Characteristics of Bone Marrow Cells under Conditions of Impaired Innervation in Patients with Spinal Trauma

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We studied quantitative and functional parameters of bone marrow stem cells and mature lymphocyte population under conditions of impaired innervation in patients with injuries to the cervical and thoracic portions of the spinal cord. Our findings indicated the absence of deficiency of quantitative and proliferative potentials of stem cells and demonstrated intact subpopulation structure of mature lymphocytes and T-cell proliferative activity similar to that in donors. The content of $CD34^+$ cells in patients did not differ from that in donors. The percentage of $CD34^+CD38^-$ hemopoietic stem cells was elevated in patients, presumably due to increased proliferative activity of hemopoietic stem cells. The possibility of derivation and *in vitro* culturing of fibroblast-like cells with mesenchymal stem cell phenotype was demonstrated.

Key Words: *bone marrow; stem cells; spinal injury*

Regulation of hemopoiesis in the bone marrow involves numerous microenvironmental cells mediating their effects through cytokine production [3]. Innervation of the bone marrow and expression of receptors to neurotransmitters by hemopoietic precursors suggest nervous regulation of hemo- and immunopoiesis [2,9,10,12], which can be regarded in several aspects. Impairment of nervous regulation in patients with spinal injury can be the cause of suppression of bone marrow hemopoiesis. Some authors noted changed differentiation potential of hemopoietic stem cells (HSC) and lymphocyte function in patients with spinal injury [7,8]. The study of functional integrity of stem cells and hemopoietic precursors under conditions of impaired nervous regulation is important for the use of autologous bone marrow for replacement cell therapy in patients with spinal injury.

Recent studies showed the possibility of differentiation of bone marrow stem cells in the neuronal direction *in vivo* and *in vitro* [1,4-6,11]. Therefore stem cells derived from patient's bone marrow can be used for the treatment of spinal injury. However, the characteristics of bone marrow cells in patients with spinal injury remain little studied.

We studied phenotypical and functional characteristics of bone marrow stem cells and lymphocytes in patients with spinal injury during delayed period of the disease.

MATERIALS AND METHODS

The study was carried out in 16 patients (12 men and 4 women aged 18-56 years), examined during delayed period of traumatic disease of the spinal cord. The disease duration varied from 8 months to 5 years (mean duration 26 months). Nine and seven patients presented with spinal injury at the

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level of cervical and thoracic vertebrae, respectively. Neurophysiological studies showed complete blockade of conduction via the somatosensory fibers from foot nerves in spinal injury at the thoracic level and complete blockade of conduction from arm nerves in patients with injury at the level of cervical intumescence. Magnetic resonance tomography of the spinal injury zone showed structural rupture of the spinal cord with cystic degeneration (intramedullary cysts of different degree) in all patients.

The bone marrow was aspirated from the iliac crests. Fraction of mononuclear cell (MNC) was isolated by standard centrifugation of the aspirate cells in Ficoll-verografin density gradient (1.078 g/liter) for 20 min at 3000 rpm. Bone marrow MNC collected from the interphase were washed 3 times in phosphate buffered saline and resuspended in RPMI 1640. The total count of MNC, percentage of $CD34^+$ and $CD34^+CD38^-$ HSC and lymphocyte subpopulations ($CD3^+$, $CD4^+$, $CD8^+$, $CD16^+$, $CD20^+$) were evaluated by flow cytofluorometry (FACS-Callibur, Becton Dickinson) using respective monoclonal antibodies. Mesenchymal stem cells (MSC) were isolated by incubation of bone marrow cells in plastic flasks (Nunclon) in DMEM (Sigma-Aldrich) with 15% FCS (ICN) at 37°C and 5% CO_2. After 24 h nonadherent cells were removed, adherent cell fraction was washed in Hanks solution and incubated until the formation of a confluence. Enzymatic dissociation of cells was carried out using 0.25% trypsin and 0.02% EDTA solutions (Sigma-Aldrich). Surface markers were evaluated in MSC cultures after 1-2 passages. The panel of studied markers included linear antigens (CD3, CD20, CD16, CD14), HSC antigens (CD34), and markers characteristic of MSC: CD90 (Thy1) and CD73 (SH-3 and SH-4). Apoptosis intensity and cell cycle in subpopulations of hemopoietic stem $CD34^+$ cells and $CD3^+$ T-lymphocytes were evaluated by double-color flow cytometry by the content of DNA using propidium iodide staining (4 μg/ml; Sigma-Aldrich). To this end, the content of cells with diploid (cells in the G_0/G_1 phases of cell cycle), hyperdiploid (cells in the S/G_2M phases of cell cycle), and hypodiploid (apoptotic cells) DNA set (orange fluorescence) among $CD34^+$ or $CD3^+$ cells was evaluated. Proliferative activity of bone marrow MNC was evaluated radiometrically (by incorporation of ^3H-thymidine, 1 μCi/well) after 72-h culturing. To this end, the cells (0.1×10^6/well) were cultured in 96-well round-bottom plates for immunological studies in RPMI-1640 with 0.3 mg/ml glutamine, 5 mM HEPES, 100 μg/ml gentamicin, and 10% inactivated donor serum (AB (IV) group). Proliferation of T-cells was stimulated by monoclonal anti-CD3 antibodies (anti-CD3, 1 μg/ml; Medbiospectr).

