Detection of Circulating Stromal Stem Cells with Osteogenic Potential in the Blood of Coronary Patients by Laser Flow Cytometry

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The number of cells expressing antigen to osteonectin was appreciably increased in the blood of coronary patients, but no cells of this kind were detected in donors. The number of $CD34^+$ stem cells in these patients virtually did not differ from that in normal subjects. A close relationship between atherosclerosis development and presence of stromal stem cells with osteogenic potential in the blood is hypothesized.

Key Words: *atherosclerosis; stem cells; osteonectin; laser flow cytometry*

Our data on the involvement of bone marrow stem cells (CFU) in human atherogenesis, obtained for the first time, prompted this study. Using cultural colony-forming tests, we detected stem CFU of not only hemopoietic, but also of stromal differentiation in the population of intimal cells from intima fragments of human atheromatous aorta (autopsy material) [1,2]. Moreover, stromal CFU were found in the blood of patients with hyperlidepemia (HLP) and coronary sclerosis [2]. This confirmed the bone marrow origin of stem CFU detected in the intima, but was in disagreement with the common concept on the impossibility of circulation of stromal stem cells in the bloodstream, in contrast to hemopoietic cells. A. Ya. Friedenshtein demonstrated for the first time that bone marrow reticular cells included stromal CFU-fibroblasts (CFU-F), capable of *in vitro* transforming into true fibroblasts, but retaining the polypotency of chondrogenic, osteogenic, and adipogenic differentiation [4]. However, it was believed at that time that stromal CFU, in contrast to hemopoietic ones, did not circulate in the bloodflow, and only few reports indirectly pointed to their circulation [4].

Using laser flow cytometry, we attempted to clear out, whether high counts of circulating stromal precursors expressing osteogenic differentiation marker (osteonectin) were characteristic of coronary patients, neglecting in our analysis the risk factors favoring the development of this disease. Previous data on the appearance of stromal CFU-F with osteogenic potential in the blood of patients with primary HLP and coronary sclerosis were confirmed by the clone method [2].

MATERIALS AND METHODS

Seven coronary patients and four healthy volunteers (controls) were examined. Clinical examinations of coronary patients included ECG recording at rest and during exercise (bicycle ergometer), daily ECG monitoring, myocardial scintigraphy at rest and after myocardial ischemia induced by bicycle ergometry, echocardiography, and coronaroangiography.

The disease course, findings of exercise tests, and daily ECG monitoring indicated coronary insufficiency of different severity in the patients. Coronaroangiography of the heart revealed critical stenosing atherosclerosis of at least two coronary arteries or their main

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branches in all patients. The severity of coronary involvement prompted aortocoronary shunting.

Blood for analysis was collected from the ulnar vein in patients and donors after overnight 14-h fast and stabilized with EDTA. The expression of different antigens by blood cells was studied 2 h after blood collection using phycoerythrin-labeled monoclonal antibodies to CD34 (CD34-PE, Caltag), FITC-labeled monoclonal antibodies to human CD45 (CD45-FITC, Caltag), and unlabeled polyclonal rabbit antibodies to human osteonectin (kind gift from Dr. Fisher, Bethesda, USA). FITC conjugate of goat antibodies to rabbit Ig(G+A+M) (IMTEK) were used as second antibodies. In the control respective isotypical antibodies (murine IgG1-PE, Caltag, and IgG1-FITC, Caltag, rabbit IgG, IgA, IgM, IMTEK) were used. Cell aliquots were incubated with antibodies for 30 min in the darkness at 20°C. Binding to second antibodies was carried out under the same conditions after centrifugation and single washing in phosphate buffer (0.1 M, pH 7.4). After the end of the reaction lyzing solution (FACS Lysing Solution, Becton Dickinson) was added to the samples; after 15 min the cells were centrifuged at 200*g* (15 min) and washed twice in phosphate buffer (0.1 M, pH 7.4), fixed in 1% paraformaldehyde, and analyzed on a FACS Calibur flow cytofluorometer (Becton Dickinson). The data were processed using CELLQuest (Becton Dickinson) and WinMDI software. A total of 100,000 events per sample were analyzed.

