Stromal Clonogenic Precursors of Hemopoietic Microenvironment and Their Rank in the Hierarchy of Mesenchymal Stem Cells

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The hierarchy of stromal precursor cells was studied. Changes in the number of fibroblast CFU in foci forming in irradiated recipients were analyzed. These precursor cells are most close known descendants of mesenchymal stem cells, while inducible precursors rank lower in the hierarchy and are the cells directly enlarging the hemopoietic area in irradiated recipients.

Key Words: *mesenchymal stem cells; fibroblast colony-forming units; focus of ectopic hemopoiesis; precursor hierarchy*

Stromal cells of hemopoietic microenvironment descend from mesenchymal stem cells (MSC), multipotent precursors capable of multilineage differentiation (giving rise to osteoblast, adipocyte, chondrocyte, fibroblasts, and myocytes) [4]. The capacity of human MSC to self-maintenance is not proven [10], but some data indicate that these cells are characterized by high proliferative potential in culture. Mouse MSC can transfer the hemopoietic environment *in vivo* at least 9 times, this proving their capacity to self-maintenance [6]. Opinions on the set of pnehotypical markers of MSC vary [15]. Therefore, stromal precursor cells are characterized by physiological methods, similarly as previously in studies of hemopoietic stem cells (HSC). Single-cell suspension of bone marrow cells placed into a culture flask yields discrete colonies forming after 10-14 days. Each of these colonies is a clone produced by a single precursor — fibroblast CFU (CFU-F) [8]. It is assumed that CFU-F are cells of mesenchymal origin, but not HSC [5,12]. The CFU-F are heterogeneous, due to which some of these cells, with sufficiently high proliferative potential and capable of differentiation are attributed to MSC [14]. Transfer of a pool of these colonies from the culture into the body leads to the formation of various tissues, including the bone and adipose tissue [3,9]. Study of factors essential for the growth of CFU-F showed that at least 4 factors are obligatory: growth factor isolated from platelets (PDGF), βGFG, transforming growth factor-α (TGF-α), and EGF [13]. Proliferative potential of CFU-F is not yet sufficiently studied. It is known that the majority of CFU-F do not divide *in vivo* and are in the G_0 cell cycle phase [11], but being transferred into culture they start to proliferate.

Similarly as HSC, MSC have a differentiation hierarchy. It was for the first time shown in experiments on *in vivo* formation of an ectopic hemopoiesis focus. An ectopic hemopoietic focus forms at the site of transplantation 6 weeks after transfer of donor bone marrow fragments under the recipient renal capsule; stromal cells in this focus belong to the donor, while hemopoietic cells belong to the recipient. The hemopoietic microenvironment in this case is constructed *de novo* by stromal precursor cells. Linear relationship between the size of transferred bone marrow cylinder and number of

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cells in the forming focus of ectopic hemopoiesis is higher than for HSC; radioresistance of cells responsible for the formation of the new focus and their capacity to retransplantation and formation of functional hemopoietic microenvironment suggest that these stromal precursor cells can be regarded as MSC. Hence, the quantity of MSC in the bone marrow can be evaluated by the method of ectopic hemopoietic focus formation.

The focus of ectopic hemopoiesis, forming under the renal capsule in an irradiated recipient evaluated by the number of nuclear cells is 2-3-fold larger than in an intact recipient. However, transplantation of this focus to an intact recipient results in the formation of a focus of usual size, and hence, the number of MSC in this focus does not change; the focus in the irradiated recipient is enlarged at the expense of other precursor cells lacking self-maintenance capacity, one of two principal characteristics of MSC [1]. This precursor, called "inducible", occupies a place similar to that of multipotent descendants from HSC. The hierarchical ratio of "inducible" precursors and CFU-F is unknown. Study of the concentration and count of CFU-F in foci of ectopic hemopoiesis formed in normal and irradiated mice will clear out this problem. The following variants were expected: the concentration of CFU-F in enlarged foci formed in irradiated recipients could increase, decrease, or remain the same as in the control. If CFU-F concentration does not change, while their count increases 2-3-fold in the focus, enlarged 2-3-fold in irradiated recipients, this means that CFU-F descend from "inducible" precursors. If the concentration and count of CFU-F in the focus formed in an irradiated animal decrease, we can hypothesize that these precursors characterized by low self-maintenance capacity were irreversibly spent for the formation of a greater number of inducible precursors. Accordingly, CFU-F rank next after MSC and are higher than the "inducible" precursors in the "genealogical" tree of stromal precursors. If the content of CFU-F in an irradiated focus does not change, it seems that these precursors do not participate in the construction of hemopoietic microenvironment. The CFU-F to MSC ratio is disputed, as, though it has been shown, that CFU-F transferred into the body, differentiate, we do not know, whether they or their descendants transfer the stromal microenvironment.

Evaluation of the concentration and number of CFU-F in foci of ectopic hemopoiesis in normal and irradiated mice showed that CFU-F descend from MSC and their rank among the stromal SC is higher than that of "inducible" precursors.

