Functional Disturbances in Hemopoietic Microenvironment in Various Forms of Myelodysplastic Syndrome

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We studied the ability of stromal sublayer of long-term bone marrow cultures and peripheral blood macrophages from patients with various forms of myelodysplastic syndrome to maintain the growth of normal granulocyte-macrophage colony-forming units in mixed cultures. There were changes in the hemopoietic microenvironment in these patients: decreased cellularity of the bone marrow and impaired formation of sublayers in long-term bone marrow cultures, production of growth factors, maintaining the growth of normal granulocyte-macrophage precursors by stromal cells. Dysfunction of macrophages in the stromal microenvironment was probably related to the presence of pathological macrophages.

Key Words: *myelodysplastic syndrome; stromal sublayer; macrophages; hemopoietic microenvironment*

Stromal microenvironment plays an important role in the regulation of proliferation and differentiation of hemopoietic cells. Bone marrow stromal cells express cytokines, including colony-stimulating factors and inhibitors of hemopoiesis, which affect various regulatory functions. In addition, normal hemopoiesis depends on the interaction between hemopoietic cells, stromal cells, and extracellular matrix. Disturbances in their interaction and cytokine expression cause severe hemopoietic disorders [4].

Myelodysplastic syndromes (MDS) include various diseases: refractory anemia (RA), refractory sideroblast anemia (RSA), RA with excess blasts (RAEB), RA with excess blasts in transformation (RAEBT), and chronic myelomonocytic leukemia (CML). MDS are caused by damages to hemopoietic stem cells [9], but little is known on dysfunction of the stromal microenvironment in MDS. It was shown that the content of bone marrow stromal precursors decreases [1], and cytokine production by the stroma [8,16] and its ability to maintain normal hemopoiesis in long-term bone marrow cultures (LTBMC) are impaired in patients with MDS [7].

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Here we studied the ability of the stromal sublayer of LTBMC and peripheral blood macrophages from patients with various forms of MDS to maintain the growth of granulocyte-macrophage colony-forming units (CFU-GM) from the bone marrow of healthy individuals in mixed cultures.

MATERIALS AND METHODS

Bone marrow cells were isolated from 27 patients with various forms of MDS (FAB classification): RA (n=6), RSA (n=5), RAEB and RAEBT (n=7), and CML (n=9). Sternal bone marrow punctate from 10 healthy individuals served as the control.

The punctate (1-2 ml) and 10 ml peripheral blood were placed in flasks containing 50 U/ml heparin. Mononuclear cells were isolated on a Ficoll gradient (1.077 g/cm^3) and explanted in *in vitro* cultures.

The stromal sublayer was obtained by culturing bone marrow cells [6] in flasks (2×10^7 cells/flask) or 24-well plates (1.25×10^6 cells/well). On the 3rd-4th week of culturing the sublayer was irradiated (20 Gy), and nonadherent cells were removed and used in mixed cultures. The sublayer was treated with trypsin, and stromal cells were counted. Macrophages were obtained by culturing of adherent peripheral blood mono-

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nuclear cells in 24-well plates (10^6 cells/well). The ability of the stromal sublayer and macrophages to maintain the growth of CFU-GM was studied in mixed cultures. Bone marrow cells from healthy individuals in agar (10^5 cells/well) were explanted to irradiated stromal sublayer or macrophages from patients with MDS and healthy individuals [1] and cultured without colony-stimulating factors. Bone marrow cells cultured without sublayers in the presence of a standard growth factor (U5637 cell-conditioned medium) served as the control. The results were analyzed by Student's *t* test.

RESULTS

Bone marrow cells from MDS patients and healthy donors formed stromal sublayers, which were confluent and multilayer in healthy individuals, but loosened and single-layer (not confluent) in patients with MDS. On week 3-4, cell count in the stromal sublayer from patients with MDS (n=18) was lower than in healthy individuals (n=10): 12.7±2.1×10^5 and 24.9±4.8×10^5 cells/flask, respectively (p<0.01). The cellularity of the stromal sublayers in patients with RA and RSA only slightly decreased (insignificant, Fig. 1, *a*), while in patients with RAEB and CML this parameter was much lower than in healthy individuals (p<0.01).