RESULTS

The count of MNC isolated from 1 ml of bone marrow aspirate tended to increase in all patients with spinal injury, being significantly increased in patients with injury to the cervical portion of the spinal cord (Table 1). The percentage of $CD34^+$ cells in patients was similar to that in donors. However, their absolute count increased as a result of increased output of MNC, particularly in subgroup 2. It is noteworthy that CD34 molecule is expressed not only on HSC, but also on committed precursors. On the other hand, more primitive HSC are characterized as $CD34^+CD38^-$ cells. The percentage of this cell subpopulation significantly increased in all groups of patients. Hence, bone marrow cell compartment in patients with spinal injury is characterized by increased absolute count of $CD34^+$ cells and increased percentage of HSC with $CD34^+$ $CD38^-$ phenotype among them.

TABLE 1. Characteristics of Bone Marrow Cells in Patients with Spinal Injury

Parameters	Donors (n=10)	Patients with spinal injury		
		total (n=16)	subgroup 1, cervical level (n=9)	subgroup 2, thoracic level (n=7)
MNC, 10^6/ml	7.5±2.2	11.0±1.1	8.8±1.6	13±1*+
$CD34^+$ cells, %	5.40±1.35	5.4±0.6	5.00±0.66	5.8±1.0
$CD34^+$ cells, $*10^6$	0.27±0.07	0.54±0.08	0.40±0.09	0.71±0.10*+
$CD34^+CD38^{-}$, %	0.38±0.2	1.23±0.20*	1.20±0.22*	1.5±0.4*
$CD34^+$ (G_0/G_1), %	77.0±3.4	59.0±4.9*	58.0±8.7*	60±6*
$CD34^+$ (S/G_2M), %	21.0±3.9	38.0±5.4*	40±9*	37.0±6.9*
$CD34^+$ apoptosis, %	0.94±0.47	3.6±1.1*	2.4±0.9	4.7±2.1*

Note. $p<0.05$ compared to: *donors; +cervical level (Student's t test).

TABLE 2. Characteristics of Bone Marrow Lymphocytes in Patients with Spinal Injury

Parameter	Donors (*n*=7)	Patients (*n*=16)
Subpopulation composition, %		
$CD3^+$ T-lymphocytes	51.4±6.9	48.8±3.2
$CD4^+$ T-lymphocytes	30.0±5.3	23.6±1.7
$CD8^+$ T-lymphocytes	20.4±3.2	23.5±1.5
$CD16^+$ NK cells	7.8±0.5	9.1±1.3
$CD20^+$ B-cells	16.2±1.6	13.3±1.4
Intact T-cells, %		
G_0/G_1	75.0±3.5	68.5±3.0
S/G_2M	13.0±5.2	16.4±2.5
apoptosis level	10.0±5.2	15.0±2.7
Anti-CD3 activated T-cells, %		
G_0/G_1	58.0±8.5	52.0±2.2
S/G_2M	29.0±5.9	36.0±2.7
apoptosis level	13±4	11.0±1.6
Proliferation, cpm		
spontaneous	24,510±3120	29,680±4290
anti-CD3-induced	35,120±2620	35,830±4400