RESULTS

The pool of stem cells expressing CD34 marker characteristic of hemopoietic stem cells does not include stromal stem cells, according to some authors. However, other authors detected stromal cell precursors among $CD34^+$ cells [6]. Their phenotype is characterized by expression of types I and III collagens, osteonectin, fibronectin, Stro-I, and some adhesion molecules [5]. Therefore, we used osteonectin and CD34 as markers of stromal and hemopoietic stem cells.

The count of $CD34^+$ hemopoietic stem cells was evaluated by flow cytometry according to ISHAGE protocol (International Society of Hematotherapy and Graft Engineering). This protocol recommends using 4 parameters: direct and lateral light scattering, CD45-FITC and CD34-PE fluorescence [7]. The strategy of $CD34^+$ stem cell verification consisted in consecutive logical gating of accumulated events (100,000 per sample) by their light scattering characteristics and slight fluorescence of CD45-FITC. Gates containing mainly lymphocyte-like cells were distinguished on diagrams showing the relationship between latent and direct light scattering. Cells with low, but positive CD45-FITC fluorescence and CD34-PE positive fluorescence were detected by the parameters of CD34-PE and CD45-FITC fluorescence. The procedure ruled out from analysis nonlyzed erythrocytes, platelet aggregations, debris, and mature lymphocyte-like cells nonspecifically stained by anti-CD34. Cells meeting these requirements by the type of light scattering and fluorescence parameters are $CD34^+$ stem cells. Their count in the peripheral blood is not high, usually 0.01-0.10% of total number of nuclear cells. Our findings indicate that the number of $CD34^+$ stem cells in coronary patients did not differ from that in normal subjects (0.400±0.006% *vs.* 0.031±0.004%).

The number of stromal precursor cells with osteogenic potential in the peripheral blood was evaluated

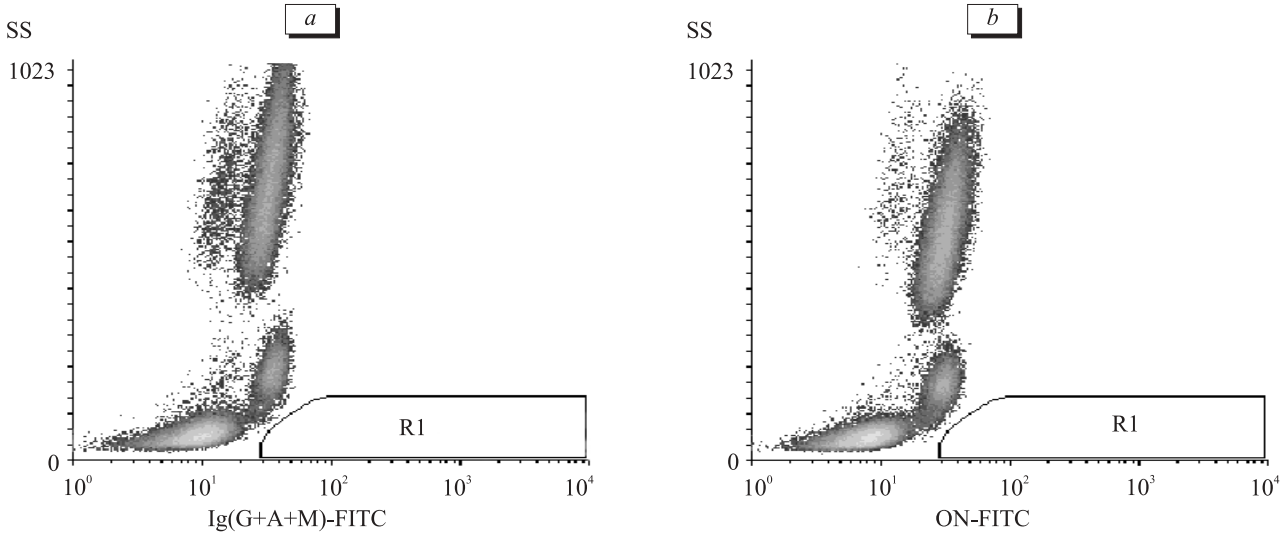

Fig. 1. Diagrams reflecting relationship between lateral light scattering (SS) and fluorescence intensity of cells labeled with isotypical controls (*a*) and antibodies to osteonectin (*b*) for a healthy volunteer.

