Involvement of Bone Marrow Fibroblasts in the Recovery of Granulocytopoiesis after Cytostatic-Induced Myelosuppression

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The effects of anticancer drugs whose action is mediated by different mechanisms on activity of bone marrow fibroblasts and their role in the regulation of granulocytopoiesis recovery after cytostatic treatment were studied. The antimetabolite 5-fluorouracil strongly suppressed, while the anthracycline antibiotic adriamycin and the alkylating agent cyclophosphamide stimulated function of stromal cells. This largely accounted for different dynamics of recovery of the bone marrow neutrophil lineage.

Key words: *fibroblasts; bone marrow; cytostatic agents; regeneration; hemopoiesis*

Stromal components of the hemopoiesis-inducing microenvironment of the bone marrow play an important role in the regulation of hemopoiesis [1,7]. For example, fibroblasts, the most functionally active stromal cells, are important factors of the regulation of granulocytopoiesis because of their preferential binding to granulocyte-macrophage precursors during the formation of hemopoietic islets (HI) and production of a variety of specific hemopoietins [1,5,7,10,11]. However, the function of stromal elements of the hemopoietic tissue under extreme conditions, such as cytostatic treatment, remains poorly investigated. This study was designed to examine the functional activity of bone marrow fibroblasts and their role in recovery of the granulocyte lineage of the hemopoietic tissue after treatment with different anticancer drugs.

MATERIALS AND METHODS

Experiments were performed on 120 male CBA/CaLac mice weighing 18-22 g and aged 2-2.5 months obtained from the collection of Laboratory of Experimental Biomodeling, Tomsk Research Center. The animals received fluoropyrimidine antimetabolite 5-fluorouracil (5-FU, Darnitsa), an alkylating cytostatic, cyclophosphamide (Biokhimik), or anthracycline antibiotic adriamycin (Walter Bushnell). All anticancer drugs were injected intraperitoneally in maximum tolerated doses determined by probit analysis (228, 250, and 6 mg/kg, respectively). The mice were killed by cervical dislocation under ether anesthesia on days 2, 4, 6, 8, 10, or 12 postinjection. The control group consisted of 10 intact mice.

Bone marrow myelokaryocytes were counted by conventional methods, and differential counts were performed in smears. Stromal myelocyte precursors fibroblast colony-forming units) in the bone marrow were counted in methylcellulose culture. To this end, the concentration of viable nonadherent myelokaryocytes in test suspension was adjusted to 20×10^5 cells per ml semisolid culture medium. The medium contained 79% RPMI-1640, 1% methylcellulose, 20% embryonic calf serum, 280 mg/ml L-glutamine, 4 μM 2-mercaptoethanol, 50 mg/ml gentamicin, and 0.5 ng/ml human recombinat granulocyte-macrophage colony-stimulating factor (Sigma). Aliquots (0.5 ml) of the cell suspension were transferred to 24-well plates and cultured for 8 days in a CO_2 incubator at 37°C, 5% CO_2, and 100% humidity. The colonies were counted and subjected to morphological examination under a LOMO inverted microscope (Russia). Fibroblast co-

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lonies were determined as cell aggregates consisting of more than 20 spindle-shaped cells. Colony-stimulating activity (CSA) of conditioned media from adherent elements of the hemopoiesis-inducing microenvironment was determined in experiments with intact mouse megakaryocytes grown on a semisolid culture medium [2]. HI were counted in the bone marrow dissociated with 0.05% collagenase at 37°C for 40 min [2]. The cell suspension was mixed with an equal volume of 0.5% neutral red. Fibroblast-containing HI were determined as cell associations with stain-negative central cell.