MATERIALS AND METHODS

The study was carried out on 12-16-week-old female F_1(CBA×C57Bl/6) mice. The animals were irradiated on a ^{137}Cs IPK device (Hematology Research Center) at dose power of 16 sGy/min.

For the analysis of CFU-F, bone marrow cells (10^6) were put into a plastic flask with 25 cm^2 bottom area in 5 ml α-MEM (ICN) with 20% FCS (HyClone) and 5 ng/ml bFGF (kind gift from M. E. Gasparyan, Cand. Biol. Sci., Laboratory of Protein Engineering, Institute of Biochemistry) and cultured at 37°C and 5% CO_2. After 14 days the resultant fibroblast colonies were stained by 0.1% Crystal Violet in 20% methanol and counted in an inverted phase microscope.

In order to obtain individual fibroblast colonies, bone marrow cells were placed into a 96-well plate (30,000-50,000 nuclear cells per well) in the above culture medium. The concentration of 30,000 cell/well was preferable for this culturing system (Table 1). The incidence of CFU-F was calculated by Poisson's formula:

$$\text{incidence of CFU-F} = -\ln \left(\frac{\text{number of empty wells}}{\text{total number of inoculated wells}}\right).$$

Clones were transferred from the wells of 96-well plates into wells of 24-well plates, then into 6-well plates, and then into flasks with 25-cm^2 bottom area. The cells for transfer were treated with versene and then harvested with 0.05% trypsin in versene. Proliferative potential of CFU-F was evaluated by their capacity to form a confluent sublayer with

TABLE 1. Evaluation of CFU-F Incidence in Mouse Bone Marrow

Cells per well inoculated	Empty wells	One clone	Two clones	More than two clones per well	Number of CFU-F in cells inoculated per well
30,000	22	30	6	2	1.02
40,000	8	15	10	27	2.04
50,000	1	8	1	50	4.13

consecutive enlargement of the area available for growth. The number of mitoses realized by descendants of CFU-F was evaluated by admitting that the number of cells in the confluent monolayer increased in proportion with bottom area. For example, the area of a well bottom in a 96-well plate is 0.32 cm^2, in a 24-well plate 1.88 cm^2, in a 6-well plate 9.4 cm^2, and in a T25 flask 25 cm^2; when confluent cells are transferred from a 96-well into a 24-well plate, the bottom area is enlarged 6-fold, while after transfer from a 24-well plate into a 6-well one the bottom area is increased 5-fold, and upon transfer from a 6-well plate into a flasR3the bottom area is enlarged 2.3 times more. Hence, in order to fill the bottom area of a 6-well plate, the cells divide 5 times (starting from the well in the 96-well plate) and one more mitosis is needed for attaining confluence in the flask.

The focus of ectopic hemopoiesis from the bone marrow was obtained as described previously [1]. The mouse femoral bone marrow cylinder was implanted under the renal capsule of syngeneic mice, intact or irradiated in a dose of 6-10 Gy. Animals exposed to 10 Gy were intravenously injected with syngeneic bone marrow (at least 10^6 cells/mouse). After 1.5 month the size of the resultant focus of ectopic hemopoiesis was evaluated by the number of hemopoietic cells in it. The foci were isolated under sterile conditions, nucleated cells in them were counted, and the number of CFU-F was analyzed by the standard method.

The data were analyzed using Student's *t* test.

RESULTS

Six weeks after irradiation of mice in a dose of 6 Gy the concentration of CFU-F in their bone marrow did not differ from that in intact animals. The concentration of CFU-F per 10^6 bone marrow cells was 68.4±8.3, after irradiation 80.6±7.4.

The CFU-F is a heterogeneous group of precursors, differing by the proliferative potential. Analysis of the capacity of cells from fibroblast colonies to 6 and more mitoses showed that only 45% clones were capable of it. After irradiation only 6.25% clones retained this capacity, presumably because precursors, which replenished the total concentration of CFU-F, virtually completely lost their proliferative potential after surviving the irradiation. Unfortunately, the standard method for CFU-F estimation fails to show great differences in their proliferative activity.

Transplantation of the resultant confluent sublayers from flasks under the renal capsule showed that these cells did not tolerate the hemopoietic microenvironment, because no ectopic focus formed in any of the cases (according to the data of 5 transplantations in intact and 4 irradiated recipients). Hence, after more than 6 mitoses in culture CFU-F loose their proliferative potential and are incapable of differentiation *in vivo*.

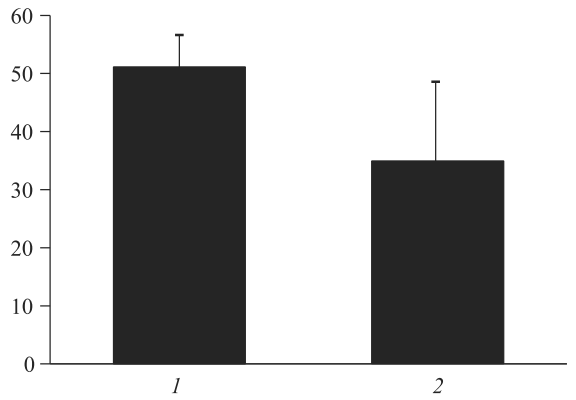

Fig. 1. Concentration of CFU-F in the bone marrow of intact mice (*1*) and in a focus of ectopic hemopoiesis (*2*). Ordinate: number of CFU-F per 10^6 cells.

