Circulating Bone Marrow Osteoclast Precursors and Osteoclastogenesis in Patients with Type IIA and IIB Hyperlipidemias

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Osteoclasts, the major source of calcium released during bone resorption, and their precursors preosteoclasts derive from bone marrow hemopoietic stem cells, granulocyte-macrophage colony-forming units. The factors responsible for commitment of colony-forming units into osteoclast precursors remain unknown. Studies of osteoclastogenesis in cultured blood mononuclear cells from patients with hyperlipidemia accompanied by atherosclerosis and calcification of vessels revealed high content of preosteoclasts in the blood.

Key Words: *hyperlipidemia; osteoclastogenesis; calcium; osteoporosis*

Hyperlipidemia (HLE) is a factor contributing to the development of sclerotic changes in blood vessels. Calcification of the vascular wall is a culmination of atherosclerotic process. Augmented calcification of atheromatous vessels and decreased bone mineral density attract much recent attention [3,10]. Intensive calcification of the coronary arteries in patients with osteoporosis, atherosclerotic damages to vessels of lower extremities, and post-menopause (compared to individuals with normal bone density) accompanies the decrease in bone mass [10]. The decrease in bone mass is induced by disturbances in its reconstruction. The equilibrium between bone development from osteoblasts and bone resorption by osteoclasts (OC) is shifted toward resorption. OC are large or giant cells formed from bone marrow hemopoietic stem cells [2,4, 6,7]. OC precursors (pre-OC) develop from pluripotent granulocyte-macrophage colony-forming units (CFU-GM), which are usually differentiated into monocytes and macrophages [11]. Our previous studies revealed the presence of stromal osteogenic cells in the blood of patients with HLE (but not normolipidemic donors) [1,14]. These cells *in vitro* formed the osteoid bone tissue surrounded by OC (normal bone tissue cells).

Here we identified pre-OC in the blood from patients with HLE and coronary arteriosclerosis. It was shown that under normal conditions the count of blood pre-OC is low. However, factors responsible for commitment of CFU-GM into pre-OC remain unknown [11].

MATERIALS AND METHODS

Experiments were performed on blood mononuclear cells (MNC) from patients with moderate type IIa and IIb HLE. The control group included normolipidemic donors. Plasma lipid content was measured on a Technicon RA-XT analyzer at the Laboratory of Clinical Biochemistry of Lipid Metabolism (Russian Research-and-Production Center for Cardiology).

Peripheral blood MNC were obtained by centrifugation in a Ficoll-Paque density gradient (ρ=1.077, Flow Lab.) and cultured in medium 199 containing 20% fetal bovine serum (FBS, Gibco BRL) using 35-mm plastic dishes and/or cover glasses (10^6 nuclear cells/ml medium). For reasons beyond our control, the density of cultured cells did not correspond to the

