Stromal Regulation of Hemopoietic Stem Cells in Long-Term Human Bone Marrow Tissue Cultures under the Effect of Parathyroid Hormone

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We studied the interaction between different categories of hemopoietic precursors with parathyroid hormone-activated stromal microenvironment. Improved survival of early precursors capable long-term hemopoiesis maintenance and increased number of later short-term repopulating precursors was demonstrated on the model of co-culturing of human bone marrow cells on a layer of adherent cells of long-term bone marrow cultures treated with parathyroid hormone. These changes correlate with increased expression of genes involved in the maintenance of the hemopoietic stem cells in the sublayer activated by parathyroid hormone. Simultaneously, the expression of some stromal differentiation genes, adhesion molecules for hemopoietic stem cells, and growth factors increased in adherent cell layers treated with parathyroid hormone. These findings attest to activating effect of parathyroid hormone on cells forming the niches for both early and later hemopoietic precursors, and hence parathyroid hormone can be used as a potential agent promoting expansion of early hemopoietic stem cells *ex vivo*.

Key Words: *parathyroid hormone; hemopoietic stem cells; genes maintaining hemopoietic stem cells; stromal microenvironment*

The niche for stem cells consists of microenvironment cells maintaining their viability and preserving tissue homeostasis [7]. Hemopoietic stem cells (HSC) are regulated by specialized stromal cells and extracellular matrix and growth factor produced by these cells. Direct and continuous contact with the niche is essential for preserving the resting state of HSC, its self-maintenance and differentiation capacity. Spindle-shaped osteoblasts producing H-cadherin are the only cell type in the hemopoietic microenvironment whose role in the self-maintenance of HSC is proven [2,12]. These cells express a set of proteins necessary for the regulation of HSC, *e.g.* Ang-1 (holds HSC in the niche), Bmi-1 (is responsible for cell proliferation), Jagged-1 (determines self-mainenance) *etc*. [10]. Parathyroid hormone (PTH) activates osteoblasts and stimulates their division, increases expression of Jagged-1 on the surface of osteoblasts and indirectly (via the interaction between Notch-1 and Jagged-1) regulates physiological status of HSC [11]. Experiments on mice showed that PTH treatment of adherent cell layers from long-term bone marrow culture considerably improves HSC survival on these layers [1]. The potency of PTH to stimulate proliferation of early hemopoietic precursors can be used for the expansion of HSC. Despite successful use of PTH for the treatment of osteoporosis, the mechanisms underlying its effects on human HSC and stromal cells are poorly studied.

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Here we studied a correlation between the expression of genes essential for HSC survival in response to PTH-treatment of stromal microenvironment and expansion of HSC on these cells.

MATERIALS AND METHODS

Experiments were performed on donor bone marrow cells, obtained by aspiration biopsy from the iliac bones for subsequent transplantation at the Department of Bone Marrow Transplantation, Hematological Research Center, Russian Academy of Medical Sciences. The samples were obtained from 23 donors (11 women and 12 men) aged 19-54 years (median 32 years).

For preparing long-term bone marrow culture, 10^6 bone marrow cells were placed in a well of 24-well plate containing complete nutrient medium consisting of 80% αMEM (ICN), 10% fetal calf serum (HyClone), 10% equine serum (GibcoBRL), 2 mM L-gluthamine (ICN), 100 U/ml penicillin, 50 μg/ml streptomycin (both from Ferane), 2×10^{-5} M 2-mercaptoethenol (Sigma), and 10^{-6} M hydrocortisone (Sigma). The first 3 days the cells were cultured at 37°C at 5% CO_2, and then at 33°C. The medium was half-replaced weekly. Cobblestone area-forming cells (CAFC) were determined as described earlier [8] on a MS5 cell feeder maintaining CAFC growth [5]. The test samples were implanted in 4 serial dilutions (usually 50, 25, 12.5, and 6.25 thousands cells per well, 15 wells for each dilution). The incidence of CAFC was determined by the number of negative wells (not containing CAFC) [8].

Human PTH_{1-34} (GenScript Corp.) was added to long-term bone marrow cultures in concentrations of 10^{-8}, 5×10^{-8} and 10^{-7} M during seeding and medium replacement.

Adhesion of CAFC to PTH-treated and irradiated (40 Gy) sublayers was determined 2-5 days after implantation of bone marrow cells on tested adherent cell layers. The cells were harvested with a scrapper and after 4-5 min the upper fraction was collected. The number of precursors of different maturity was analyzed by counting CAFC.