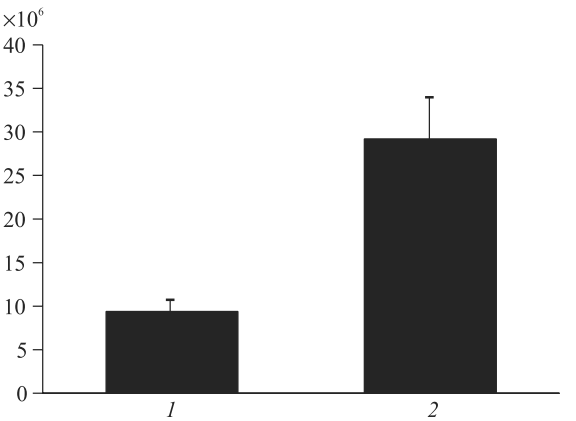

Fig. 2. Focus of ectopic hemopoiesis. *1*) intact animals; *2*) chimerical mice. Ordinate: number of nuclear cells per focus.

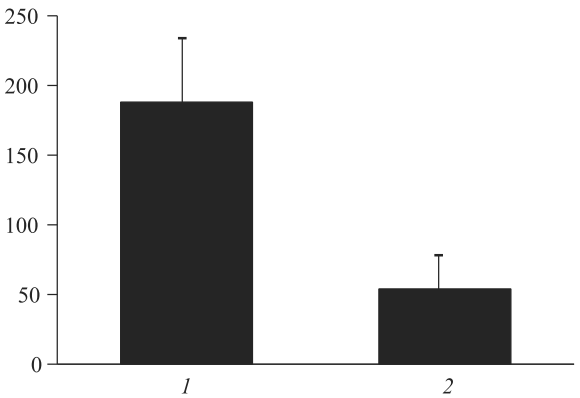

Fig. 3. Number of CFU-F in ectopic hemopoietic focus. *1*) normal animals; *2*) chimerical mice. Ordinate: number of CFU-F per focus.

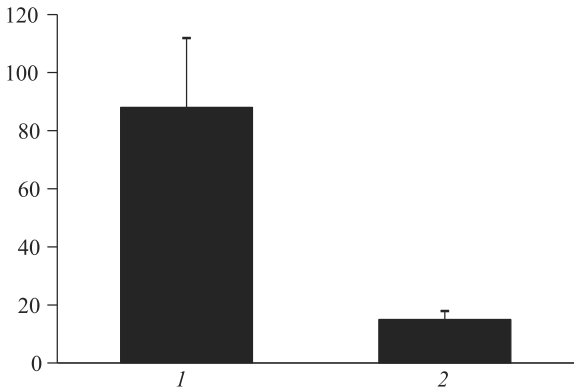

Fig. 4. Effect of mouse serum on CFU-F. Ordinate: CFU-F concentration per 10^6 bone marrow cells. *1*) mouse serum; *2*) irradiated mouse serum.

The characteristics of CFU-F in a focus of ectopic hemopoiesis were never studied. The concentration of these precursors in the focus was low in comparison with that in the bone marrow (Fig. 1, *a*). The concentration of CFU-F in ectopic hemopoietic focus formed in irradiated recipients was 20-fold reduced in comparison with that in the focus in intact recipients. As the focus in irradiated recipients was significantly larger than in intact animals (Fig. 2), the total number of CFU-F in the focus in irradiated recipients was only 3-fold less than in the foci of normal recipients (Fig. 3).

Hence, presumably CFU-F rank higher in the hierarchy of stromal stem cells than "inducible" precursors, but lower than MSC.

The effect of an irradiated mouse serum on the growth of CFU-F also suggests a lower rank of "inducible" precursors, directly realizing the enlargement of hemopoietic area in response to irradiation, in comparison with CFU-F. It is known that stromal growth factor, due to which a larger focus forms in irradiated recipients, is produced in the bones and released into the blood [7]. Addition of mouse serum to mouse CFU-F culture medium increases the cell count [2]. Addition of 2.5% serum of irradiated mice to culture medium results in a drop of CFU-F count per flask (Fig. 4). A great number of dissociated cells is seen in this case at the bottom of the flask, which is untypical of this method. We can speculate on the causes of reduction of CFU-F number in the presence of irradiated animal serum. On the one hand, it is probable that not all colonies are discernible in the presence of dissociated cells, on the other hand, presumably, stromal growth factor, present in irradiated animal serum, stimulates the differentiation of CFU-F descendants, as if enlarging the hemopoietic area, similarly to the *in vivo* enlargement of the focus in irradiated animals.

These data determine the place of CFU-F in the hierarchy of stromal precursor cells. These heterogeneous cells, incapable of self-maintenance, but characterized by very high proliferative potential, descend from MSC. "Inducible" precursors, stimulated by stromal growth factor in irradiated animals and providing enlargement of hemopoietic area in ectopic hemopoietic focus, rank lower in this hierarchy.

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