EXPERIMENTAL BIOLOGY

Thymodepressin Inhibits Migration of CD34$^+$ Cells from Bone Marrow in Normal and Granulocyte CSF-Stimulated Hemopoiesis

O. V. Semina, T. N. Semenets, I. A. Zamulaeva, E. I. Selivanova, T. P. Il'ina, Ya. V. Malyutina, D. Yu. Semin, V. I. Deigin, and A. S. Saenko

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We studied the effect of thymodepressin on migration and adhesion of mouse hemopoietic CD34$^+$ cells under normal conditions and under the effect of granulocytic CSF. It was found that the peptide reduced the absolute number of CD34$^+$ hemopoietic cells in the peripheral blood, increased the percent of cells bound to fibronectin and expressing receptor for integrin β_1 (CD29$^+$) in the bone marrow of mice under normal conditions and after stimulation with granulocytic CSF, and reduced the relative number of cells carrying CXCR4 receptor for stromal factor-1 (CD184$^+$) in the bone marrow (CD34$^+$CD184$^+$) and blood (CD184$^+$) of mice stimulated with granulocytic CSF. The results suggest that thymodepressin can inhibit migration of CD34$^+$ cells from bone marrow into peripheral blood under conditions of normal and granulocytic CSF-stimulated hemopoiesis.

Key Words: *CD34$^+$ cells, γ-d-Glu-d-Trp dipeptide (thymodepressin), granulocytic colony-stimulating factor; migration; adhesion*

Dipeptides, structures formed as a result of protein proteolysis, modulate *in vivo* proliferation of bone marrow hemopoietic stem cells in experimental animals. It was found that optic isomerism plays an important role in biological (hemo- and immunomodulating) effects of dipeptides [1,3]. However, the mechanisms of the effect of low-molecular-weight peptides are poorly studied. It was hypothesized that adhesion interactions of hemopoietic precursors with cells and extracellular components of the hemopoietic microenvironment in the bone marrow and the response to chemokines are involved into mobilization, homing, and proliferation of these cells. Binding of stem cells with proteins of the extracellular matrix, *e.g.* fibronectin, mediated by adhesion molecules, in particular, by integrins, is crucial for proliferation and differentiation [6,10,11]. Taking these data into account we hypothesized that the effects of optic isomers of low-molecular-weight peptides can be determined by changes in adhesion properties and receptor apparatus of bone marrow cells. For verification of this hypothesis we studied the effect of dipeptide γ-d-Glu-d-Trp consisting of artificial d-amino acids on migration and adhesion to fibronectin of CD34$^+$ hemopoietic cells from mice treated and not treated with granulocytic CSF (G-CSF). This d-dipeptide is particularly interesting because it serves as the basis

Medical Radiological Research Center, Russian Academy of Medical Sciences, Obninsk. ***Address for correspondence:*** ovs45@mail.ru. O. V. Semina.

for Thymodepressin, a new drug approved for the use in medical practice by Pharmacological Committee of the Russian Federation in 2000 as a hemo- and immunosuppressor inhibiting proliferation of hemopoietic precursors.

MATERIALS AND METHODS

Experiments were carried out on 2-3-month-old C57Bl/6 (CBA×C57Bl/6)F_1 female mice weighing 20-22 g (Stolbovaya nursery). The animals were divided into 4 groups (15 rats per group). Group 1 mice were controls; groups 2 and 3 received CSF and γ-d-Glu-d-Trp, respectively; and group 4 received G-CSF in combination with the peptide. The dipeptide γ-d-Glu-d-Trp synthesized in Peptos Peptide Engineering Center was administered intraperitoneally in a daily dose of 500 μg/kg for 3 days with or without G-CSF (Neipogen, F. Hoffmann La Roche). G-CSF was injected subcutaneously in a dose of 250 μg/kg daily for 4 days [8]. In case of combined treatment with γ-d-Glu-d-Trp and G-CSF, the dipeptide was injected after the 2nd injection of G-CSF according to the above scheme.

Bone marrow samples (washed with medium 199 from the femur) and peripheral blood were obtained one day after the last injection of the preparations. The number of nucleated cells in the bone marrow and peripheral blood was determined.