The *in vitro* effects of anticancer drugs on fibroblasts of the hemopoietic environment were determined by feeder activity (maintenance of colony growth from intact nonadherent myelokaryocytes) of bone marrow adherent elements treated with cytostatic agents directly in the culture. Adherent cells were preincubated with 0.2 μg/ml adriamycin, 20 μg/ml cyclophosphamide, or 18 μg/ml 5-FU (the dose causing a 2-fold decrease in the number of granulocyte-macrophage colony-forming units in intact bone marrow *in vitro*). After 2-h incubation, the cells were washed and covered with a semisolid medium containing 2×10^5 intact myelocytes per ml medium. After 7-day of incubation under standard conditions, granulocyte colonies were counted.

Statistical analysis was performed by Student's *t* test.

RESULTS

The strongest and longest depression of the granulocytopoiesis developed after administration of 5-FU (up to 10-12 days, Fig. 1). The animals injected with adriamycin displayed active recovery of the bone marrow neutrophil counts starting from days 3-4 postinjection, which was characterized by a biphasic time course. Regeneration of the granulocyte lineage following the period of its strong suppression was especially rapid in mice injected with cyclophosphamide. In this group, there was an overshoot of immature (days 5-14) and mature (days 7-10) bone marrow neutrophils.

Our previous studies [3] indicate that accelerated differentiation of committed hemopoietic precursors is the main mechanism of recovery of the bone marrow after administration of adriamycin and cyclophosphamide, whereas their retarded maturation in the presence of 5-FU accounts for long-term cell depletion from the bone marrow.

The content of stromal mechanocyte precursors in the bone marrow of test mice receiving cytostatics changed considerably (Fig. 2). The yield of fibroblast colonies from bone marrow suspension from mice injected with adriamycin increased significantly on day 2, peaked on day 4, almost 4-fold surpassing the initial level, and then slightly decreased but remained above the control level throughout the study period. The decrease in fibroblast precursors after cyclophosphamide administration (to zero on day 6) was followed by a rapid increase to a maximum 3 times surpassing the initial count by day 10 of observation. However, the growth of colonies from bone marrow stromal precursors of 5-FU-treated mice was completely suppressed between days 2 and 6 postinjection, then slowly increased, but did not reach the control level (Fig. 2).

Functional activity of mature bone marrow fibroblasts displayed different responses depending on the

