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Content of Circulating Extracellular DNA, Plasma Activities of Matrix Metalloproteinases, and Ultrastructure of the Myocardium in Hypothyroid Rats with Hypercholesterolemia

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Free circulating extracellular DNA, plasma activities of matrix metalloproteinases in hypothyroid rats, and ultrastructural changes in the myocardium were studied under conditions of experimental hypercholesterolemia. For suppression of thyroid function, the animals received antithyroid drug mercazolyl under conditions of cholesterol diet. Hypercholesterolemia in hypothyroid rats (thyroxine concentration 2-fold below the normal) was paralleled by a pronounced increase of the concentration of free circulating extracellular DNA and total matrix metalloproteinases 2 and 7 activity. These changes were associated with lytic and destructive changes in cardiomyocytes and blood capillary endotheliocytes. Changes in the cardiomyocyte and endotheliocyte ultrastructure were more pronounced in hypothyroid rats.

Key Words: *hypercholesterolemia; hypothyroidism; extracellular DNA; plasma matrix metalloproteinases (MMP-2, MMP-7); myocardial ultrastructure*

The interest of scientists to free circulating nucleic acids has increased significantly in recent years. Circulating nucleic acids (DNA, mRNA, microRNA) primarily have endogenous origin and are released from nucleated cells after their death (apoptosis and necrosis), from erythrocytes and platelets during their maturation, and as a result or secretion of specific organelles (exosomes, microvesicles, apoptotic bubbles) containing nucleic acids [3]. Extracellular nucleic acids circulating in the blood are bound to plasma proteins or form complexes with certain components of the cell surface [3,4]. Free circulating extracellular DNA (ecDNA) in low concentrations is normally present in the plasma, its concentration increasing significantly in diseases associated with destruction of extracellular matrix and cell death [4,12].

Destruction of the extracellular matrix is mainly caused by high activities of matrix metalloproteinases (MMP) expressed by tissue monocytes/macrophages, stellate cells, and less so by smooth muscle cells and endotheliocytes [5,8]. MMP constitute a family of calcium- and zinc-dependent endopeptidases with catalytic activity towards connective tissue extracellular matrix proteins at physiological pH [13]. Controlled degradation of the extracellular matrix is essential for many processes, including angiogenesis, embryogenesis, morphogenetic and involution processes, *etc*. Disturbances in controlled degradation of the connective tissue extracellular matrix can trigger the development and induce complications of many diseases, including atherosclerosis, arthritis, nephritis, tumors, and ulcerative processes [7].

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We studied the content of free circulating ecDNA, plasma activities of MMP-2 and MMP-7, and ultrastructural reorganization of rat myocardium under conditions of experimental hypercholesterolemia combined with the hypothyroid status.

MATERIALS AND METHODS

Experiments were carried out on male Wistar rats (390-560 g; n=18) for 68 days. The animals were kept in individual cages with free access to water. The rats were divided into 3 groups (6 animals per group). Group 1 animals (control) were kept under vivarium conditions and received standard daily ration *ad libitum*. Group 2 animals with intact thyroid function (euthyroid control) received atherogenic diet (alimentary hypercholesterolemia model): cholesterol (Pancreac Quimica) in a dose of 25 mg/100 g added to the standard ration. Group 3 animals received, in parallel with high cholesterol diet, antithyroid drug mercazolyl (Acrikhin) in a dose of 1 mg/100 g, added to the standard ration for inhibition of thyroid function. The animals were fed according to the scheme: atherogenic diet (group 2) or atherogenic diet+mercazolyl (group 3) every other day and fasting on the rest days; water was supplied *ad libitum*. Experiments were carried out in accordance with regulations and recommendations of the European Convention for Protection of Vertebrates Used in Experimental Studies.

Thyroid status was evaluated by plasma thyroxine (T_4) and triiodothyronine (T_3) concentrations, measured by the immunochemiluminescent method on an LM-01A luminometer (Beckman Coulter) using Immunotech kits.

Plasma concentration of free circulating ecDNA was evaluated by fluorescence of DNA-bound Hoechst 33258 stain (Fluka) [3] on an RF-5301 PC spectrofluorometer (Shimadzu) after DNA isolation as described previously [9]. Plasma concentration of ecDNA was calculated by the regression equation for the calibration curve constructed using calf thymus DNA (Sigma).