Gene expression in the long-term bone marrow culture sublayer was semiquantitatively evaluated using reverse transcription-polymerase chain reaction. The adherent sublayer was washed with phosphate buffered saline (ISC), treated with denaturating solution, and total RNA was isolated as de-

TABLE 1. Primers used for identification of genes

Gene	Primer sequence
Aggrecan	sense-GGGTCAACAGTGCCTATCAG
	antisense-GGGTGTAGCGTGTAGAGATG
Ang-1	sense-GGTCAGAAGAAAGGAGCAAG
	antisense-TGGTAGCCGTGTGGTTCTGA
β-Actin	sense-CCAAGGCCAACCGCGAGAAGATGAC
	antisense-AGGGTACATGGTGGTGCCGCCAGAC
Bmi-1	sense-GAGAATTATAACTGATGAT
	antisense-AGTCCATCTCTCTGGTGACTG
FGF-2	sense-GCTACAACTTCAAGCAGAAG
	antisense-CCAATTATCCAAACTGAGCTAT
Jagged-1	sense-GAGGACAACACCACCAACAA
	antisense-TCCAAGTCTCTGTTGTCCTG
ICAM-1	sense-CCG GAA GGT GTA TGA ACT G
	antisense-TCC ATG GTG ATC TCT CCT C
Notch-1	sense-GCCGTCATCTCCGACTTCAT
	antisense-TCCAGCAGCCTCACGATG
Osteopontin	sense-CTAGGCATCACcTGTGCCATACC
	antisense-CAGTGACCAGTTCATCAGATTCATC
VCAM-1	sense-GAA GGA TGC GGG AGT ATA TGA
	antisense-GAC ATA GAT GGG CAT TTC TTT
VEGF	sense-TTATGCGGATCAAACCTCAC
	antisense-TCACCGCCTCGGCTTGTC

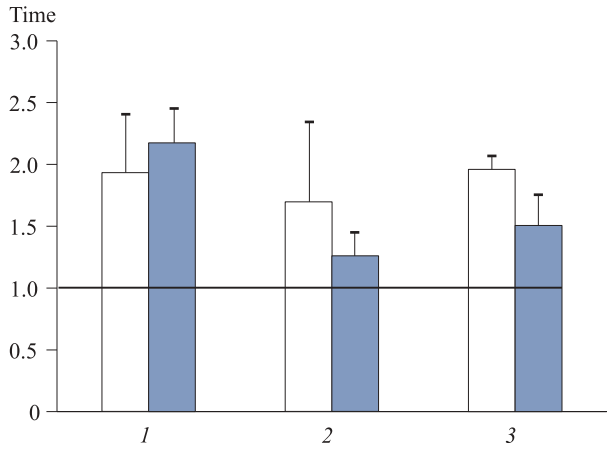

Fig. 1. Dynamics of the incidence of CAFC 7 (light bars) and CAFC 28-35 (dark bars) after 2-5 day culturing of bone marrow cells of adherent cell sublayer treated with PTH. Abscissa: PTH concentration. Ordinate: Horizontal line: control level (without PTH treatment). PTH concentration: *1*) 10^{-8}, *2*) 5×10^{-8}, *3*) 10^{-7} M.

scribed previously [3]. After annealing of poly-T primers the fist DNA chains were synthesized using reverse transcriptase (M-MLV, Promega). Expression of genes was evaluated by polymerase chain reaction with specific primers (Table 1).

The relative level of expression of some genes in PTH-treated and non-treated sublayers was evaluated by Southern blot of fragments obtained after polymerase chain reaction [6]. The yield of polymerase chain reaction products in the exponential phase was determined by the number of fragments after 20-30 cycles. We used 3 variants with a 20-cycle step. The expression was analyzed using PhosphoImager device (Cyclone, Packard Bell). The images were processed using the applied software of the same company. Gene expression was standardized by β-actin expression. The data were processed statistically using Student *t* test.

RESULTS

Both the early precursors CAFC 28-35 and later polypotent cells CAFC 7 better survived of PTH-treated layer of adherent cells than on non-treated cells. 1). They were 2-fold more incident after treatment with 10^{-8} M PTH. Increasing the concentration of PTH in our experiment did not considerably affect CAFC survival.