Note. Level of apoptosis in $CD3^+$ T-cells and their distribution by cell cycle were evaluated in 24-h cultures of bone marrow MNC without stimulation (intact T-cells) and after stimulation with anti-CD3 antibodies (anti-CD3 activated T-cells) by double-color flow cytometry.

The count of bone marrow HSC, similarly to other cells in the body, is determined by the ratio of intensities of the proliferation/apoptosis processes. Comparative analysis of apoptosis and cell cycle for the $CD34^+$ cell subpopulation showed that the percentage of apoptotic cells in the whole group and in patients with thoracic level of injury was significantly higher than in donors. On the other hand, proliferative potential of HSC was increased in patients with spinal injury irrespective of the level of injury. The percentage of silent G_0/G_1 phase $CD34^+$ cells decreased significantly, while the mean content of actively dividing S/G_2M-phase cells 2-fold surpassed the normal. Hence, one more characteristic of HSC in patients with spinal injury is increased percentage of proliferating cells. Presumably, increased proliferative activity of HSC is responsible for their more intensive apoptosis. Abnormal migration activity of HSC as a factor promoting their accumulation in the bone marrow also cannot be excluded.

Evaluation of the content of lymphocyte subpopulations among bone marrow mononuclear cells showed no appreciable changes in the lymphocyte subpopulation structure (Table 2). The percentage of $CD3^+$ and $CD8^+$ T-cells, $CD20^+$ B-lymphocytes, and $CD16^+$ NK cells in patients did not differ from those in donors. The moderate decrease in the content of $CD4^+$ T-lymphocytes was statistically negligible. It was also found that the intensity of T-cell apoptosis and distribution by the cell cycle in non-stimulated and anti-CD3-stimulated cultures derived from patients were similar to those in donors. No differences in the level of proliferative response of bone marrow T-cells to stimulation with anti-CD3-antibodies were detected.

The possibility of MSC derivation was evaluated as an additional functional characteristic of bone marrow cells in all patients. The growth of fibroblast-like mesenchymal cells was observed in 12 of 16 patients. The period needed for confluent fusion (till the zero passage) varied from 7 to 28 days. Phenotypical characterization of MSC was carried out in 4 patients after 1-2 passages (Table 3). MSC did not express linear markers of T-cells (CD3), B-cells (CD20), NK cells (CD16), HSC (CD34), and HLA-DR molecules, but the overwhelming majority carried on their surface CD90 (Thy1 antigen) and CD73 (SH-3 and SH-4), characteristic of mesenchymal cells.

These data confirm preserved count of HSC, intact structure and functions of mature lymphocytes, and possibility of MSC generation in patients during delayed period of traumatic disease of the spinal cord. The detected increase in the count of HSC associated with an increase in their proliferative potential can be due to attenuation of the negative control from the nervous system, resultant from impaired innervation of the spinal cord after the injury. Disorders in stem cell migration from the bone marrow to the periphery are also possible. Autologous bone marrow of patients with spinal

TABLE 3. Phenotypical Characteristics of MSC from the Bone Marrow of Patients with Spinal Injury (*n*=4) after Long-Term Culturing

Markers, %	Patients			
	No. 1	No. 2	No. 3	No. 4
$CD3^+$	0.46	0.5	0.6	0
$CD20^+$	0.46	0.7	1.8	0.1
$CD16^+$	0.82	0.9	1.7	0.1
$CD34^+$	1.01	0.8	0.74	0.8
$HLA\text{-}DR^+$	1.1	2.0	13	1.4
$CD90^+$ (Thy-1)	72	81	56	49
$CD73^+$ (SH-3 and SH-4)	97	99	97	62

injury seems to be a good source of HSC and MSC, which can be used for cell replacement therapy.

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