Ex Vivo Expansion of Hemopoietic Precursor Cells on a Sublayer Treated with Parathyroid Hormone

D. A. Svinareva, I. N. Nifontova, I. L. Chertkov, and N. I. Drize

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 140, No. 9, pp. 320-324, September, 2005
Original article submitted September 2, 2004

The kinetics of hemopoietic precursor cells was studied in cultures treated with parathyroid hormone in a concentration of 10^{-7} M. Long-term culturing of bone marrow with parathyroid hormone did not change the number of mature cells, while the number of precursors forming colonies in semisolid media increased 7-fold and the number of cells forming "cobblestone" areas on day 28 increased 9-10-fold. After 24 h culturing of bone marrow cells on an irradiated sublayer pretreated with parathyroid hormone for 8 and 12 weeks, the number of early hemopoietic precursor cells forming "cobblestone" areas on day 28 of culturing increased 2- and 5.5-fold, respectively. The expression of Bmi-1 gene responsible for self-maintenance of stem hemopoietic cells increased in cultures treated with parathyroid hormone. It seems that parathyroid hormone can be used for expansion of hemopoietic stem cells *ex vivo*, which is essential for their transplantation to patients.

Key Words: *parathyroid hormone; hemopoietic stem cells; long-term bone marrow culture; "cobblestone" area forming cells*

Interactions with stromal cells forming "hemopoietic niches" are essential for normal function of hemopoietic stem cells (HSC). Osteoblasts are a component of these "niches" [3,10]. Stimulation of osteoblast proliferation and activation stimulates *ex vivo* growth of HSC [3]. Parathyroid hormone (PTH) regulates the osteoblast/osteoclast ratio in the bone tissue: depending on the dose and conditions of treatment, PTH can increase the bone bulk (by activating osteoblasts) or induce bone resorption (by activating osteoclasts) [9]. The problem of increasing the number of HSC *ex vivo* is important for the treatment of patients requiring HSC transplantation, particularly when HSC from the umbilical blood are transplanted, because the content of these cells in the umbilical blood is usually insufficient for the treatment of adult patients. Presumably, PTH can be used for HSC expansion *ex vivo* under adequate conditions. We studied the kinetics of hemopoietic precursor cells in long-term bone marrow cultures treated with PTH in a concentration of 10^{-7} M; changes in the stromal sublayer of these cultures were characterized.

Hematology Research Center, Russian Academy of Medical Sciences, Moscow. ***Address for correspondence:*** ndrize@yandex.ru. N. I. Drize

MATERIALS AND METHODS

Male C57Bl/6 mice aging 9-26 weeks were used in the study. Bone marrow culturing was carried out as described previously [6]. Cells forming "cobblestone" areas (CFCA) were detected in long-term culture as described previously [8], colonies in semisolid media (CFU-C) by a previously described method [4]. The percentage of proliferating cells was determined by the method of cell "suicide" with hydroxyurea [1]. Rat synthetic PTH (1-34; Bachem) in a concentration of 10^{-7} M was added into long-term bone marrow culture during inoculation and during every medium replacement. In order to evaluate changes in the adhesive cell sublayer, the culture was treated with PTH for 4, 8, or 12 weeks, after which the sublayers were exposed to 40 Gy and 10^6 bone marrow cells/2 cm^2 sublayer were explanted on them. Bone marrow cells were used after preliminary adhesion for 2 h on an FCS-coated plastic surface. After 24-h culturing on