Total activity of plasma MMP-2 and MMP-7 was evaluated spectrofluorometrically [10] using methylcoumarylimide MCA-Pro-Leu-Gly-Leu-DPA-Ala-Arg-NH_2 (Calbiochem) as the substrate. The fluorescence intensity was measured on an RF-5301 PC spectrofluorimeter at λ_{ex}=325 nm and λ_{em}=393 nm. Enzyme activities were expressed in μmol MCA/liter/h.

Myocardium specimens for electron microscopy were fixed in 4% paraformaldehyde, postfixed in 1% OsO_4, and embedded in epon and araldite mixture. Ultrathin sections were sliced on an LKB III and Leica Ultracut EM UC7 ultratome and contrasted with uranyl acetate and lead citrate. The specimens were examined under a JEM 1400 electron microscope (Jeol) at accelerating voltage of 80 kV. Photos were made with a Veleta digital camera using iTEM software (Olympus).

The results were statistically processed by the common method using Student's t test. The differences were considered significant at p<0.05.

RESULTS

Cholesterol diet (group 2) was inessential for the thyroid status of animals: plasma levels of T_3 and T_4 remained virtually unchanged (Fig. 1, *a*, *b*). Addition of mercazolyl to the ration little changed T_3 content in comparison with the controls, while T_4 level decreased almost 2-fold, which confirmed the hypothyroid status and indicated the efficiency of this model of hypothyroid status.

Plasma concentration of free circulating ecDNA in controls was 16.68±2.48 ng/ml (Fig. 1, *c*), which was in line with our previous data obtained on rats [2]. Cholesterol diet in animals with intact thyroid function (euthyroid control; group 2) was inessential for plasma level of ecDNA throughout the entire experiment. This parameter increased by 72% in group 3 animals receiving exogenous cholesterol and mercazolyl (p<0.05 in comparison with groups 1 and 2).

Another possible cause of free circulating ecDNA appearance in the plasma in diseases, including dyslipidemias, are cell damage and cell death [3,12]. We detected changes of this kind for cardiomyocytes and endotheliocytes of cardiac blood vessels.

According to electron microscopy findings, long-term cholesterol diet caused significant changes in all the main intracellular compartments of cardiomyocytes, capillary endotheliocytes, connective tissue cells, and extracellular space. Diffuse and focal lysis of myofibrillar bundles of different intensity, from moderate to significant (mainly in the intercalated disc regions) was found in many cardiomyocytes in group 2 rats (Fig. 2, *a*). Pronounced changes in the mitochondria (focal lysis of the matrix, irregular dilatation, destruction of crists and their lesser number, focal myelin-like transformation) were found in just few cardiomyocytes, while dilatation of the agranular sarcoplasmic reticulum vesicles was found in the majority of cells.

Changes in the cardiac microcirculatory bed manifested by significant flattening of the endothelium with its intact pinocytic activity, in formation of luminal cytoplasmic protrusions (Fig. 3, *a*), and thickening of the basal membrane. The endotheliocyte cytoplasm contained osmiophilic lipid inclusions, similar to those in cardiomyocytes, in addition to well-developed granular cytoplasmic reticulum and small mitochondria (with focal destruction of crists). On the other hand,