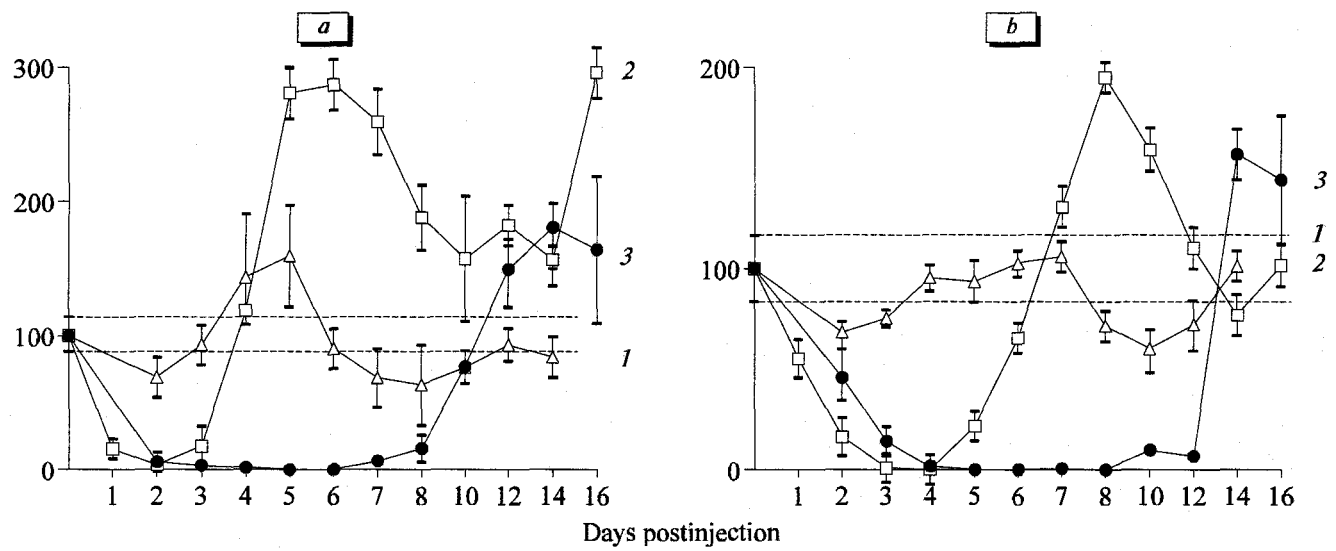

Fig. 1. Content of immature (*a*) and mature (*b*) neutrophil granulocytes in the bone marrow of CBA mice injected with adriamycin (*1*), cyclophosphamide (*2*), or 5-fluorouracil (*3*). Ordinate: cell content in the bone marrow. Dotted lines show confidence intervals at $p=0.05$.

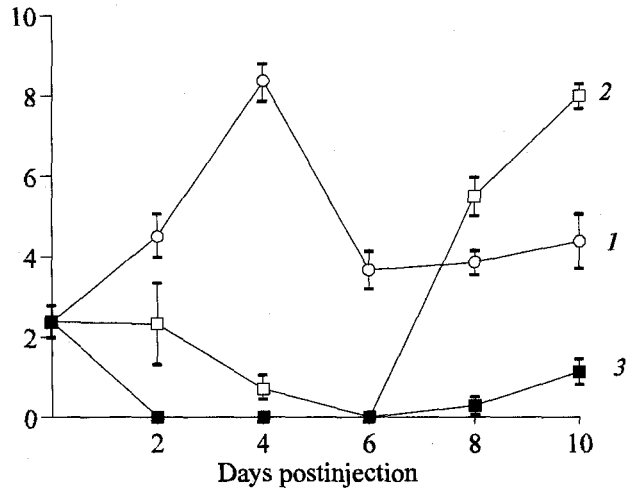

Fig. 2. Number of fibroblast colony-forming units (CFU-F) in the bone marrow of CBA mice injected with adriamycin (*1*), cyclophosphamide (*2*), or 5-fluorouracil (*3*). Ordinate: number of CFU-F per 10^5 myelokaryocytes.

cytostatic agent used. In mice injected with adriamycin or cyclophosphamide, the HI-forming capacity of these cells rapidly recovered (after a short period of depression) by days 5-6 (Fig. 3). In contrast, after 5-FU administration the number of granulocyte associations containing a fibroblast as the central cell in the bone marrow remained strongly decreased over the whole period of observation. Our previous studies suggest that structural and functional responses of the bone marrow to cytostatic agents are due to a decreased affinity of stromal cells for hemopoietic precursors [6].

The time course of secretory activity of stromal mechanocytes slightly differed from the time course of the yields of fibroblast-containing HI, most probably because of morphofunctional heterogeneity of these cell [4,8,9]. For example, cyclophosphamide 8-fold increased CSA production by fibroblasts immediately postinjection and this index remained high during the whole observation period (Fig. 3). Adriamycin induced a biphasic in this index (on days 2 and 6-8 postinjection). It should be noted that the rise of CSA after injections of adriamycin and cyclophosphamide were immediately followed by an increase in the count of immature neutrophils in the bone marrow. In turn, 5-FU did not stimulate CSA secretion by fibroblasts up day 8 postinjection. Thus, functional depression of bone marrow stromal cells caused by 5-FU (Fig. 3) was most probably responsible for long-term and severe depletion of the granulocyte lineage.