0007-4888/05/1403-0334

sublayers treated and not treated with PTH the cells were removed and the counts of precursors of different maturity were evaluated by the CFCA method. Expression of genes in the sublayer of long-term bone marrow culture was studied semiquantitatively by reverse transcription-PCR. The adhesive cell sublayer was removed with a scraper and RNA was isolated by the standard method [5]. After construction of poly-T primer, the first DNA strand was completed using reverse transcriptase (M-MLV; Promega). The presence of the expected gene product was detected by PCR with specific primers: β-actin: direct 5'-ACCG TGAAAAGATGACCCAG-3', reverse 5'-CGTTGCC AATAGTGATGACC-3'; SDF-1α (CXCL12): direct 5"TTGTCCCTGAGTCCTATA-3', reverse 5"ATACA CCGTGGCTGACAC-3'; c-kit: direct 5'-TACCAGA CTGTCACCAGTT-3', reverse 5'-ACCCACATGTAA CGTGAC-3'; VEGF: direct 5'-GATCCTTCGAGGA GCACTTTG-3', reverse 5'-CGAGCTCTACAGGAA TCCCA-3; COMP: direct 5"CAGAGTGACAGTGA TGGTGA-3'; reverse 5'-CTGAAGTCGGTGAGGGT GAC-3'; CXCR4: direct 5'-CTGTAGAGCGAGTGTT GCCA-3', reverse 5'-TTGACTTGTTGGTGGCGTG GA-3'; BMI-1: direct 5'-CACAAAACCAGACCACT CCT-3', reverse 5'-TCACTTTCCAGCTCTCCAGC-3'; osteocalcin: direct 5'-CCAGACCTAGCAGACA CCAT-3', reverse 5'-CTGTGACATCCATACTTGCA-3'; IBSP: direct5'-GCTCAGCATTTTGGGAATGG-3', reverse 5'-CTTCTTGGGCAGTTGGAGTG-3'; F VIII: direct 5'-TGGCACGTGATTGGAATGGG-3', reverse 5'-CGAGGTAGGAACTGAAGGTG-3'.

For immunochemical staining for extracellular matrix proteins and for evaluating the expression of other markers, the cells were fixed with 4% paraformaldehyde. Rat antimurine antibodies to CD44 (BD Pharmingen) and rabbit antihuman antibodies to types I, II and IV collagens and fibronectin (Imtek) were used. FITC-conjugated goat antirat Ig (BD Pharmingen) and phycoerythrin-conjugated goat antirabbit antibodies (Sigma) served as second antibodies.

Statistical analysis was carried out using Student's *t* test.

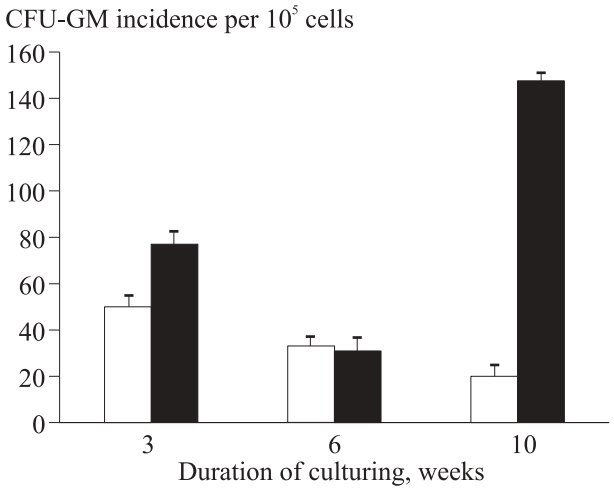

Fig. 1. Changed incidence of CFU-GM in long-term bone marrow culture under the effect of parathyroid hormone (PTH). Here and in Fig. 2: light bars: control; dark bars: PTH.

RESULTS

Long-term administration of PTH in a dose presumably inducing activation and proliferation of osteoblasts to mice increased the concentration of primitive hemopoietic CFCA 28, 35 (28, 35 days) in the bone

TABLE 1. Expression of Marker Genes in PTH-Treated and Untreated Sublayer Cells in Long-Term Bone Marrow Culture

Marker gene	3 weeks		6 weeks		10 weeks	
	control	PTH	control	PTH	control	PTH
c-kit	+++	++++	+++	+	±	+
SDF-1	+++++	+++++	+++	++	++++	+++
CXCR4	+++	+++	++++	++	±	±
Bmi-1	±	+++	—	+		
Osteocalcin	+	++	+	+		
Osteopontin	++++	+++++	+++++	+++++		
COMP	+	±	+	—		
IBSP	+++++	++++	+++++	+	±	++
PECAM	+	++	+++	+		
F VIII	+	++	+	—		
VEGF	±	++	±	+++		
FGF1	±	+	+	+		

Note. +++++, ++++, +++, ++, +: expression evaluated qualitatively visually; ±: very low expression; "—": no expression.

