# MICROBIOLOGY AND IMMUNOLOGY

# Origination of Stromal Precursor Cells Replenishing the Population of These Cells in Heterotopic Mouse Bone Marrow Transplants after Curettage in Recipients

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Curettage of bone marrow cavities of two bones (femoral and crural) in recipient mice causes a drastic (more than 7-fold) increase in the count of stromal precursor cells in heterotopic bone marrow transplants. Stromal colonies in cell cultures from these transplants consist of fibroblasts with an appreciable admixture of macrophages. All Y chromosome-typed colonies from cultures of female donor bone marrow transplants in recipient males (intact and subjected to curettage) contained cells carrying and not carrying Y chromosome. Quantitative results of Y chromosome typing of cells from colonies corresponded to the fibroblast/macrophage ratio in colonies and the predominant localization of the label corresponded to predominant localization of macrophages (at the periphery of colonies). The results indicate that the pool of bone marrow stromal precursor cells under conditions of increased demands originates from local sources, which confirms ample data on inability of these cells to migration.

**Key Words:** *bone marrow stromal precursor cells; heterotopic transplantation; capacity to migration*

There are published data that human and animal bone marrow contains osteogenic precursor cells and precursor cells of many other tissues [4,5,8]. Based on these data, attempts at the treatment of some diseases and defects of the bone, nervous, and muscle tissues by transplantation of stromal stem cells from the bone marrow were made. The method of transplantation is very important: intravenous injection or transplantation directly into the site of the defect (for example, infarction or bone fracture). An important question here is whether stromal stem cells residing in hemopoietic and lymphoid organs are able to migrate via the vascular network to the site of the defect with subsequent repopulation of this area, or the increase in the count of these cells in case of defect and in response to increased demands is replenished solely from local sources.

In order to answer this question we used curettage of the bone marrow cavity in recipient males with bone marrow transplants from donor females, with subsequent identification of the genotype of stromal precursor cells (CFC-F) replenishing the pool of CFC-F in the bone marrow transplants. It was reported that destruction of the contents of

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bone marrow cavity in femoral and crural bones by curettage was associated with a systemic increase in CFC-F count in the bone marrow in both subjected to curettage and contralateral limbs (up to 6 times after repeated curettage) [2].

## MATERIALS AND METHODS

Experiments were carried out on 2-3-month-old CBA mice (males and females) and 4-5-month-old guinea pigs (females) from Laboratory Animal Breeding Center, Kryukovo. For heterotopic transplantation, $^1/_2$ of femoral bone marrow from CBA females was placed under the renal capsule of CBA males as described previously [1]. The suspension of bone marrow cells from 8-month transplants was prepared as follows: the transplant contents was scraped out with a scalpel into αMEM with 5% FCS (Paneco), passed several times through a syringe with needles of reducing diameters, and filtered through 4 layers of Capron. Suspensions of femoral bone marrow cells were prepared as described previously [7]. The cells of the transplant ($2\text{-}4\times10^5$) and femoral bone marrow ($2\text{-}3\times10^6$) were then explanted into Slide Flasks (Nunc) for Y chromosome typing or into 25-$cm^2$ flasks in 5 ml αMEM culture medium (Sigma) containing 5% FCS (Paneco). After 2 h the medium with nonadherent cells was discarded, the cultures were washed twice with αMEM, and complete culture medium containing 80% αMEM, 20% FCS, and antibiotics (penicillin and streptomycin, 100 μg/ml each) was added. Bone marrow cells ($10^7$) from guinea pigs exposed in a dose of 60 Gy ($^{60}$Co, 10 Gy/min) were added to all cultures as a feeder. Guinea pig bone marrow cell suspension was prepared with a syringe as described previously [7]. All cultures were grown for 7-8 days in a $CO_2$ incubator at 37°C, after which they were washed with medium, fixed in methanol, colonies consisting of at least 30 fibroblasts were counted under a binocular microscope, and the fibroblast/macrophage ratio in the colonies was estimated. Cloning efficiency (CFE-F, the number of colonies formed by $10^5$ explanted cells) was evaluated by the number of colonies.

Curettage of two bones (femoral and crural) in recipient males carrying bone marrow transplants from females was carried out 24 h before explantation of the transplant cells into cultures as described previously [2]. The CFC-F colonies formed in the bone marrow cell cultures were typed for Y chromosome. Cell typing for Y chromosome was carried out by fluorescent *in situ* hybridization (FISH) using DNA probe to mouse whole Y chromosome (Cambio) according to the instruction for whole painting probe hybridization. The sensitivity of the FISH method evaluated as the percent of fluorescent label incorporation as a result of hybridization in control experiments was 96%.

## RESULTS

Curettage of two recipient bone marrow cavities (femoral and crural) drastically (more than 7-fold) increased the number of CFC-F in the bone marrow transplants as soon as after 24 h (Table 1), which confirms the data on the systemic response of the bone marrow stromal tissue to curettage [2]. The content of nucleated cells in the transplants changed negligibly (Table 1). The number of cells in heterotopic transplants explanted into cultures for typing was chosen so that solitary colonies grew in cell cultures of heterotopic bone marrow transplants from intact recipients, while in similar cultures from recipients subjected to curettage the number of colonies increased 8-fold as soon as after 24 h (Table 2). Hence, the overwhelming majority (88%) of colonies in cultures of the latter group were newly formed "additional" CFC-F colonies, whose precursors appeared in the transplant territory from an unknown source as soon as 24 h after curettage.

