less than 50 cells was counted in them. Depending on the number of fibroblast clones, the number of stromal precursor cells in the explanted populations of hematopoietic cells was deduced. Since it has been shown that [1] cloning efficiency of bone marrow from the right and left limbs of the same animal is equal, bone marrow from the corresponding contralateral zone, treated in the same way except with medium No. 199 or RPMI 1640 instead of the trypsin solution, served as the control. When the effect of trypsinization on bone marrow of cancellous bone was studied, the flat portion of the pelvic bones was used, from which the bone marrow was scraped with a scalpel, after preliminary splitting of the bone along the surface.

To study the radiosensitivity of the CFFC liberated with the aid of trypsin, the suspension of bone marrow cells was irradiated on the ÉKU-50 apparatus at 4°C.

EXPERIMENTAL RESULTS

The cloning efficiency of normal guinea pig femoral marrow averaged 4.8 ± 2.1 CFFC/10^5 explanted cells (from 1.2 to 10.7), within the limits of individual variations among animals. After treatment with trypsin the cloning efficiency was increased about tenfold, to 49.7 ± 8.4 CFFC/10^5 cells (from 31.8 to 72.0). The same result was obtained after trypsinization of marrow from the other long bone — the tibia, but from the flat part of the pelvic bones the increase was fourfold. After trypsinization the dimensions of the colonies were unchanged, i.e., the proliferative potential of the "trypsin-independent" and "trypsin-dependent" CFFC was probably equal. Trypsinization of mouse bone marrow also led to an increase in cloning efficiency (Table 1). When trypsin solution with EDTA was used the cloning efficiency was increased compared with the control, but not more than after trypsinization alone.

The results show that after treatment of fragments of hematopoietic tissue with trypsin there was no appreciable increase in the total number of nucleated cells, and for that reason the increase in the number of CFFC in the bone marrow was due entirely to an increase in the concentration of CFFC in the cell suspension. Meanwhile, during trypsinization there was no sign of death of any considerable number of bone marrow cells, which, given the high survival rate of the CFFC, might have been the cause of the sharp increase in their concentration. The increase in the number of CFFC after trypsinization could take place either

TABLE 2. Colony-Forming Ability of Guinea Pig Bone Marrow Cells after Treatment of Cell Suspensions with Trypsin

Expt. No.	Duration of treatment, min	Trypsin concentration, %	No. of CFFC per 10^5 cells
1	—	—	8,2
	10	0,25	6,5
	30	0,25	7,2
	60	0,25	9,7
2	—	—	0.4
	30	0.25	0,5
	30	0.025	0,4
	30	0.0025	0.7
3	—	—	0.2
	10	0.0025	0,2
	10	0,00025	0,3
	10	0,000025	0.2

Legend. Young (experiment 1) and old (experiments 2 and 3) animals with high and low cloning efficiency on their bone marrow, respectively, were used as bone marrow donors.

on account of additional liberation of CFFC from the pieces of bone marrow into the cell suspension or on account of conversion of nonclonogenic cells into clonogenic under the influence of trypsin, i.e., trypsin activates the transition from noncolony-forming cells of the bone marrow suspension into the CFFC category. However, the hypothesis on the activating action of trypsin was not confirmed, for treatment with trypsin did not cause any increase in concentration of CFFC in the suspensions of bone marrow prepared by the ordinary mechanical method — forcing fragments of bone marrow from syringes through needles of decreasing diameter (Table 2).

Trypsin thus did not activate cells which were already in suspension, but it evidently liberated an additional population of CFFC from the bone marrow fragments, which was not included among the cells readily eluted from the hematopoietic tissue structures. This was confirmed by an experiment to compare the efficiency of cloning of bone marrow treated in its entirety with trypsin and of bone marrow in which only firmly adherent structures were treated with trypsin, the readily eluted cells having first been separated. For this purpose, fragments of bone marrow from the femur were incubated for 30 min in medium No. 199 with periodic shaking. A suspension of readily eluted cells, the number of which amounted to 71% of the total number of hematopoietic cells in the femur, was transferred into a separate flask. The remaining fragments of bone marrow were treated with trypsin. The cloning efficiency of the mixture of trypsinized and easily eluted bone marrow cells from the femur was 12 CFFC/10^5 cells.