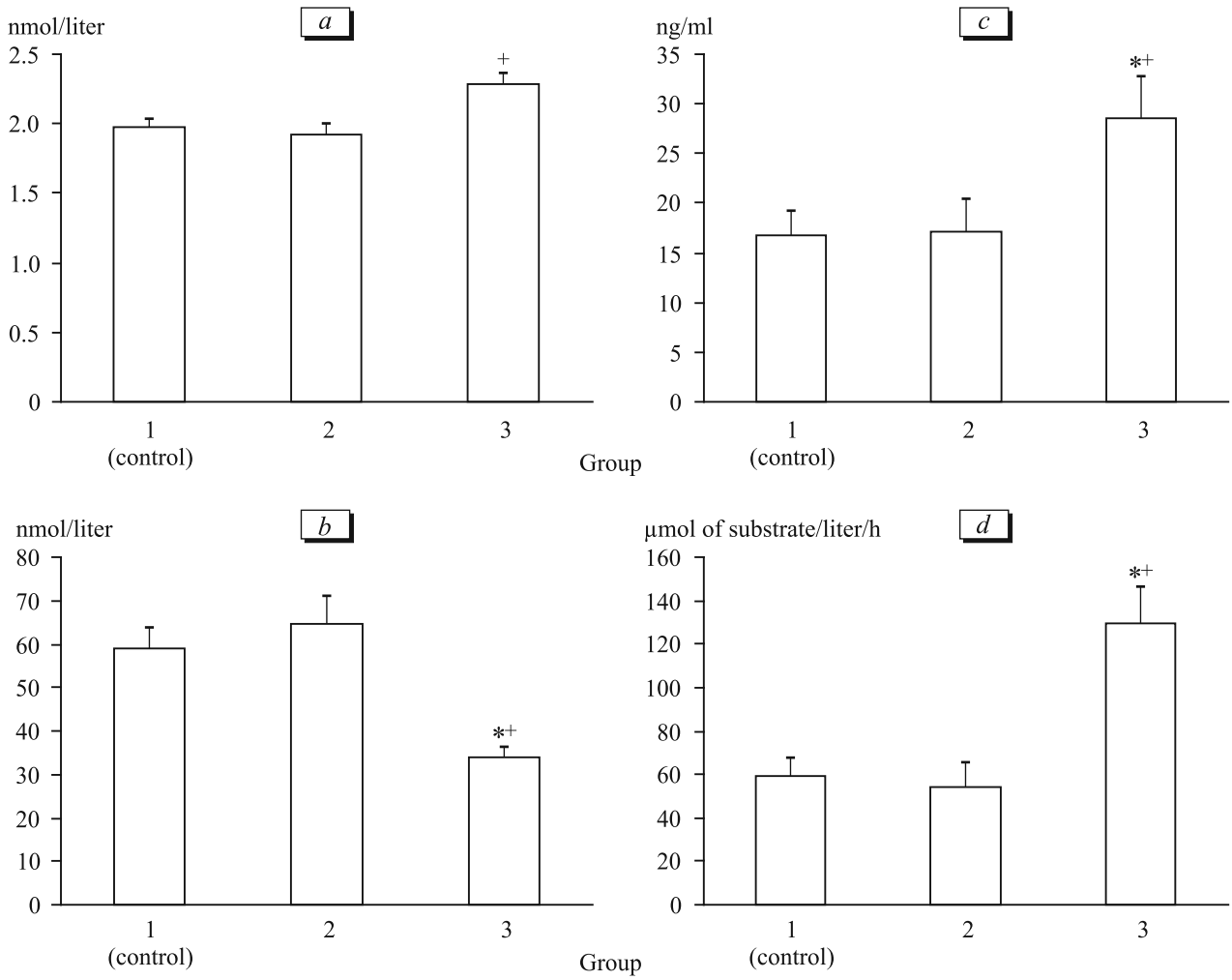

Fig. 1. Plasma levels of T_3 (*a*), T_4 (*b*), and circulating ecDNA (*c*) and MMP activities (*d*) in hypothyroid and euthyroid rats with experimental hypercholesterolemia. $p<0.05$ in comparison with *group 1, +group 2.

some cells were in a state of necrobiosis (marked edema; Fig. 3, *b*), their cytoplasmic matrix was greatly lyzed, the number of pinocytic microvesicles below the normal, and the cells acquired a spherical shape.

Capillary lumens and cell–cell spaces were usually filled with floccular substance and contained numerous heterogeneous vacuole-like and myelin-like structures (Fig. 2, *b*). Numerous macrophages with heterogeneous lipid incorporations and residual bodies were seen in the interstitium.

The ultrastructural changes in cardiomyocytes were more pronounced in group 3 rats (exogenous cholesterol+mercazolyl). Focal and diffuse lytic lesions in the myofibrils were more often recorded in them. The mitochondria were markedly polymorphic, with focal destruction. Significant lytic changes in the sarcoplasmic matrix, mitochondrial destruction, and formation of vacuole-like structures filled with granular contents were found in the cardiomyocyte perinuclear zones (Fig. 2, *c*). Accumulations of myelin-like structures, released into the cell–cell spaces, were seen near the intercalated discs (Fig. 2, *d*).