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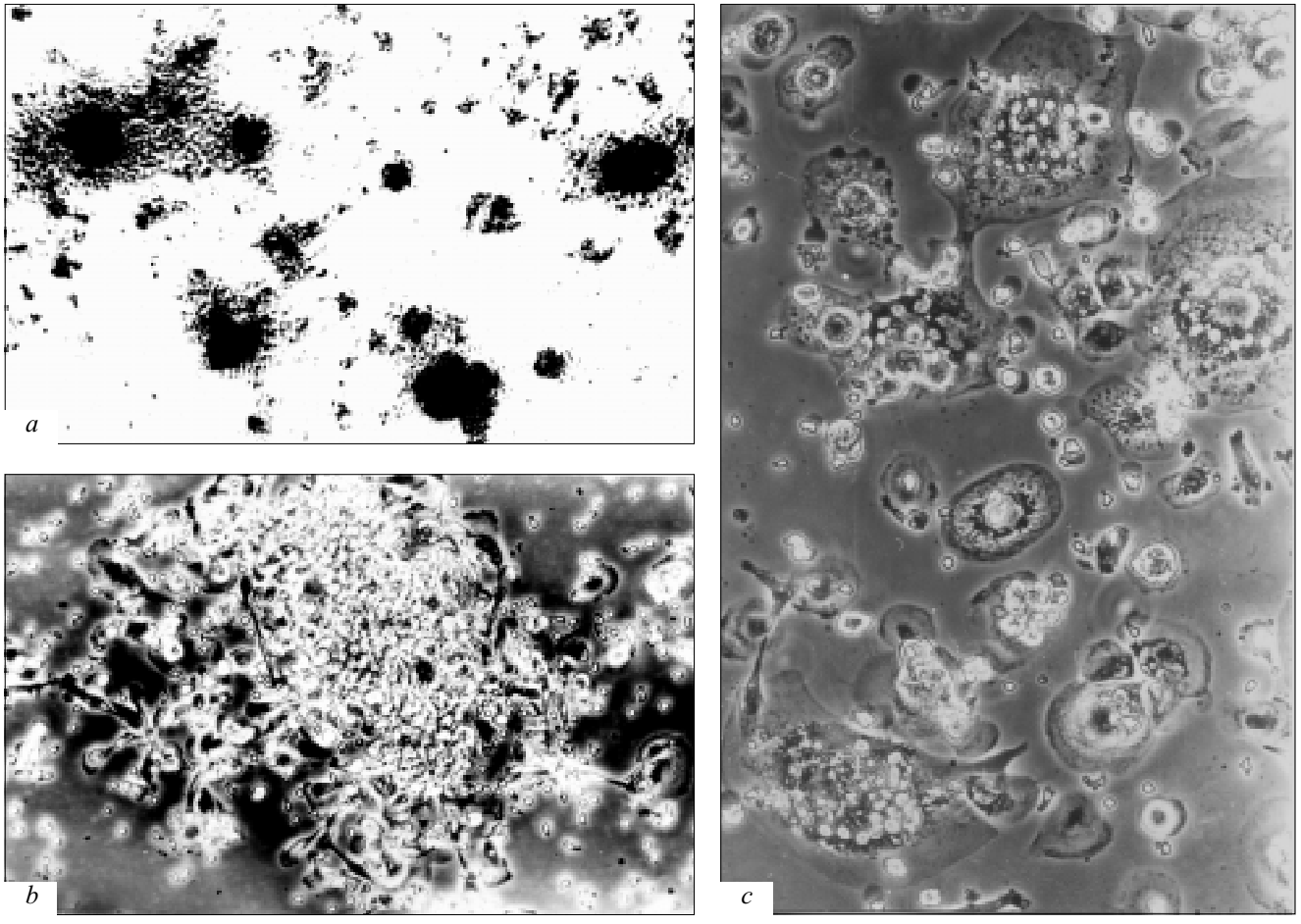

Fig. 1. Formation of colonies with osteoclasts in liquid culture of blood mononuclear cells from patients with hyperlipidemia (72 h-21 days): aggregates of cells adherent to plastic (*a*, 72 h, ×80); colony of spindle-shaped and round adherent cells in cooperation with numerous small lymphocyte-like cells (*b*, ×160); colony of giant multinuclear cells (*c*, ×200). Phase contrast microscopy.

optimum value estimated in studies of osteoclastogenesis in patients receiving colony-stimulating factors (CSF, $\times 10^6$ nuclear cells/dish) [11]. Nonadherent cells were removed 72 h after the start of culturing. Adherent cells were cultured in a medium of the same composition. The culture medium was replaced 2 times a week.

Morphological and phenotypic assays were performed by histochemical, immunochemical, and ultrastructural methods on days 3, 7, 10, 14, and 21 of culturing. The number of clusters/colonies containing multinuclear cells, polykaryons containing more than 5 nuclei, and the count of individual polykaryons were estimated. The OC marker tartrate-resistant acid phosphatase (TRAP) was detected by the method of Gomori in the presence of sodium tartrate. The monocyte/macrophage marker nonspecific esterase was detected histochemically. Expression of monocyte, macrophage, and total leukocytic antigen markers was studied using monoclonal antibodies against CD14 (Daco), CD68 (Daco), and CD45 (Chemicon), respectively. Goat anti-mouse IgG conjugated with horseradish peroxidase (Daco) were used as secondary antibodies. FBS (1%, Sigma) in 0.1 M phosphate buffer (pH 7.4) or non-immune serum (instead of primary antibodies) served as the control. Peroxidase activity was measured using diaminobenzidin (0.6 mg/ml). Ultrathin sections of colonies embedded into Epon were examined under a JEM-100CX electron microscope. The results were analyzed by Student's *t* test.