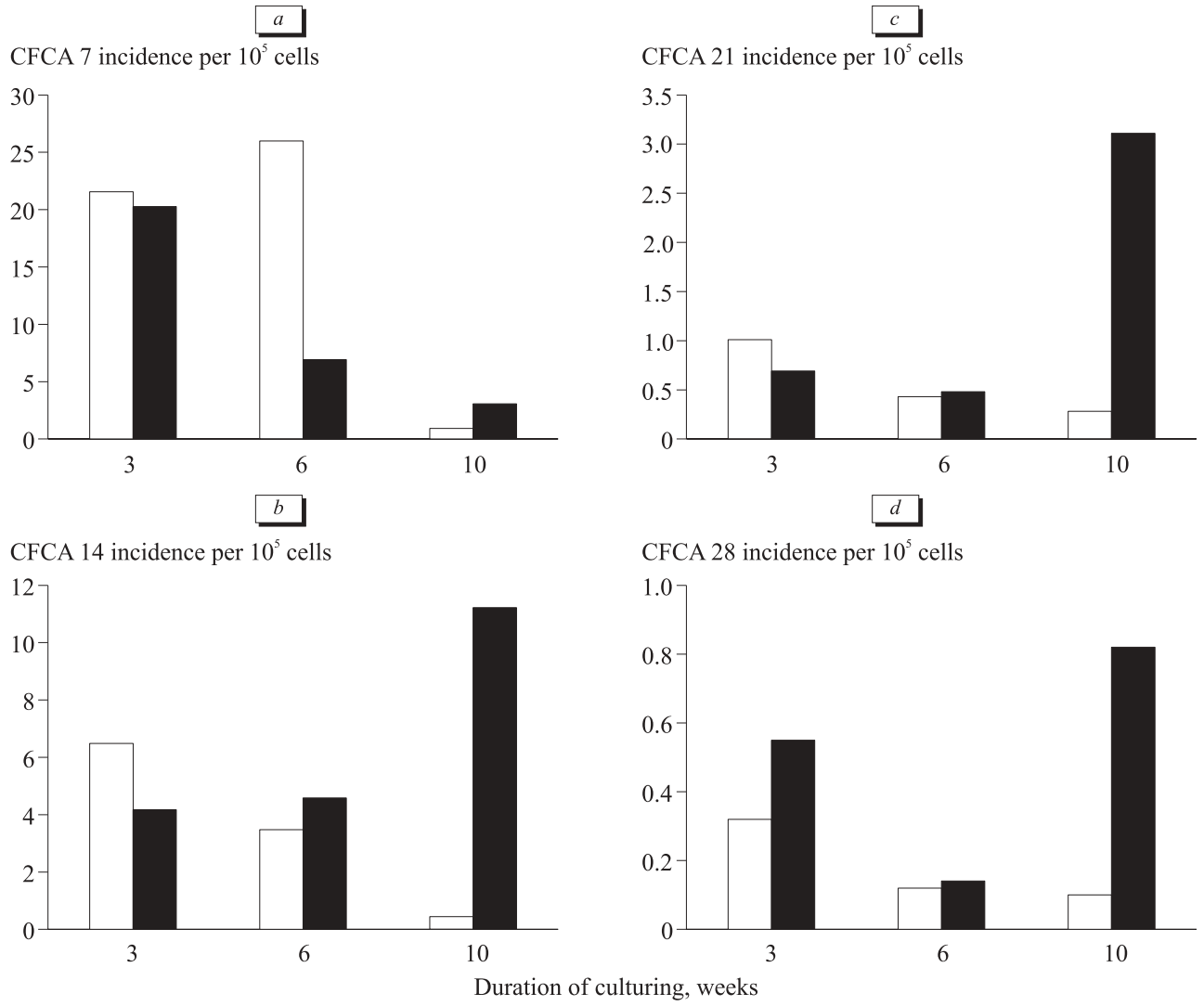

Fig. 2. Changed incidence of cells forming "cobblestone" areas (CFCA) at different periods of observation in long-term bone marrow culture under the effect of PTH. *a*) CFCA 7; *b*) CFCA 14; *c*) CFCA 21; *d*) CFCA 28.