Blood capillaries were formed mainly by flat endotheliocytes with moderately electron-dense cytoplasm (mature forms), the luminal surface of endotheliocytes forming slight protrusions (Fig. 3, *c*). There were also capillaries lined with endotheliocytes with electron-transparent cytoplasm; the number of organelles in these cells was less than normally, they contained heterogeneous vacuole-like structures, the luminal surface was smooth without protrusions (edematous forms) (Fig. 3, *d*). Capillary lumens were filled with floccular substance and often contained erythrocytes, platelets, and vacuole-like structures (Fig. 3, *c*). The interstitium round the capillaries always contained myelin-like and vacuole-like structures, floccular substance, and bundles of collagen fibrils. The most numerous of the cellular elements were macrophages and mast cells.

We showed in a previous study [1] that long-term atherogenic diet led to reduction of the total count of

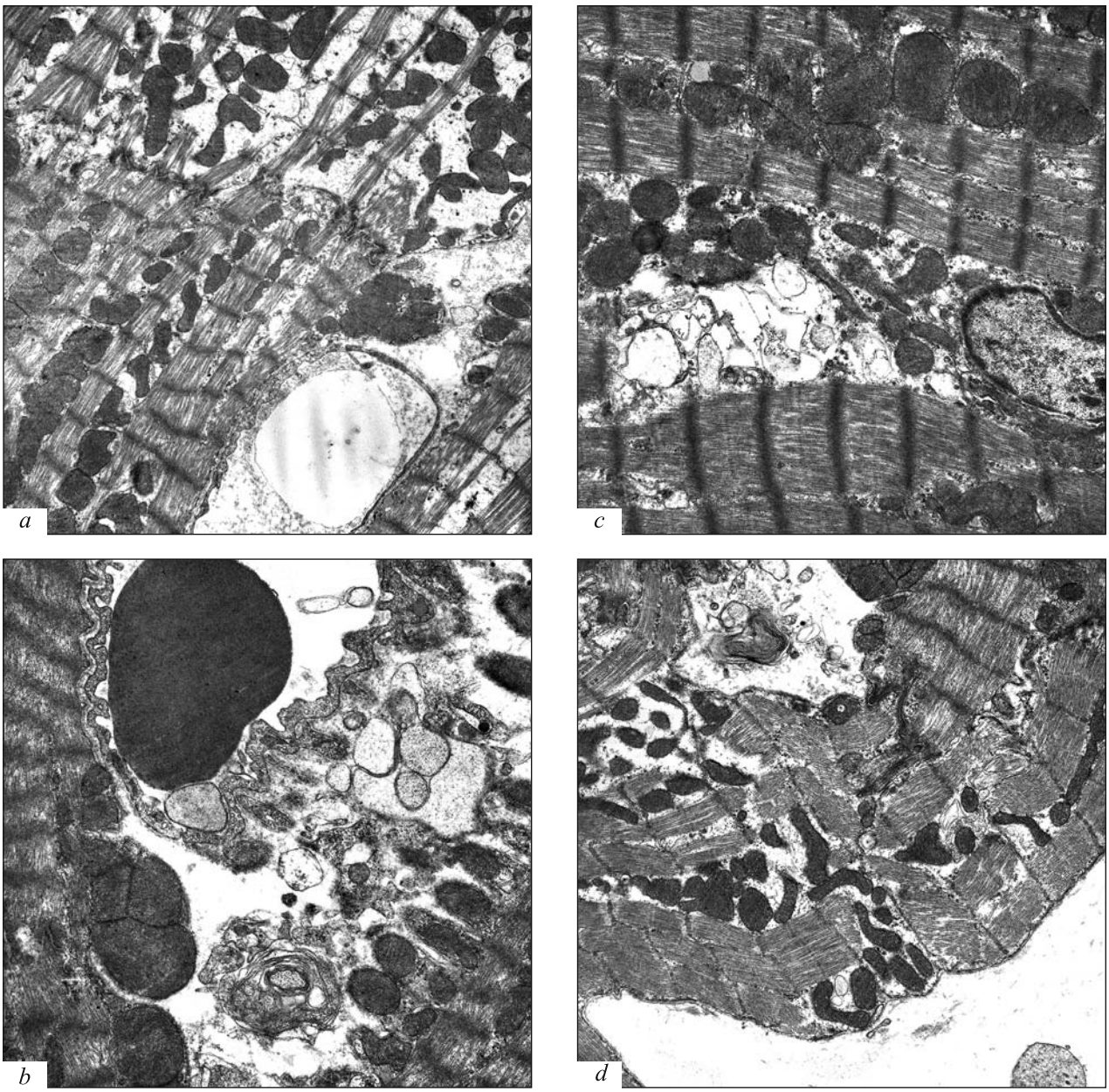

Fig. 2. Ultrastructural changes in cardiomyocytes of euthyroid and hypothyroid rats receiving cholesterol diets for a long time. *a*) Pronounced lysis and thinning of myofibrillar bundles, lysis of sarcoplasmic matrix in intercalated disc region in a rat from group 2, ×15,000; *b*) myelin-like and vacuole-like structures, filled with floccular substance, in extracellular space in a rat from group 2, ×15,000; *c*) destruction of mitochondria, lysis of sarcoplasmic matrix, and formation of heterogeneous vacuole-like structures in perinuclear zone of cardiomyocyte in a rat from group 3, ×15,000; *d*) accumulation of myelin-like structures near cardiomyocyte intercalated disc in a rat from group 3, ×12,000.

cardiomyocytes (by 21%) due to their apoptotic death. Cell death was as a rule paralleled by activation of micro- and macrophages migrating into the focus in order to absorb the cell detritus. Cell migration to foci of lesions and cell death were possible under conditions of extracellular matrix degradation, maintained by MMP [14,15]. *In vitro* and *in vivo* experiments showed that the MMP family caused degradation of virtually all components of the extracellular matrix at the expense of their catalytic activity. Importantly, MMP (including MMP-2 and MMP-7) were involved in remodeling of the vascular wall extracellular matrix and thus modulated the atherogenesis model, as well as the formation and destruction of atherosclerotic plaques.

Cholesterol diet in group 2 animals with preserved thyroid function (euthyroid animals) in our experiments was virtually inessential for total activity of MMP-2 (gelatinase A specifically active towards laminin, collagens I, II, and III – main structural components of organs and tissues) and of MMP-7 (matri-