Expression of genes responsible for the maintenance of HSC under the effect on PTH also increased in cells of the stromal sublayer independently of the increase in hormone concentration. PTH in a concentration of 5×10^{-8} M increased the expression of Bmi-1, Notch-1, and Jagged-1 (Fig. 2, *a*). The absence of enhanced reaction of hemopoietic precursors and increased gene expression in microenvironmental cells in response to higher concentration of PTH can be explained by the fact that PTH depending on its concentration can activate not only osteoblasts, but also osteoclasts. The balance between the number of activated osteoblasts forming niches for hemopoietic cells and osteoclasts destroying these niches is thus maintained in the organism and, to a lesser extent, in cell culture. Self-maintenance of HSC is regulated via the interaction of Notch-1 and Jagged-1. The expression of Jagged-1 in the stromal sublayer can be induced by PTH [11], while Notch-1 is expressed more intensively in HSC remaining in the adherent cell sublayer during isolation of stromal cells. Expression of Bmi-1 (transcription factor respon-

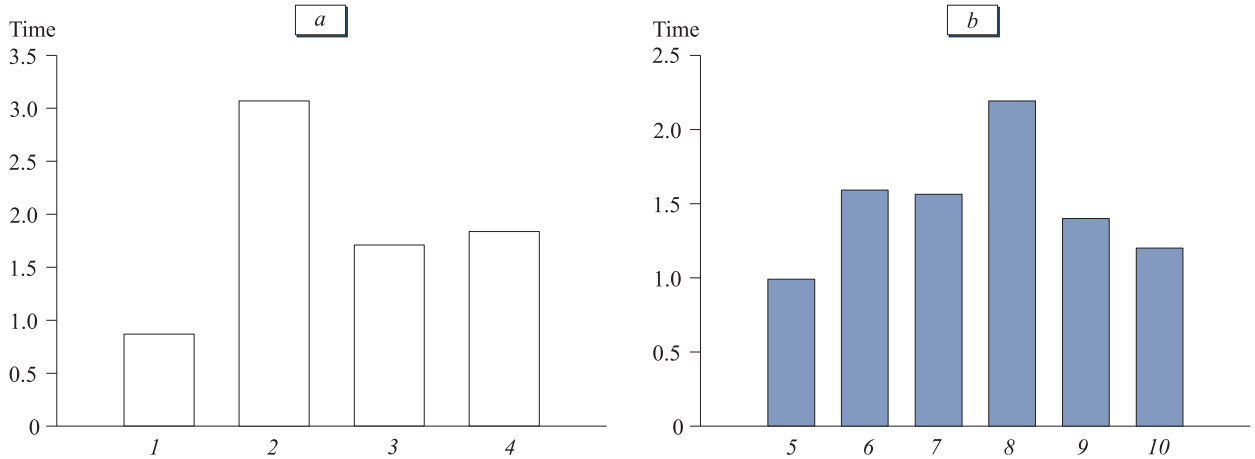

Fig. 2. Gene expression in cells of stromal sublayer under the effect of PTH. *a*) genes responsible for HSC maintenance (light bars): *1*) Ang-1, *2*) Bmi-1, *3*) Notch-1, *4*) Jagged-1; *b*) gene of stromal differentiation, adhesion molecules, anf growth factors (dark bars): 5) fibroblast growth factor-2; *6*) vascular endothelium growth factor; *7*) Osteopontin, *8*) Aggrecan, *9*) ICAM-1, *10*) VCAM-1. Ordinate: changes in gene expression compared to sublayer not treated with PTH.

sible for HSC proliferation [4]) also most likely increases in hemopoietic precursors remaining in the sublayer.

PTH increases the expression of osteopontin and aggrecan, markers of bone and cartilaginous differentiation (Fig. 2, *b*).

Under normal physiological conditions HSC exist in niches: early HSC capable of long-term maintenance of hemopoiesis, whose incidence correlates with the incidence of CAFC 28-35, are located in microenvironment consisting primarily of osteoblasts, while later short-term repopulating HSC corresponding to CAFC 7 proliferate and differentiate in the stroma related to vascular endothelium [12]. Enhanced expression of adhesion molecules ICAM-1 (adhesion to osteoblasts) and VCAM-1 (adhesion to endothelium) together with activation of expression of vascular endothelium growth factor can explain improved survival of CAFC 7 and CAFC 28-35 on PTH-activated sublayers.

We present the dynamics of HSC number after co-culturing with the adherent cell sublayer treated with PTH (Fig. 3, *a*). In parallel, we analyzed the expression of genes maintaining HSC in cells of this sublayer (Fig. 4). Increased expression of Bmi-1 and Jagged-1 activated proliferation of short-living HSC, which 3.5-4-fold increased their number and promoted survival of early precursors CAFC 35. PTH treatment (in a concentration of 10^{-7} M) did not decrease the number of CAFC 35 (as it did in other variants of cell culturing), but increased it 1-5-fold, which was probably related to enhanced expression of Ang-1 responsible for the maintenance of HSC in the niche in the resting state. *Ex vivo* expansion of HSC usually decreases the number of long-term repopulating HSC, which limits the use of HSC in clinical practice. PTH treatment of stromal microenvironment cells improved survival of early HSC and stimulated proliferation of more mature HSC *ex vivo*, which can be important in case of low number of HSC for successful transplantation of the bone marrow.

