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Endothelial Changes in Rat Aorta after Terminating an Atherogenic Diet: A Scanning and Transmission Electron Microscopic Study

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Summary. **This work undertook an evaluation of ultrastructural examinations (by transmission electron microscopy (TEM) and samples for scanning electron microscopy (SEM)) of the endothelium and subendothelial space in the aorta of Wistar rats one, two and three months after ceasing an atherogenic diet rich in animal fat, cholesterol and methyl thiouracil.**

Changes corresponding to the early stage of the experimental atherosclerosis coexisting with formation of parietal platelet microthrombi were observed after three months of feeding the animals with the diet.

No distinct signs of a regression of atherosclerotic changes were observed in the internal membrane one month after stopping the atherogenic diet. An increased adherence of blood white cells to impaired endothelium could be noticed. Two and three months after ceasing the applied diet, the atherosclerotic changes were still observed in 1/3 of the animals. However, in 2/3 of the animals, there was a distinct regression of the atherosclerotic changes connected with the reconstruction of small and shallow defects in the endothelial lining and a decrease in the thrombotic changes. Regenerated cells of the endothelium ("dark endothelial cells") included a relative increase in the number of some cellular organelle (mainly micropinocytotic vesicles, Weibel-Palade bodies) and numerous cytoplasmic processes. The subendothelial space below the regenerating endothelium was generally widened, and included a microfibrillar substance from which the basal membrane of the endothelial cells probably arose during the process of regeneration.

Key words—endothelium, atherosclerosis, regeneration, ultrastructural studies, rat aorta.

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INTRODUCTION

Microscopic examination of the regression of atherosclerotic changes comprises an important subject in many scientific centers,[6,13,16-18,20,34] a fact directly relating to the very complicated and unclear morphogenetic background of the main disorder, i.e. atherosclerosis.[1,4,5,14,27,31,41]

It is known that many changes of a morphologic, biochemical and immunologic character take place in endothelial cells during the process of regeneration of the vessel internal membrane when the stimulus impairing the vessel wall is removed.[9,12,21,22,36,38,39] According to Jackman, Anderson and Sheridan[13] regeneration of the endothelial cells is a very complicated phenomenon, even in the simplest experimental model.

This work, a small contribution to the ongoing investigation, is an attempt to evaluate the ultrastructural form of the endothelial cells and subendothelial space in the aorta of the rat after stopping the applied atherogenic diet—a diet supplemented with animal fat, cholesterol, and methyl thiouracil.

It should be added that this work is a continuation of our previous examinations concerning changes in the inner membrane of aorta in the early period of experimental atherosclerosis.[29-32]

MATERIALS AND METHODS

Experiments were carried out on 24 Wistar rats, 3-month-old males weighing 150 g. The animals were kept in standard conditions and were divided into 3 groups: Group I-a control group consisting of 8 rats