Direct adhesion to fibronectin was evaluated as follows [7]: fibronectin (Sigma) in a concentration of 10 μg/ml was placed into wells of Nunc plates (100 μl per well) and incubated overnight on cold. Then the plates were filled with 1% BSA. Mouse bone marrow cells washed by centrifugation were suspended in a buffer and added to wells (5×10^5 cells per well). After 2-h incubation at 37°C, nonadherent cells were removed, while cells bound to fibronectin were fixed with 100% methanol for 5 min (methanol was then removed from the wells). The cells were stained with azure II in 0.2 M acetate buffer (pH 4.1) for 5 min. After removal of the dye, the wells were thoroughly washed in a buffer and absorbed dye was dissolved in 50% ethanol in 0.1 M sodium citrate. Optical density was measured on a Uniplan reader at λ=650. Cell adhesion capacity was expressed as the ratio of the number adherent cells to the total number of cells in a well.

$CD34^+$ cells were identified after staining of blood samples (25 μl) and bone marrow samples (2.5×10^5 nucleated cells) with phycoerythrin- and FITC-labeled monoclonal antibodies (Pharmingen Becton Dickinson). The samples were analyzed on a FACS Vantage flow cytofluorometer (BDIS) equipped with Spectra-Physics 177-G1202 (Spectra-Physics) within 2 h after staining. The data were processed using Cell Quest software (BDIS). The area corresponding to undamaged nucleated cells with low side scatter was determined on cell distribution diagram. Among these cells, $CD34^+$ hemopoietic precursors characterized by weak expression of CD45 were identified, their percent and intensity of fluorescence were determined. The relative numbers of $CD29^+$, $CD184^+$, and $CD34^+CD184^+$ cells were determined similarly. Nonspecific binding was controlled routinely using species-specific immunoglobulins (Caltag Laboratories) belonging to the same isotype and labeled with the same fluorochromes as the above specified antibodies to surface markers.

The data are presented as means of 5 experiments. The data were processed statistically using Student *t* test.

RESULTS

Treatment with peptide γ-d-Glu-d-Trp decreased the number of $CD34^+$ hemopoietic precursors and in parallel increased the mean density of this marker in the peripheral blood of both G-CSG-stimulated and not stimulated mice (Table 1). No significant changes in the number of $CD34^+$ cells were observed in the bone marrow. At the same time, the density of the marker on the surface of $CD34^+$ cells significantly increased compared to the control only in mice treated with the dipeptide (Table 2). Hence, peptide γ-d-Glu-d-Trp inhibited migration of $CD34^+$ cells (with parallel increase in the percent of polypotent precursors) from the bone marrow into the peripheral blood under conditions of both normal and G-CSF-stimulated hemopoiesis.

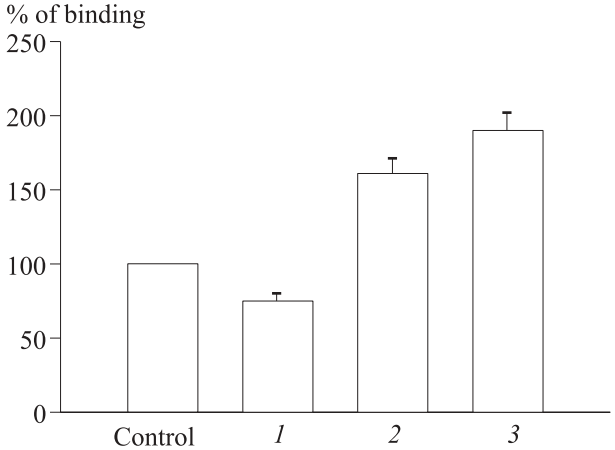

Fig. 1. Binding of mouse bone marrow cells with fibronectin. *1*) treatment with G-CSF, *2*) treatment with γ-d-Glu-d-Trp, *3*) treatment with γ-d-Glu-d-Trp+G-CSF.

TABLE 1. Effect of Peptide γ-d-Glu-d-Trp on the Number of $CD34^+$ and $CD184^+$ Cells in Peripheral Blood of Mice Stimulated and Not Stimulated with G-CSG (*M*±*m*)

Group	Absolute number of $CD34^+$ cells per 1 μl	Intensity of $CD34^+$ cell fluorescence, rel. units	$CD184^+$ cells, %
Control	23.8±2.2	94.1±9.6	2.9±0.1
G-CSF	35.3±1.6*	103.7±8.3	4.1±0.3*
γ-d-Glu-d-Trp	15.3±0.7***	141.3±10.6*	2.5±0.3
G-CSF+γ-d-Glu-d-Trp	14.8±1.2^{++}	140.5±9.1**	2.8±0.1$^+$

Note. Here and in Table 2: *$p<0.05$, **$p<0.01$, ***$p<0.001$ compared to the control; $^+p<0.05$, $^{++}p<0.001$ compared to G-CSF treatment.