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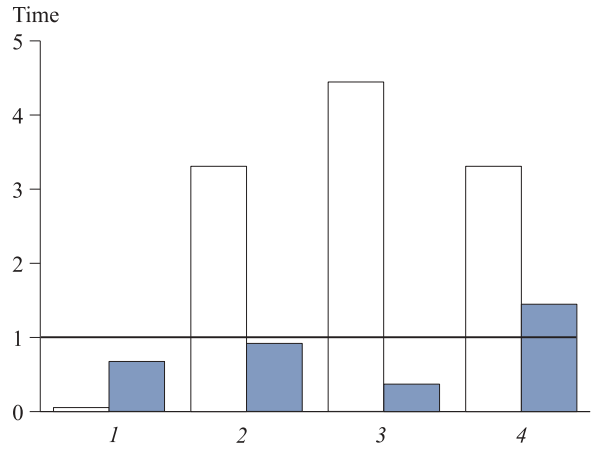

Fig. 3. Number of CAFC 7 (light bars) and CAFC 35 (dark bars) after culturing of bone marrow cells on PTH-treated sublayer. Ordinate: changes in the number of CAFC 7 and CAFC 35 compared to the number of explanted precursors. Horizontal line: number of CAFC in the initial bone marrow. Control (*1*), PTH in concentration of 10^{-8} (*2*), 5×10^{-8} (*3*), 10^{-7} M (*4*).

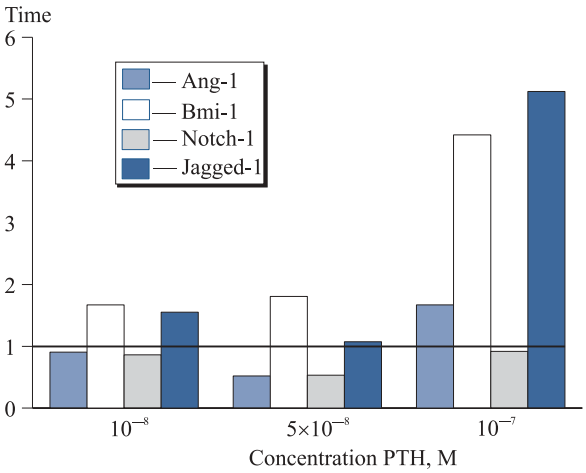

Fig. 4. Expression of genes responsible for HSC maintenance on stromal cell sublayer. Ordinate: changes in gene expression compared to sublayer not treated with PTH. Horizontal line: control level (without PTH treatment).

REFERENCES

1. D. A. Svinareva, I. N. Nifontova, I. N. Chertkov, and N. I. Drize, *Byull. Eksp. Biol. Med*., **140**, No. 9, 320-325 (2005).
2. L. M. Calvi, G. B. Adams, K.W. Weibrecht, *et al. Nature*, 425, 841-846 (2003).
3. P. Chomczynski and N. Sacchi, *Anal. Biochem*., **162**, 156-159 (1987).
4. A. Iwama, H. Oguro, M. Negishi, *et al., Immunity*, **21**, 843-851 (2004).
5. L. Kobari, A. Dubart, F. Le Pesteur, *et al*., *J. Cell Physiol*., **163**, 295-304 (1995).
6. T. Maniatis, E. F. Fritsch, and J. Sambrook, Molecular Cloning, a Laboratory Manual. New York (1982).
7. Moore K. A. and I. R. Lemischka, *Science*, **311**, No. 5769, 1880-1885 (2006).
8. R. E. Ploemacher, J. P. van der Sluijs, J. S. Voerman, and N. H. Brons, *Blood*, **74**, 2755-2763 (1989).
9. J. T. Swarthout, R. C. D'Alonzo, N. Selvamurugan, and N. C. Partridge, *Gene*, **282**, Nos. 1-2, 1-17 (2002).
10. R. S. Taichman, *Blood*, **105**, No. 7, 2631-2639 (2005).
11. J. M. Weber, S. R. Forsythe, C. A. Christianson, *et al*., *Bone*, **39**, No. 3, 485-493 (2006).
12. T. Yin and L. Li, *J. Clin. Invest*., **116**, 1195-1201 (2006).
13. J. Zhang, C. Niu, L. Ye, *et al*., *Nature*, **425**, 836-841 (2003).