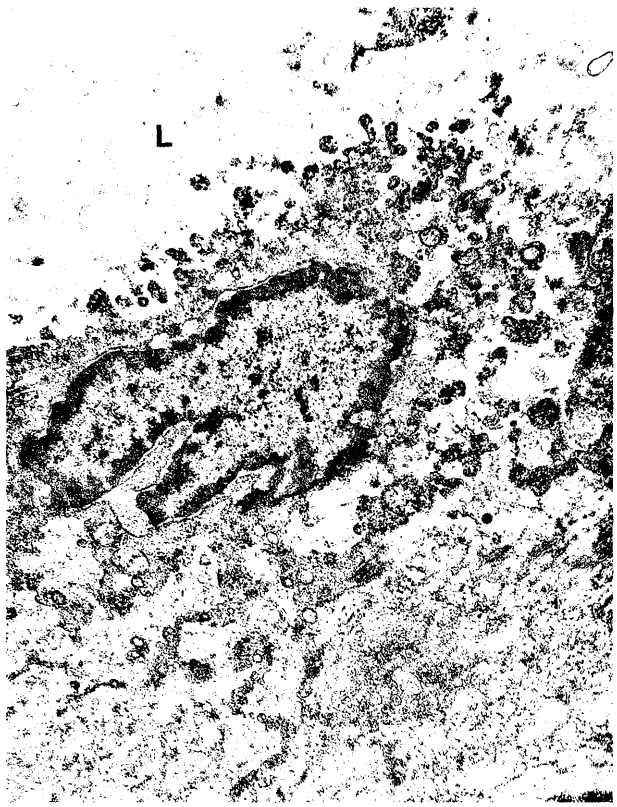

Fig. 1. TEM micrograph of a disintegrated endothelial cell of the thoracic aorta from a rat on an atherogenic diet for three months. The subendothelial spaces-widened and filled with a fibrillar fine-granular substance. L; the vessel lumen. ×7,000

of the same age as the experimental animals; they were given a standard laboratory rat chow ("Murigran") and water ad libitum; Group II-(4 rats) fed a typical atherogenic diet containing 40% animal fat, 3% cholesterol (cholesterol FP IV) and 0.3% methyl thiouracil (Methylothiouracilum "Polfa") every day for 3 months; each animal received 10 g of the diet during 24 h (according to 29-32), and a standard rat chow ad libidum; Group III-(18 animals) was given the same diet as Group II for 3 months and then the diet was stopped. The rats were sacrificed 1, 2 and 3 months after termination of the atherogenic diet (time subgroups IIIa, IIIb, IIIc consisted of 4 animals each).

Under sodium pentobarbital anesthesia supplemented with ether, all rats for the ultrastructural examinations were perfusion-fixed with 2, 5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.4. The buffered glutaraldehyde was infused at a pressure of 100 mm Hg for 20 min via the left ventricle of the heart. The thoracic segment of the aorta including the aortic arch and the upper 1/3 descending part was carefully removed from the animal to avoid distortion, and then gently opened.

Samples for transmission electron microscopy (TEM) of the volume 1 mm^3 were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, and post-fixed in buffered 1% osmium tetroxide. After dehydratation in increasing concentrations of alcohol the specimens were embedded in Epon 812 and cut on a LKB ultramicrotome. Ultrathin sections were further stained with uranyl acetate and lead citrate and examined in an Opton 900 PC transmission electron microscope.

Rectagular samples for scanning electron microscopy (SEM), measuring approximately 1.0 cm × 0.5 cm, were fixed with 1.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.4 and post-fixed with buffered 1% osmium tetroxide, dehydrated through a series of graded alcohol, critical-point dried in liquid CO_2, and sputter-coated with gold/palladium. The specimens were viewed in a JEOL scanning microscope.

RESULTS

TEM analysis of the normal rat thoracic aorta demonstrated flat and continuous endothelial cells with interlacing junctions. The subendothelial space was very thin. SEM analysis showed that the endothelial surface structure was uniform and smooth, and that cells were typically elongated with a slight nuclear bulging parallel to the longitudinal axis of the nuclear bulging noted. The presence of intercel-