Bone marrow from the contralateral bone was divided into two halves. One half of the bone marrow was wholly treated with trypsin, and its cloning efficiency was 14.3 CFFC/10^5 cells. The cloning efficiency of hematopoietic cells from the other half (used as the control for the trypsinized bone marrow from both bones) was 2.2 CFFC/10^5 cells. Consequently, in bone marrow treated in its entirety with trypsin, for every 10^5 cells an additional 12 "trypsin-dependent" CFFC (2.2-14.3) were liberated. The number of CFFC additionally liberated from bone marrow in which only cells firmly bound into structures were treated with trypsin was no less (12.0-2.2 = 10.8 CFFC), only about 30% of the total number of cells. This means that the trypsin-dependent population of CFFC was not eluted for practical purposes into the medium together with the readily liberated hematopoietic cells, but it remained in the composition of structures formed from firmly bound bone marrow cells.

The radiosensitivity of the CFFC from trypsinized bone marrow fragments remaining after removal of the readily liberated cells was characterized by the formulas $D_o = 127 \pm 7$ R and $n = 1.8$.

Depending on the character of their connections with hematopoietic tissue structures the clonogenic stromal bone marrow cells thus form two fractions. One is "trypsin-independent," i.e., it includes cells which are liberated together with hematopoietic cells during mechanical suspension of bone marrow fragments. The second fraction — "trypsin-de-

pendent" — consists of cells more firmly bound with tissue structures, and trypsinization is necessary for their liberation. The role of each of these fractions of stromal precursors in transfer of the microenvironment is a matter for further study.

LITERATURE CITED

1. N. V. Latsinik, S. Yu. Sidorovich, Yu. F. Gorskaya, et al., Radiobiologiya, No. 6, 848 (1979).
2. S. Yu. Sidorovich and N. V. Latsinik, Byull. Éksp. Biol. Med., No. 7, 95 (1978).
3. A. Ya. Fridenshtein, R. K. Chailakhyan, and K. S. Lalykina, Tsitologiya, No. 9, 1147 (1970).
4. A. Ya. Fridenshtein, R. K. Chailakhyan, N. V. Latsinik, et al., Probl. Gematol., No. 10, 14 (1973).
5. T. M. Fliedner, Y. P. Bond, and V. D. Cronkite, Am. J. Pathol., 38, 599 (1961).

AUTORADIOGRAPHIC STUDY OF HORMONE-REGULATED CELL PROLIFERATION IN THE GOLDEN HAMSTER UTERUS DURING POSTNATAL DEVELOPMENT

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Numerous investigations have shown that estrogens stimulate proliferative processes in the uterine epithelium of experimental animals whereas progesterone has an inhibitory action. Conversely, after parenteral injection of estrogen, progesterone stimulates cell proliferation in the stroma of the uterus [1]. Both stimulation and inhibition of proliferation are realized in the tissues of the reproductive tract through interaction between sex hormones and specific receptor proteins. Several workers have found that receptors of both estrogens [9, 12] and progesterone [10] are present in cells of the uterus in rodents at birth, but ability to respond to hormonal stimulation is formed much later [4, 5, 11]. However, ideas on the gradual formation of hormone sensitivity are by no means generally accepted, and there is no unanimity on this problem [3, 8].

The object of this investigation was to study the effect of estrogen, gestagen, and antiestrogen on proliferative activity of epithelial and stromal cells in the golden hamster uterus during postnatal development.

EXPERIMENTAL METHOD

Experiments were carried out on 134 female golden hamsters aged from 1 to 20 days. In the experiments of series I (control) intact females were decapitated at the ages of 1, 3, 8, 12, and 20 days. In the experiments of series II the animals were decapitated at the same age 20 h after subcutaneous injection of 10 μg of an oily solution of octestrol. In the experiments of series III hamsters aged 1 and 3 days were decapitated 20 h after subcutaneous injection of 250 μg of an oily solution of 17-hydroxyprogesterone capronate, and animals aged 6, 12, and 20 days were decapitated after two injections of the hormone (44 h after the 1st and 20 h after the 2nd injection). In the experiments of series IV only animals aged 12 days were used. The hamsters were decapitated after two subcutaneous injections of the antiestrogens tamoxifen* (48 and 24 h thereafter).

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