The intensity of migration of stem cells from the bone marrow largely depends on adhesion interactions with extracellular matrix proteins and stromal factor 1 (SDF-1). Binding of stem cells to extracellular matrix proteins mediated by adhesion molecules, in particular, by integrins, and expression of CXCR-4 (receptor for SDF-1) is more important for migration capacity and is often crucial for proliferation and differentiation [5,13]. In light of this, we analyzed the effect of peptide γ-d-Glu-d-Trp on the expression of CXCR-4 ($CD184^+$) and integrin β_1 ($CD29^+$) receptors (the latter determines adhesion to fibronectin). Moreover, direct adhesion of bone marrow cells to fibronectin was determined in all animals. It was found that three injections of γ-d-Glu-d-Trp dipeptide significantly increased the percent of cells expressing receptor for integrin β_1 ($CD29^+$) in the bone marrow irrespective on G-CSF treatment (Table 2). The percent of bone marrow cells binding to extracellular matrix protein fibronectin also increased under the effect of the peptide in both experimental systems (Fig. 1). In parallel, γ-d-Glu-d-Trp reduced the relative number of cells carrying CXCR4 receptors for SDF-1 ($CD184^+$) in the bone marrow ($CD34^+CD184^+$) and blood ($CD184^+$) of mice stimulated with G-CSF (Table. 1, 2).

Thus, dipeptide γ-d-Glu-d-Trp (thymodepressin) more markedly suppressed migration of $CD34^+$ hemopoietic cells from the bone marrow to the peripheral blood against the background of their mobilization with G-CSF. Taking these data into account we hypothesized that the hemo- and immunosuppressor effects of the examined dipeptide observed in our previous experiments are determined by reduced response of hemopoietic precursor to SDF-1 and increased binding of cells to extracellular matrix proteins, *e.g.* fibronectin.

The release of hemopoietic stem cells into the blood is a multistep process including activation of proliferation of early precursors and subsequent migration from the bone marrow. The inhibition of CFU-8 proliferation and accumulation of low-differentiated cells in the bone marrow under the effect of thymodepressin can be explained by the effect of the peptide on the expression of integrins, in particular, β_1 integrin, and on cell binding to extracellular matrix proteins. Integrins, cell adhesion molecules, mediate binding of cells with extracellular matrix proteins, thus ensuring cell anchoring and reducing their migration, and participate in transmission of growth signals from the matrix to cells. It was demonstrated that enhanced expression of integrins, *e.g.* $\alpha_4\beta_1$, during co-culturing of $CD34^+$ hemopoietic precursors with fibronectin reduces the intensity of their proliferation and leads to accumulation of more primitive cells [2].

It should be emphasized that the capacity of γ-d-Glu-d-Trp to suppress migration of $CD34^+$ hemopoietic cells is of particular importance in light of the concept postulating similarity of migration mechanisms for hemopoietic stem cells and tumor stem cells initiating metastases [4]. A negative prognostic role

TABLE 2. Effect of Peptide γ-d-Glu-d-Trp on Total Number of $CD34^+$ $CD29^+$ and $CD34^+CD184^+$ Cells in Bone Marrow (*M*±*m*)

Group	Absolute number of $CD34^+$ cells, $\times10^3$	Intensity of $CD34^+$ cell fluorescence, rel. units	$CD184^+$ cells, %	$CD29^+$ cells, %
Control	76.6±21.2	59.1±5.6	1.0± 0.1	65.0±1.6
G-CSF	91.8±11.4	38.9±5.9*	2.4±0.2**	56.1±2.9*
γ-d-Glu-d-Trp	77.4±14.6	92.6±10.6*	1.3±0.1	71.6±1.7*
G-CSF+γ-d-Glu-d-Trp	118.1±20.9	41.5±2.1	1.4±0.1$^+$	70.7±3.5$^+$

of enhanced migration of $CD34^+$ cells in the development of tumor process was demonstrated: cytokines produced by tumor cells, *e.g.* GM-CSF, enhance proliferation and mobilization of $CD34^+$ hemopoietic cells from the bone marrow to peripheral blood followed by their settling in the tumor tissue; this process correlates with the increase in the number of metastases and the incidence of tumor relapses [2,14]. In light of this, the search for new preparations suppressing migration and adhesion of $CD34^+$ cells is important for prevention of metastatic distribution of tumor cells.

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