RESULTS

We studied the dynamics of OC formation in culture. After 72-h culturing peripheral blood MNC from patients with HLE formed aggregates adherent to the bottom (Fig. 1, *a*). After 7 days cell aggregates of various shapes, sizes, and degrees of adherence were seen. On days 7-10 of culturing aggregates/colonies primarily consisted of adherent elongated and flattened cells. Non-adherent small round-shaped lymphocyte-like cells were produced on the surface of

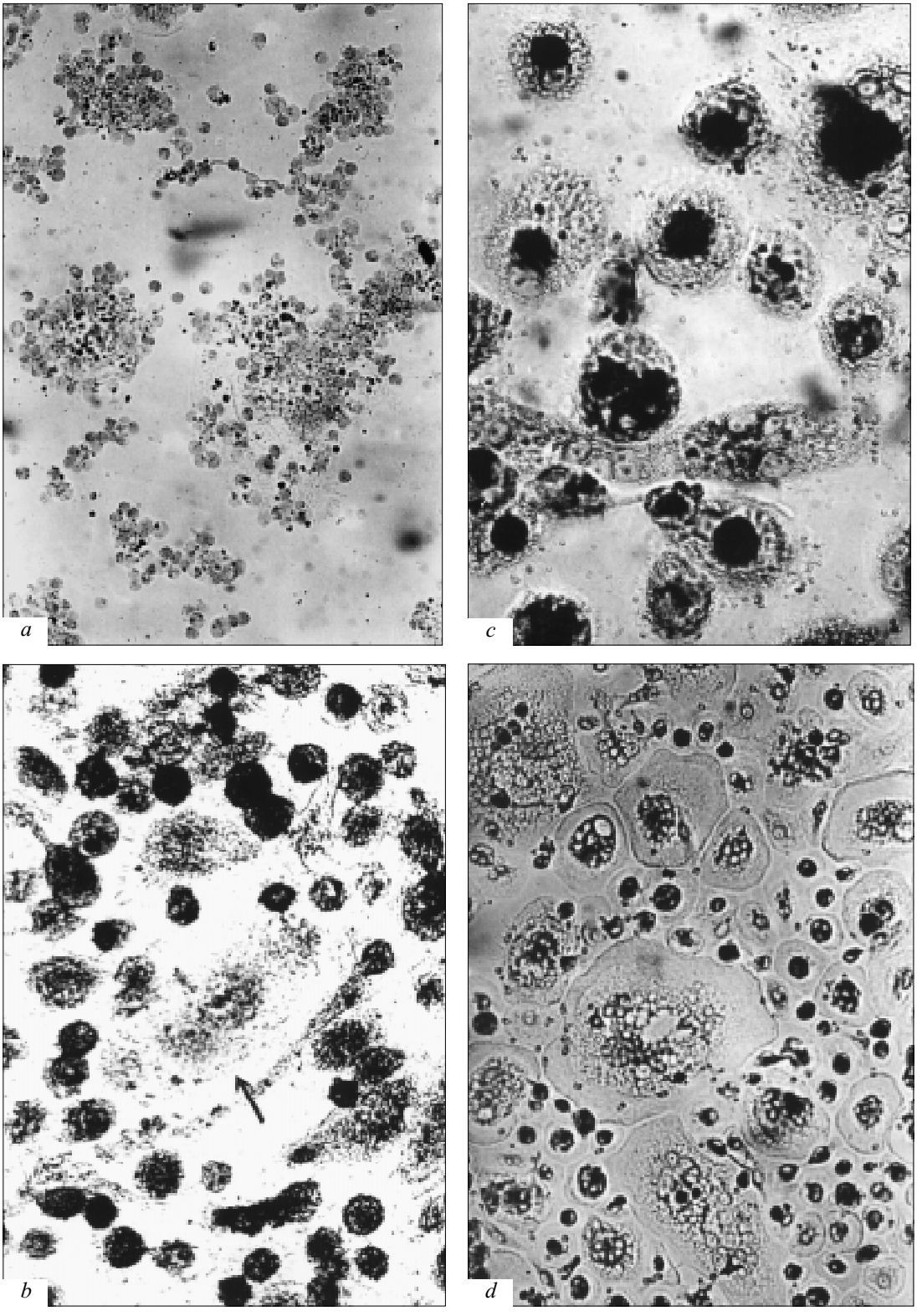

Fig. 2. Phenotyping of cells in colonies shown in Fig. 1 (post-staining with methyl green): monocyte- and lymphocyte-like cells express leukocytic antigen CD45 after 10-day culturing (*a*, ×150); intensive expression of nonspecific esterase by macrophage and preosteoclasts, insignificant expression of nonspecific esterase by multinuclear cells (arrow, *b*, ×210); pronounced staining of multinuclear cells for tartrate-resistant acid phosphatase (*c*, ×200); CD14 expression on monocyte-like cells, unstained multinuclear cells (osteoclasts, *d*, ×200).

these cells and released into the supernatant (Fig. 1, *b*). Most non-adherent cells expressed leukocytic antigen CD45 (Fig. 2, *a*). After 2-3-week culturing these aggregates contained large multinuclear or giant flattened cells (Fig. 1, *c*). These cells were formed after fusion of monocyte-like cells, precursors of OC (positive staining for nonspecific esterase). The intensity of staining for nonspecific esterase decreased with increasing the number of nuclei in polykaryons. The cells containing 5 or more nuclei differed from monocytes/macrophages by weak staining (Fig. 2, *b*). Large multinuclear cells were TRAP-positive (Fig. 2, *c*), expressed no CD14 (Fig. 2, *d*), and were weakly stained for CD68.