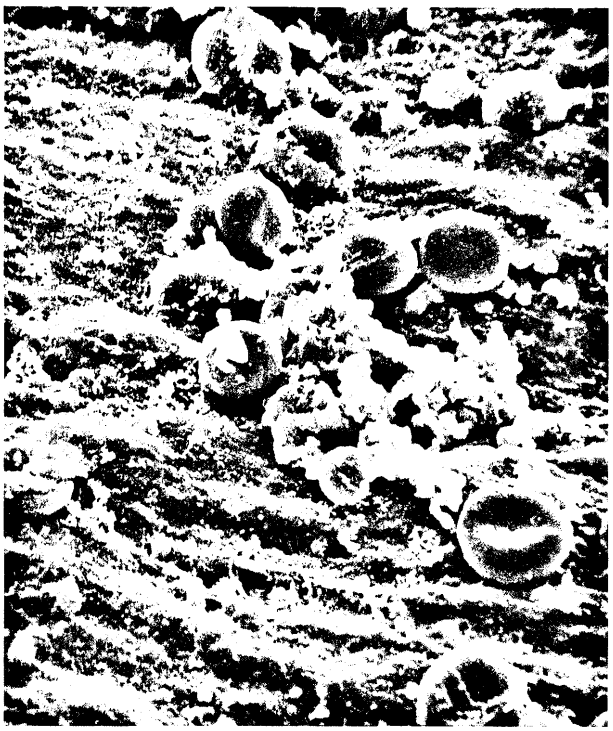

Fig. 2. SEM micrograph of the platelet microthrombus with a component of red blood cells abhering to the uneven endothelial surface covered with rough microgranular material in the thoracic aorta from a rat on an atherogenic diet for three months. ×2,700

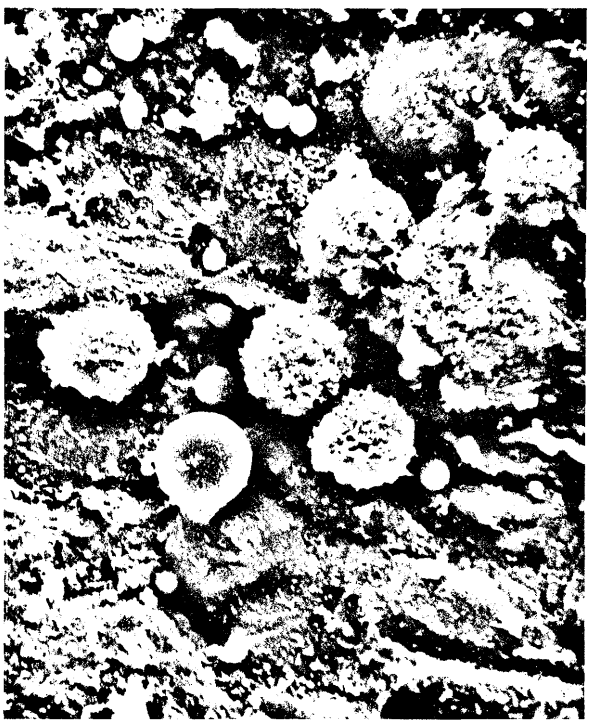

Fig. 3. SEM micrograph of white blood cell aggregates adhering to the impaired endothelial surface of a rat thoracic aorta one month after terminating the atherogenic diet; leukocytes, thrombocytes, erythrocyte and seen. ×2,200

lular bridges between neighbouring endothelial folds was observed.

Changes in the aortic intima of rats receiving an atherogenic diet (Group II) for three months were similar to those observed in the previous experiments under analogous conditions and the same diet.[29-32] Impairment of the aortic intima as seen in TEM included degenerative changes and the degradation of many endothelial cells and the prominent widening of the subendothelial space (Fig. 1). Lipid drops and cholesterol clefts were observed in the cytoplasm of the endothelial cells. The cells often had cytoplasmic processes directed both towards the vessel lumen and into the endothelial layer. The basal membrane was interrupted in a majority of the endothelial cells. The subendothelial space—markedly dilated, and of increased electron density—often included "cistern" forms surrounded by long cytoplasmic processes of the impaired endothelial cells filled with an amorphic or fibrillary microgranular substance. Single mononuclear phagocytes including lipid drops and myelinic forms were seen in this space.

Analysis of the endothelial lining using SEM showed the swelling and degradation of many transverse intercellular bridges. Lesions of different size and depth—from very small shallow and uneven depressions to extensive, crater-like defects—could be seen. The increased adherence of the blood elements, especially platelets and red blood cells to the changed endothelial surface and the presence of numerous mainly parietal microthrombi, was observed. Extensive fragments of the impaired endothelial lining were often covered with rough, microgranular material (Fig. 2). The elastic internal membrane, middle membrane and external membrane did not show significant deviations from normal conditions.