Cytostatics cause a stress response that affects a number of parameters [1]. To exclude the effects of stress-induced distant humoral factors on stromal cells, we studied the direct effects of cytostatic agents on bone marrow fibroblasts *in vitro*. Adriamycin and cyclophosphamide added to cultured bone marrow cells stimulated the ability of adherent myelokaryocytes to support the growth of granulocyte colonies (to 6.6±0.4 and 7.0±0.37 colonies, respectively, compared to spontaneous feeder activity of 5.0±0.32 colonies from 10^5 intact nonadherent myelokaryocytes). In contrast to these agents, 5-FU *in vitro* considerably decreased (to 3.6±0.4 colonies) feeder activity of adherent cells of hemopoiesis-inducing microenvironment toward granulomonocytopoietic precursors.

Thus, adriamycin caused a strong stimulatory effect, and 5-FU caused a toxic effect on bone marrow stromal cells. These effects were observed *in vivo* and *in vitro* at the earliest stages of fibroblast maturation

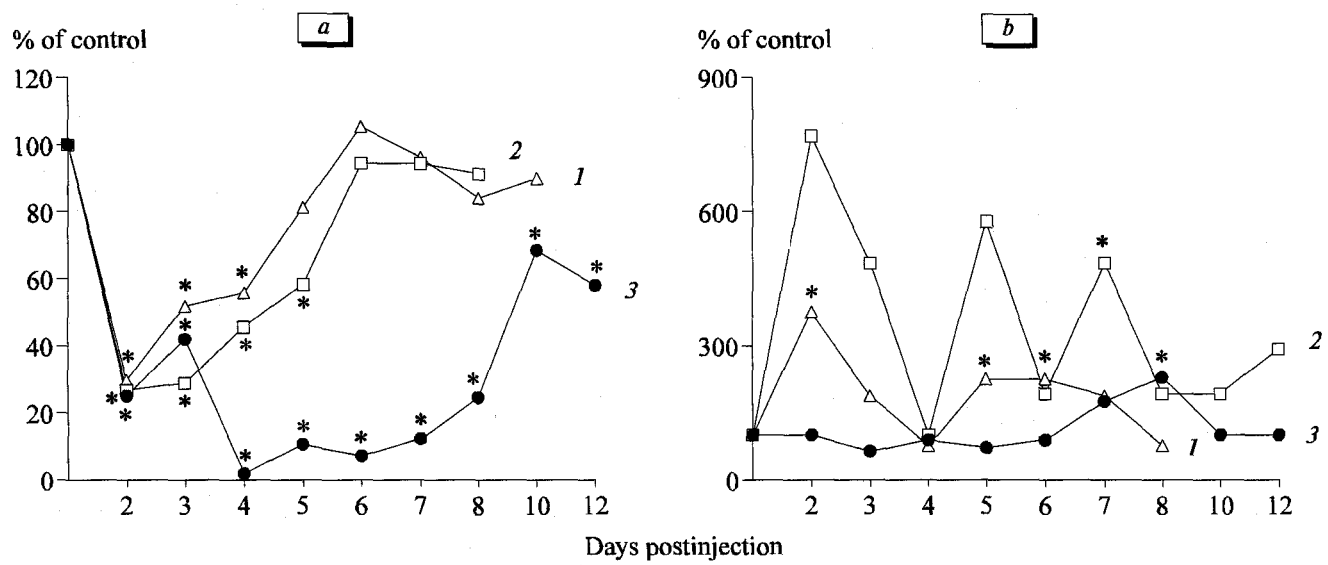

Fig. 3. Formation of hemopoietic islets (*a*) and secretion of colony-stimulating activity (*b*) by bone marrow fibroblasts from CBA mice injected with adriamycin (*1*), cyclophosphamide (*2*), or 5-fluorouracil (*3*). Ordinates: number of islets (*a*) and secretion rate (*b*). *$p<0.05$ compared to the control.

and influenced their various functional activities. The effect of cyclophosphamide on fibroblasts of the hemopoietic microenvironment was biphasic: depression caused by this cytostatic agent was followed by active recovery of the stromal mechanocyte population and their function, which resulted in rapid regeneration of the fibroblast-dependent granulocyte lineage of the bone marrow.

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