The stromal sublayer of LTBMC from patients with MDS and healthy individuals maintained *in vitro* growth of normal granulocyte-macrophage colonies. Differences in CFU-GM growth between patients with MDS and healthy individuals were insignificant (65.6± 28.0 and 84.3±12.9%, respectively) probably due to considerable variations in this parameter in patients (0-584%).

The ability of LTBMC sublayers from patients with RAEB, RAEBT, and CML to stimulate the growth of normal bone marrow CFU-GM differed from that in healthy individuals (Fig. 1, *b*). The number of granulocyte-macrophage colonies formed on LTBMC sublayers from patients with RAEB and RAEBT was 2-fold lower than in healthy individuals. At the same time, the growth of CFU-GM on the stromal sublayer of LTBMC from patients with CML was in some cases negligible, while in others this parameter surpassed that in healthy individuals by several times. The growth

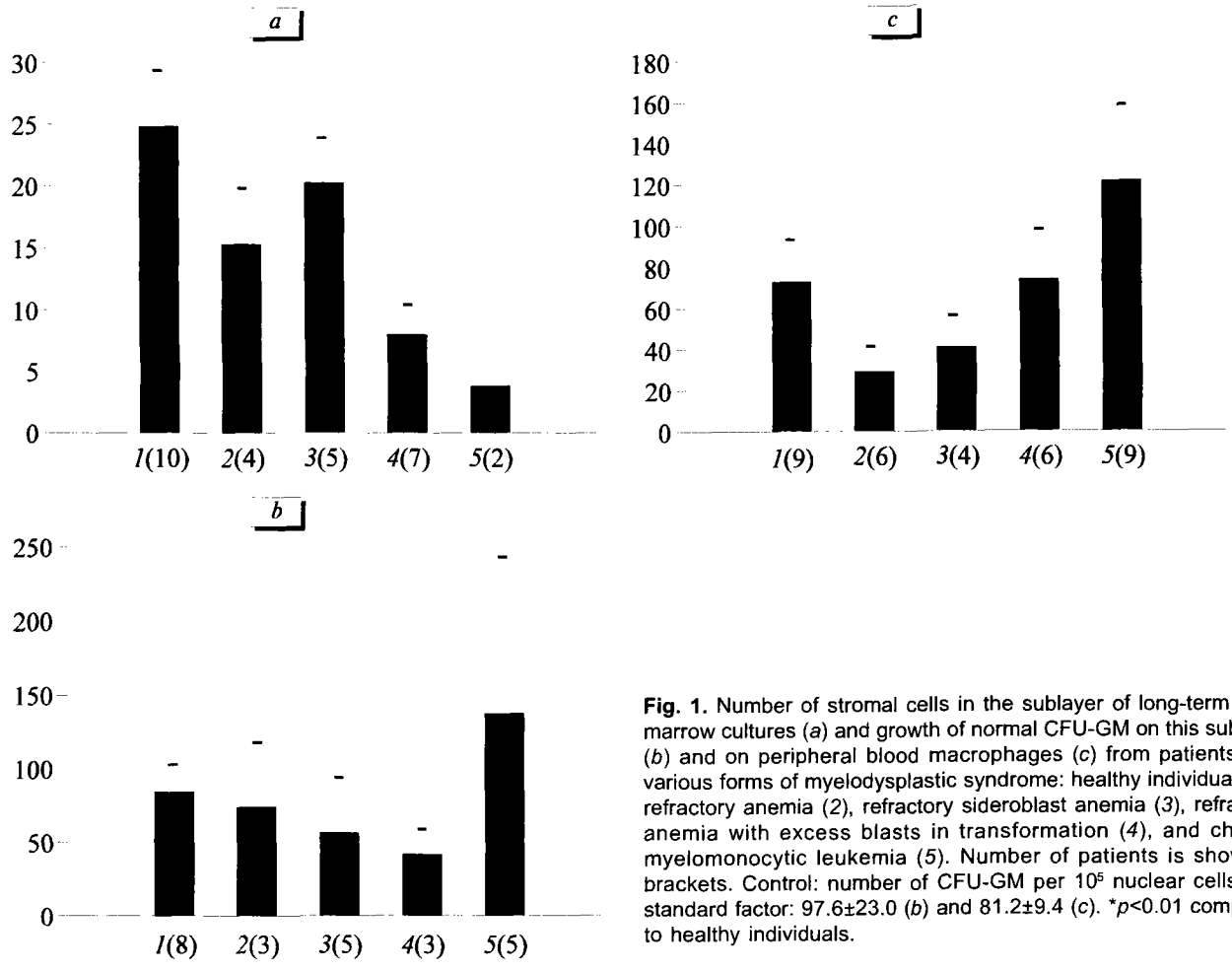

Fig. 1. Number of stromal cells in the sublayer of long-term bone marrow cultures (*a*) and growth of normal CFU-GM on this sublayer (*b*) and on peripheral blood macrophages (*c*) from patients with various forms of myelodysplastic syndrome: healthy individuals (*1*), refractory anemia (*2*), refractory sideroblast anemia (*3*), refractory anemia with excess blasts in transformation (*4*), and chronic myelomonocytic leukemia (*5*). Number of patients is shown in brackets. Control: number of CFU-GM per 10^5 nuclear cells with standard factor: 97.6±23.0 (*b*) and 81.2±9.4 (*c*). *p<0.01 compared to healthy individuals.

of CFU-GM on the stromal sublayer of LTBMC from patients with RA and RSA did not differ from the control (Fig. 1, *b*).

Thus, in patients with MDS the cellularity of the stromal sublayer in LTBMC and the ability of the stroma to produce growth factors and maintain the growth of normal hemopoietic precursors in clonogenic bone marrow cultures was impaired. It should be emphasized that these functional disturbances in the stromal microenvironment were most typical of patients with RAEB, RAEBT, and CML. Similar changes in the ability of the stromal sublayer to maintain proliferation and differentiation of $CD34^+$ cells were observed in patients with RA (S. Aizawa, *et al.* [2]).