TABLE 2. Changed Counts of Hemopoietic Precursors in the Bone Marrow Cocultured for 24 h with a Layer of Adhesive Cells Treated by PTH

Duration of PTH treatment, weeks		CFCA, days			
		7	14	21	28
4	initially	866.6	>900	122.7	46.2
	on control sublayer	524.4	439.2	139.4	27.6
	on PTH sublayer	1526.0	803.1	339.8	27.3
8	initially	>1460	705.0	99.2	34.4
	on control sublayer	736.5	236.0	103.8	40.3
	on PTH sublayer	617.8	150.4	199.0	68.6
12	initially	332.1	570.8	332.1	42.7
	on control sublayer	4060.4	3475.0	3116.7	417.9
	on PTH sublayer	998.5	588.8	666.8	236.8

marrow [2]. These precursors correspond to early HSC capable of restoring hemopoiesis in lethally irradiated recipients [8]. Long-term bone marrow culture was used as a model for the study of PTH effect *ex vivo*. Addition of PTH in a concentration of 10^{-7} M into culture for 10 weeks did not increase the total cell production. A total of 35.22×10^6 cells were produced in control cultures for 10 weeks, *vs.* 27.86×10^6 in PTH-treated cultures. The counts of granulocytic macrophagic precursor cells (CFU-GM) was similar in control and experimental cultures for 6 weeks, but to week 10 of culturing the number of CFU-GM increased 7-fold in PTH-treated cultures (Fig. 1). The production of earlier precursor cells forming colonies in semisolid media on day 21 appreciably increased (11.5 ± 1.7 in control cultures, 102 ± 2 CFU-GM per 10^5 cells in PTH-treated cultures) only after 10 weeks. The level of CFU-GM proliferation was higher in PTH-treated cultures (21% *vs.* 13% in the control). The expression of Bmi-1 gene is associated with HSC proliferation and self-maintenance [7]. The expression of Bmi-1 increased in PTH-treated cultures (in adhesive cell sublayer) after 3 weeks of culturing. After 6 weeks Bmi-1 expression remained high in PTH-treated cultures, but no expression was detected in control (untreated) cultures (Table 1). The expression of a cartilage differentiation markers (COMP: cartilaginous oligomer matrix protein) decreased in the sublayer of PTH-treated cultures, while the level of another marker, IBSP (bone sialoprotein II), changed during culturing and after 10 weeks was detected only in PTH-treated cultures. The expression of endothelial cell markers (PECAM: platelet/endothelial cell adhesion molecule; and FVIII) decreased, while the level of bone markers (osteocalcin and osteopontin) and fibroblast and vascular endothelium growth factors increased (Table 1).

No differences in the expression of CD44 surface antigen and extracellular matrix molecules, *e.g.* fibronectin, collagens I and IV, integrin β1 (CD29) were detected, while type II collagen forming the cartilage was detected after 3 weeks in the form of disseminated network in PTH-treated cultures. These data indicate changed expression of cartilage and bone differentiation markers in PTH-treated cultures.

Detection of CFCA in long-term bone marrow culture helps to evaluate a wide spectrum of hemopoietic stem cells at once, from early cells restoring hemopoiesis in lethally irradiated mice (CFCA 28, 35) to still polypotent, but more mature precursors corresponding to splenic CFU (CFCA 7) [8]. Long-term treatment with PTH more than 10-fold increased the number of CFCA 28 (Fig. 2). Changed expression of bone and cartilage markers suggests that expansion of early hemopoietic precursor cells in PTH-treated cultures is directly related to PTH capacity to activate osteoblasts and/or stimulate their proliferation.

Twenty-four-hour incubation of fresh bone marrow cells on sublayers treated with PTH for 4 and 12 weeks increased the number of CFCA 7 (1.8 and 3 times, respectively); the number of CFCA 28 decreased on a sublayer treated with PTH for 4 weeks, but increased on 8-12-week sublayers (2 and 5.5 times, respectively; Table 2).

Hence, prolonged treatment of sublayer cells with PTH leads to its functional changes mediating an appreciable increase in the content of early hemopoietic precursors not only after long-term, but even after short-term culturing thereof. The importance of HSC expansion with therapeutic purposes makes studies in this direction perspective.

The study was supported by the Russian Foundation for Basic Research (grant No. 05-04-48242).

The authors are grateful to Cand. Biol. Sci. M. A. Ershler and V. L. Surin for selection of primers.

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