One month after terminating the atherogenic diet (subgroup IIIa) the changes in the internal membrane of the aorta persisted. No distinct signs of the regeneration of the endothelial cells were observed. The increased adhesion of the blood elements, mainly white and red blood cells to the changed endothelial lining, was observed (Fig. 3). Other elements such as thrombocytes and parietal thrombi were observed more rarely.

TEM examinations showed that the basal membrane of the preserved endothelial cells was not distinct, and in some places fused with the subendo-

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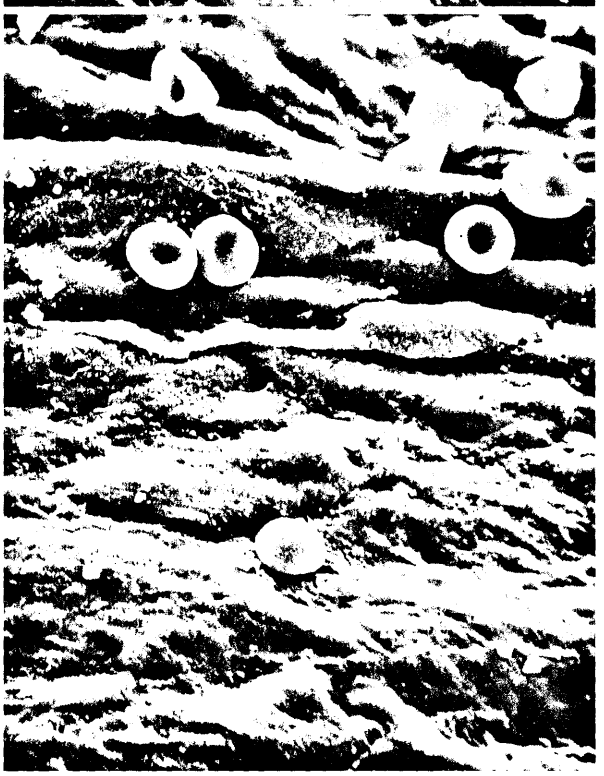

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Fig. 4. SEM micrograph of an oval shallow defect in the endothelial fold partly covered with single delicate cellular processes arising from the preserved surrounding endothelium of rat thoracic aorta two months after removing the atherogenic diet. ×1,800

Fig. 5. SEM micrograph of the impaired endothelial surface covered in some places with numerous cellular processes in the thoracic aorta of a rat three months after stopping the atherogenic diet. ×2,700

Fig. 6. SEM micrograph of partly regenerated, fairly smooth and focally flattened endothelial lining in the thoracic aorta of a rat three months after stopping the atherogenic diet; a swollen intercellular bridge above. ×1,800

thelial space. Endothelial cytoplasmic processes of different, often finger-like forms directed towards markedly thickened subendothelial space were seen. The thickened subendothelial space was filled with a microfibrillar substance of increased electron density. No fibrillary microgranular substance was seen in it.