Ultrastructural assay of multinuclear cells revealed signs typical of OC: numerous uniform nuclei with minimum heterochromatin contentand well-defined nucleoli, considerable number of lysosomes, and mitochondria in the organelle-rich cytoplasm (Fig. 3). On day 21 of culturing individual OC had 50-70 nuclei. After 2-week culturing the number of colonies/clusters containing well-defined OC was 5-30 per 10^6 peripheral blood MNC from patients with HLE. The total count of OC in colonies/clusters and individual polykaryons was 30-199 per 10^6 MNC (Table 1). MNC cultures from healthy donors contained only individual cell aggregates with multinuclear cells. The total count of polykaryons did not exceed 17 per 10^6 MNC. Polykaryons contained no more than 6 nuclei.

Thus, ultrastructural and immunohistochemical assays of cultured MNC revealed progressive formation of colonies containing multinuclear giant $CD14^{-}$ and $CD68^{\pm}$ cells, precursors of OC (positive staining for TRAP). The intensity of staining for nonspecific esterase decreases with increasing the number of nuclei in polykaryons, which is consistent with published data [6,11,13]. Low concentration of circulating OC precursors under normal conditions (less than 1 per 10^5 nuclear cells) determines the use of specific culture systems in studies of osteoclastogenesis. Animal and human blood MNC are cultured in specific systems in the presence or absence of stromal cells, growth factors, and vitamin D_3. Moreover, culturing of MNC is performed after stimulation of the release of noncommitted stem hemopoietic cells into the peripheral circulation by administration of granulocyte CSF into the blood and/or culture [8,11,13]. In our experiments the culture medium contained only FBS. The density of cultured peripheral blood MNC from patients with HLE did not correspond to the optimum value, because these patients were not treated with CSF [11]. Previously, we revealed the presence of circulating