To evaluate the role of microenvironmental elements in hemopoietic disturbances during MDS, we studied the ability of macrophages (cells of the stromal microenvironment) to initiate the growth of CFU-GM in mixed cultures. The percent of adherent peripheral blood mononuclear cells in patients with MDS surpassed that in healthy individuals (60.1±4.2 and 47.4±6.0%, $p<0.02$). The ability of macrophages from patients with MDS and healthy individuals to maintain the growth of normal CFU-GM did not differ (74.4± 17.7 and 73.2±13.0%, respectively). However, in different forms of MDS this parameter differed considerably (Fig. 1, *c*). The percent of CFU-GM on macrophages from patients with RA and RSA was 2 times lower than in healthy individuals. In patients with RAEB and RAEBT this parameter practically did not differ from normal, but in patients with CML it was above the control. These data indicate that macrophages from patients with various forms of MDS lose their ability to produce cytokines and to maintain proliferation and differentiation of normal CFU-GM in mixed cultures.

Pathogenetic mechanisms of MDS include damages to hemopoietic stem cells and hemopoietic microenvironment [3]. It is known that the number of fibroblast colony-forming units (stromal elements) in the bone marrow decreases during MDS [1]. In patients with various forms of MDS, the growth, structure, and functions of the stromal sublayer in LTBMC differ from the control [10,12]. Furthermore, in these patients the stromal sublayer loses its ability to maintain normal hemopoiesis in LTBMC [1,7]. This dysfunction of the stromal microenvironment also manifests in impaired cytokine secretion [8,11,14,15] and abnormal expression of cytokines and their receptors [5,16].

Macrophages derived from stem hemopoietic cells and expressing cytokines play a role in cytokine production. Our experiments revealed a mechanism underlying dysfunction of the stromal sublayer in patients with MDS. Macrophages from patients with various forms of MDS cannot maintain the growth of normal CFU-GM. Disturbances in the stromal sublayer and macrophages were most pronounced in patients with RAEB, RAEBT, and CML characterized by poor prognosis [13].

Our findings indicate that the nature and severity of disturbances in the stromal microenvironment differ in various forms of MDS. In MDS patients, the stromal microenvironment does not stimulate proliferation and differentiation of normal hemopoietic precursors in mixed cultures. Moreover, stromal microenvironmental cells from patients with MDS lose their ability to secrete cytokines probably due to dysfunction of macrophages. In patients with RA and RSA, there were only insignificant disturbances in the stromal sublayer, but the functions of macrophages were impaired. RAEB and RAEBT were accompanied by severe dysfunction of the stromal sublayer. Pathological changes in the stromal sublayer and macrophages were most pronounced in patients with CML.

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