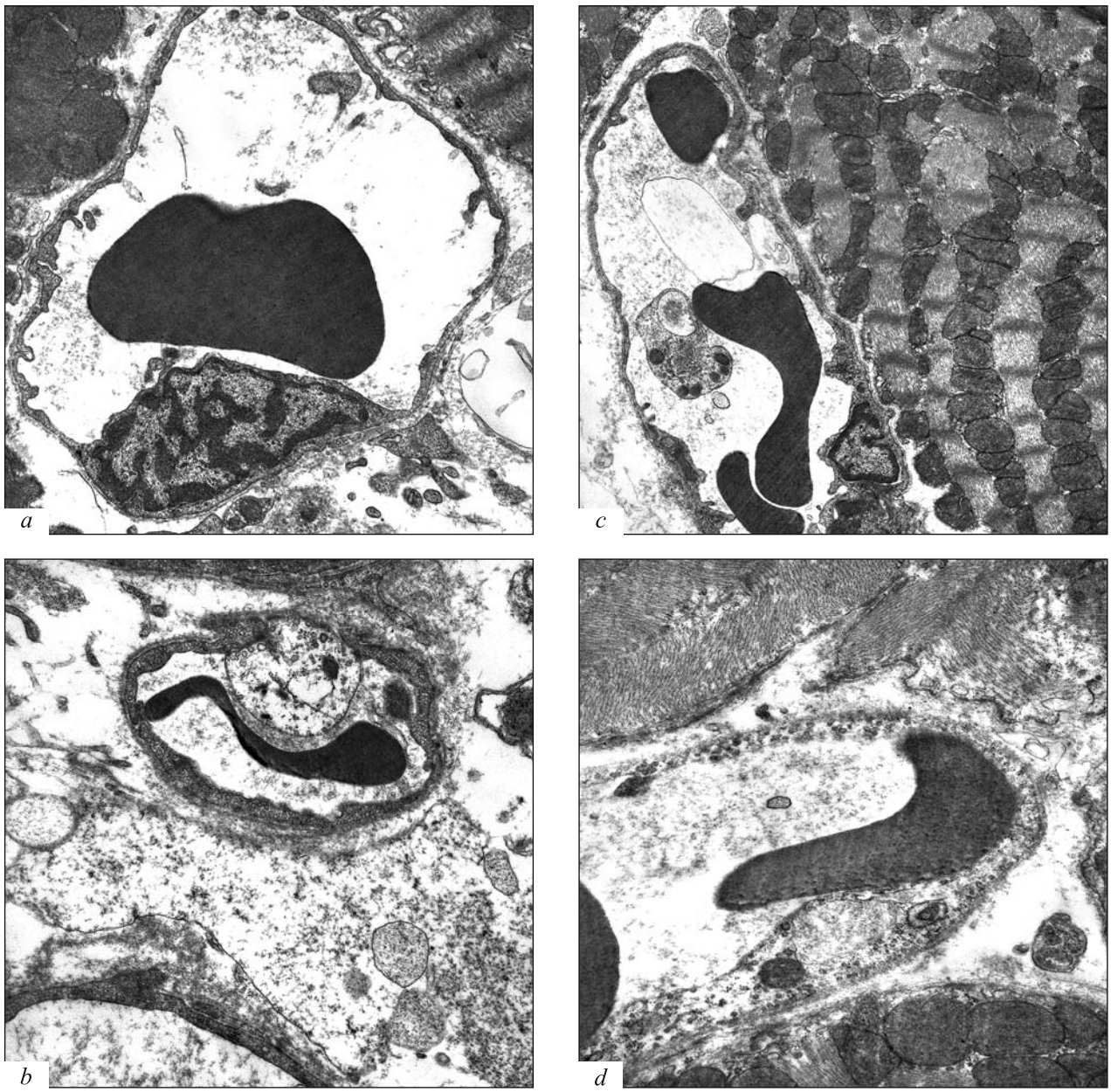

Fig. 3. Ultrastructural changes in blood capillary endotheliocytes in the myocardium of euthyroid and hypothyroid rats receiving cholesterol diets for a long time. *a*) Flat endotheliocytes with small luminal protrusions in the myocardium of a rat from group 2, ×12,000; *b*) fragments of flat and spherical (necrobiotic) endotheliocytes in the myocardium of a rat from group 2, ×20,000; *c*) floccular substance and vacuole-like structures in capillary lumen in the myocardium of a rat from group 3, ×12,000; *d*) vacuole-like structures in endotheliocytes with electron-transparent cytoplasm in the myocardium of a rat from group 3, ×25,000.

lysin 1, proteinase active in destruction of the main components of extracellular matrix, including elastin, proteoglycans, fibronectin) in comparison with the control (Fig. 1, *d*). The hypothyroid status of group 3 animals, induced by addition of mercazolyl to the atherogenic ration, was associated with a pronounced increase of MMP summary activity (by 119%, $p<0.05$ in comparison with two other groups).

Normal level of MMP expression after 68 days of exogenous cholesterol consumption and increase of their expression after exogenous cholesterol combined with mercazolyl was presumably explained by prevention of atherosclerotic plaque formation in the vessels during the early stages of the unfolding atherosclerotic process. On the other hand, experiments on transgenic mice with atherosclerosis, receiving normal or hyperlipidemic diets, showed that the development of atherosclerotic changes with macrophage infiltration of the intima was paralleled by an increase in MMP expression and activity, specifically,

of MMP-2 and MMP-9 [14]. Intravenous heparin and apolipoprotein A-1 inhibited the production of MMP-2 in the atherosclerotic foci in the aorta and led to a significant shrinkage of the foci in HDL receptor knockout mice [6,11].

In addition, proapoptotic activity of MMP, specifically MMP-7, is described. Previous studies have demonstrated significant destruction of N-cadherin, increase of MMP-7 activity, and intense apoptosis of smooth muscle cells in atherosclerotic plaques in comparison with control specimens of arteries and a 50% decrease of smooth muscle cell apoptosis intensity in atherosclerotic plaques in MMP-7 knockout mice comparison with wild-type mice [15].

Hence, a marked increase of plasma concentrations of free circulating ecDNA under conditions of hypercholesterolemia induced by atherogenic diet paralleled by the hypothyroid status could directly determine lytic lesions in cardiomyocytes and myocardial blood capillary endotheliocytes and their subsequent apoptotic death. High expression of MMP under these conditions could reflect more intense lytic processes in the extracellular matrix and its restructuring essential for migration of monocytes/macrophages to the dying cells for their resorption.

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