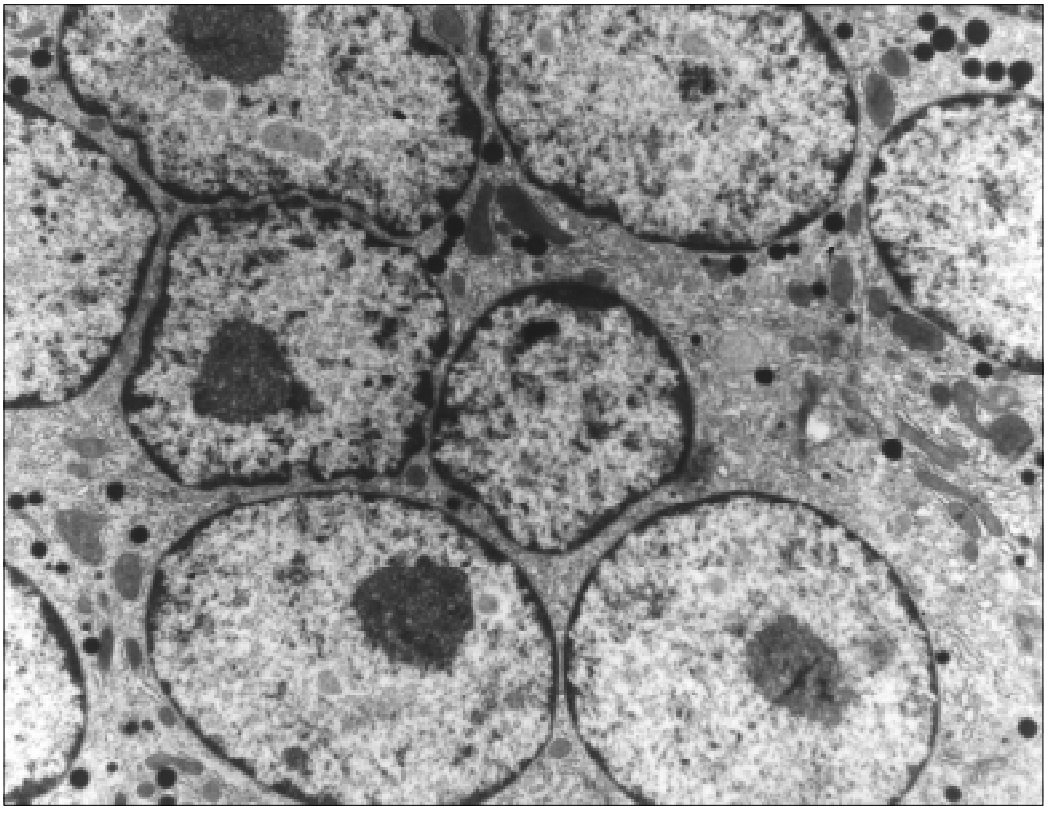

Fig. 3. Ultrastructure of osteoclasts formed in culture of blood mononuclear cells from patients with hyperlipidemia (21 days). Numerous uniform nuclei with well-defined nucleoli and low content of heterochromatin. The cytoplasm contains numerous lysosomes and mitochondria (×5200).

TABLE 1. Osteoclastogenesis (21 Days) in Liquid Cultures of Peripheral Blood Mononuclear Cells from Healthy Donors and Patients with Type IIa and IIb HLE

Patients, sex, age		Number of OC-containing colonies/clusters	Total number of OC
HLE IIa	M, 63	24	107
	M, 28	25	106
	M, 52	12	54
HLE IIb	M, 49	26	199
	W, 52	5	30
	M, 28	30	86
	M, 50	12	80
	M, 48	13	62
	M, 68	9	108
Mean, *M*±*m*		17.3±3.2*	92.4±17.2*
Donors	M, 21	2	6
	W, 35	5	17
	M, 39	1	13
Mean, *M*±*m*		2.7±1.5	12.0±4.0

Note. *$p<0.05$ compared to donors.

stem stromal cells with osteogenic activity in patients with HLE [1,14]. It can not be excluded that in our experiments the culture system also contained these cells.

The count of pre-OC was high in MNC fraction from patients with primary type IIa and IIb HLE (Table 1). Our results suggest that this disorder promotes committment of CFU-GM into pre-OC, whose content in the circulation markedly surpasses the control. CFU-GM, interleukin-1, and interleukin-3 are responsible for activation of osteoclastogenesis in cultured cells [8]. Previous studies showed that plasma lipid content in patients with neutropenia decreases after treatment with CSF, which promotes osteoporosis [9]. Thus, the measurements of CSF level in the blood of patients with HLE are of great interest.

Osteogenesis is accompanied by calcification. Intensification of osteoclastogenesis in patients with HLE is probably associated with increased calcium utilization for extra-bone calcification. We hypothesize that intensive calcification of the vascular wall is related to the presence of stromal stem cells in the intima of human atheromatous aorta. Our previous studies showed that osteogenic cells form focuses of osteogenesis in cultured cells from the aorta intima [12]. Our assumption is confirmed by intensive calcification of the vascular wall in patients with osteoporosis and young homozygotes with familial HLE [5].

These data contribute to better understanding of mechanisms underlying calcification of the vascular wall in patients with atherosclerosis.

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