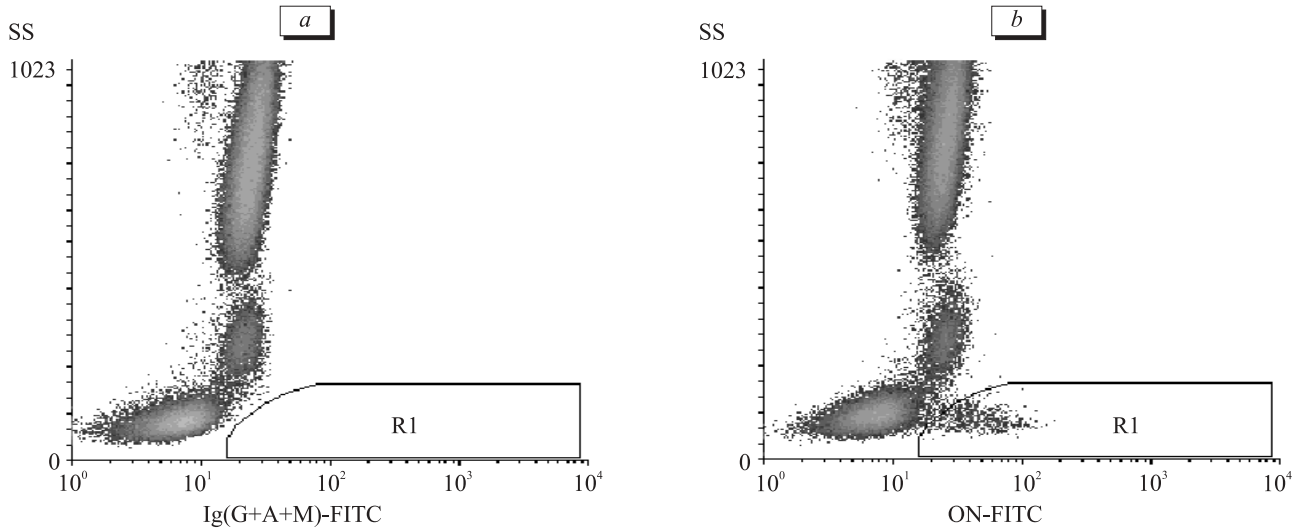

Fig. 2. Diagrams reflecting relationship between lateral light scattering (SS) and fluorescence intensity of cells labeled with isotypical controls (*a*) and antibodies to osteonectin (*b*) for a coronary patient.

using antibodies to osteonectin (noncollagen bone tissue protein). The results were analyzed using diagrams of relationship between lateral light scattering and intensity of FITC-labeled cell fluorescence (Fig. 1, *a*; 2, *a*). Gate R1 was distinguished in the slight intensity domain of lateral light scattering and ON-FITC positive fluorescence. Similar diagrams were plotted for samples stained with antibodies to osteonectin, and osteonectin-expressing stromal cells were detected in gate R1 (Fig. 1, *b*; 2, *b*). We found no osteonectin-positive cells in healthy subjects (0 cells per 100,000 events), while their number in coronary patients was appreciable, varying from 98 to 1853 per 100,000 events.

Hence, analysis of results of the peripheral blood flow cytofluorometry showed an appreciable increase in the number of cells of stromal differentiation lineage expressing antigen to osteonectin in coronary patients, but not in donors. This confirmed previous data on the absence of stromal stem CFU in normal subjects and their appearance in patients with coronary sclerosis, obtained by the classical cloning method. Presumably, atherosclerosis development is closely related to the presence of stromal stem cells in the blood, these cells penetrate into the intima and realize their polypotency by inducing different types of vascular wall sclerosis (fibrosis, chondro-osteogenesis with subsequent calcification) and formation of plaques of different origin. Recent studies showed that stromal stem cells (often called mesenchymal stem cells) participate in repair of injuries in the majority of tissues and are transported from the bone marrow through the bloodstream into other organs, where they realize their pluripotency inducing the growth of cells of different stromal phenotype. Our previous studies showed that human vascular intima can also be repopulated with circulating stromal stem cells, which has been confirmed [3].

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