The morphology of cultures from bone marrow transplants did not differ from that described previously [7]. The colonies in these cultures consisted from fibroblasts with a significant admixture of macrophages (Table 2). An appreciable number of macrophages were seen between the colonies. In the majority of colonies, fibroblasts were located in the center, while macrophages were primarily located at the periphery of colonies. The presence of an appreciable number of macrophages in the cultures seems to be due to the fact that the cultures were fixed during early (7-8 days) periods in order to prevent the appearance of multilayer colonies of

**TABLE 1.** Cloning Efficiency in Cultures of Mouse 8-Month Bone Marrow Transplants after Curettage of Femoral and Crural Bone Marrow Cavities in Recipients

| Parameter | Recipient | |
|---|---|---|
| | intact | after curettage |
| Content of nucleated cells in transplant, $\times10^6$ | 5.4±0.6 | 5.3±0.7 |
| CFE-F, $\times10^{-5}$ | 1.3±0.1 | 9.2±1.3 |
| Count of CFC-F in transplant | 71±14 | 476±40 |

**Note.** A pool of cells from 5 transplants was used for explantation.

**TABLE 2.** Y Chromosome Typing of CFC-F Colonies in Mouse Bone Marrow Cultures

| Cell source | Number of colonies in cultures | Of these, typed for Y chromosome | Number of mixed colonies by results of typing | Number of colonies containing only cells with Y chromosome | Percent of macrophages in colonies | Percent of cells with Y chromosome in colonies |
|---|---|---|---|---|---|---|
| CBA mouse bone marrow transplant from females to males* | | | | | | |
| intact recipient | 5 | 5 | 5 | 0 | 70 | 70 |
| recipient subjected to curettage | 42 | 13 | 13 | 0 | 30 | 30 |
| CBA male femoral bone marrow | 20 | 20 | 0 | 20 | 20 | 96-100 |

**Note.** *Cell pool from 5 transplants was used for explantation in each experiment.

fibroblasts (which would impede cell typing), though it is known that the content of macrophages in cultures decreases during culturing. Stromal fibroblasts and macrophages clearly differed by their morphology. The composition of colonies in cultures varied. Group A: CFC-F colonies in bone marrow transplant cultures from intact recipients contained about 30% fibroblasts and about 70% macrophages. Group B: CFC-F colonies in cultures from recipients subjected to curettage of two bones 24 h before explantation of the transplant cells contained about 70% fibroblasts and about 30% macrophages. This difference in the fibroblast/macrophage ratio can be due to the following facts: 1) appreciable part of CFC-F is characterized by osteogenic activity and, as was reported, the bone marrow regenerating after bone marrow cavity curettage produces osteogenic growth peptide [3]; 2) stromal fibroblasts produce an autocrine stimulatory factor, its content in cultures increases with increasing the number of stromal fibroblasts in the culture [7]. Presumably, that is why macrophages predominated in group A cultures with solitary CFC-F colonies, while fibroblasts predominated in group B with numerous CFC-F colonies. All colonies typed for Y chromosome (groups A and B) contained cells carrying this chromosome (70% in group A and 30% in group B) and not carrying Y chromosome (30% in group A and 70% in group B), that is, the colonies were mixed. Hence, quantitative results of Y chromosome typing of cells in colonies corresponded to the fibroblast/macrophage ratio in colonies (Table 2), the predominant localization of the label corresponding to predominant localization of macrophages (periphery of colonies).

Previous studies showed that in cell cultures of 8-month bone marrow transplants from intact recipients the macrophages are of the recipient origin, because these cells descend from hemopoietic stem cells capable of migration, while virtually all local cells with the donor genotype are stromal precursor cells and their descendants, stromal fibroblasts [5].

Typing of cells in CFC-F colonies from the cultures of bone marrow transplants developing under conditions when the count of stromal precursors in the transplants increases rapidly and sharply (in our case after curettage) can lead to different results, depending on the source of replenishment of the CFC-F pool in the transplant territory: by repopulation of these cells or their migration from local sources.

Variant 1: the CFC-F pool in the transplant is replenished due to migration and repopulation of these cells from the recipient organism. In this case all cells in virtually all colonies have the recipient genotype. Not only descendants of migrating cells, *i.e.* macrophages, but also stromal fibroblasts have Y chromosome in this case. The only exception is cultures of 2-month bone marrow transplants. Negligible admixture of macrophages with the donor genotype can be present in them, no more than 6% of total number of cells in cultures (20% of 30% macrophages) [6]. We used cultures of 8-month bone marrow transplants, where (according to some authors [5,6]) all macrophages were of the recipient's origin. Variant 2: the pool of CFC-F in the transplant is replenished from local sources. In this case, all fibroblasts in all colonies remain unlabeled during typing; the label is present only in macrophages, and hence, each colony containing fibroblasts and macrophages remains mixed by the results of typing. Variant 3: both mechanisms are involved. In this case some colonies consist of Y chromosome-labeled cells (variant 1) and others contain a mixture of labeled and unlabeled cells (variant 2).

The results of cell typing in colonies from transplant cultures originating from recipients subjected to curettage conform to variant 2: all CFC-F colonies contained unlabeled cells and there was not a single CFC-F colony in which all cells contained the "Y" label (Table 2). Typing of cells in colonies by the FISH method detects virtually all nuclei with Y chromosome. Really, at least 96% cells in 100% typed colonies were labeled in bone marrow cell cultures from the femoral bones of male recipients.

Curettage of the bone marrow cavity is assumed as an adequate model for studies of the mechanisms of posttraumatic bone tissue regeneration. The results indicate that the pool of bone marrow CFC-F under conditions of increased demands in cells of this population is replenished from local sources. This is confirmed by numerous data indicating that this category of cells is incapable of migration and repopulation [5,9] and this fact should be taken into consideration when choosing the method for stromal tissue transplantation.

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