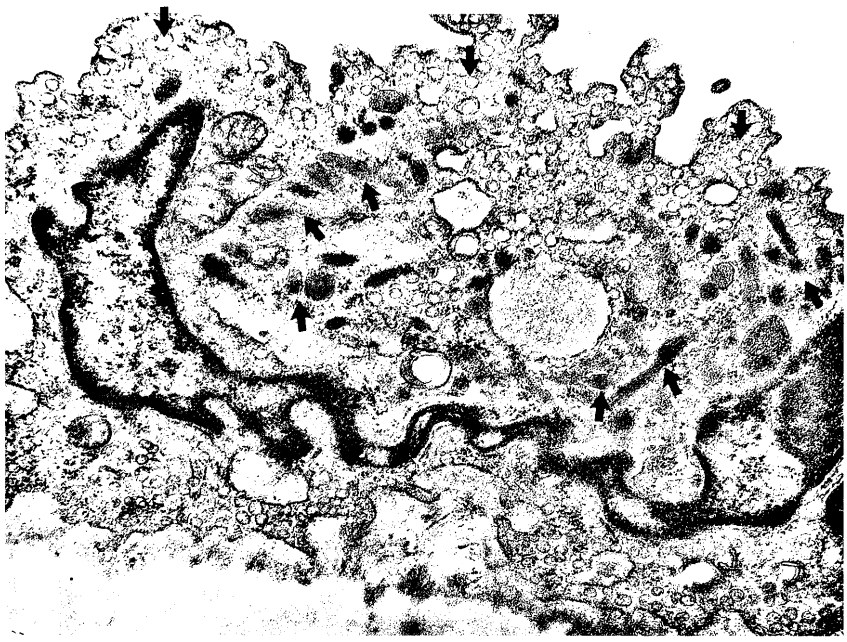
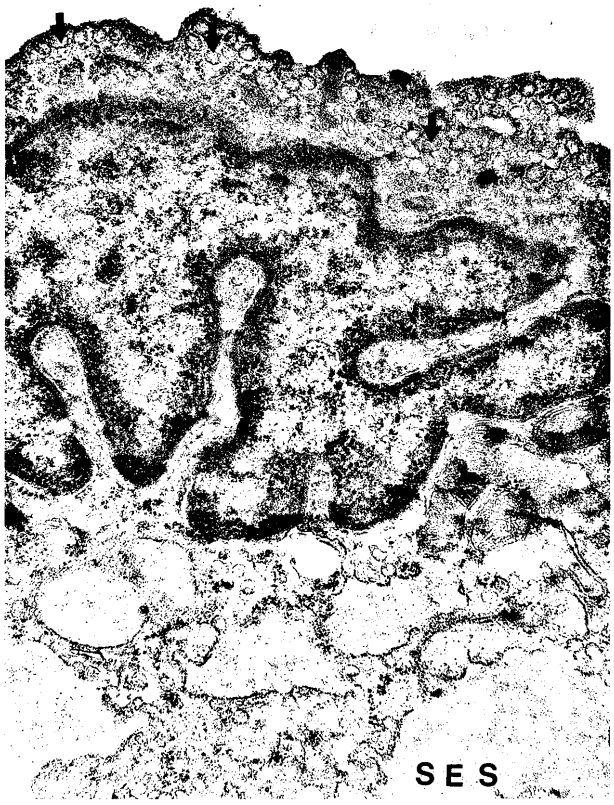


Fig. 7. TEM micrograph of rat thoracic aorta two months after stopping the atherogenic diet shows a fragment of stimulated endothelial cell, including an increased number of micropinocytotic vesicles and Weibel-Palade bodies (*arrows*). ×9,800



Two and three months after terminating the atherogenic diet (subgroup IIIb, IIIc) changes in the endothelium and subendothelial space were similar to each other. Distinct morphologic signs of the regression of the atherosclerotic changes and the process of regeneration of the impaired endothelium were observed in 2/3 of the animals. Changes in the internal membrane persisted in 1/3 of the animals; they were expressed in the same way as in subgroup IIIa. The process of endothelial regeneration usually concerned small and shallow defects covering a fragment of the endothelial cell. Numerous cytoplasmic delicate finger-like processes then protruding from the preserved endothelium towards the defects (Figs. 4 and 5) were seen by SEM. These processes covered the shallow defects as a mesh, coming into contact with the nearest fragment of the cell or with the

Fig. 8. TEM micrograph of a rat thoracic aorta two months after stopping the atherogenic diet, showing a fragment of a "dark" endothelial cell with cytoplasmic processes of different shapes directed towards prominent widened subendothelial spaces (SES). The basal membrane is discontinuous in some places, and fused with a microfibrillar substance beneath the endothelium; a part of the micropinocytotic vesicles includes an electron dense substance. ×9,800

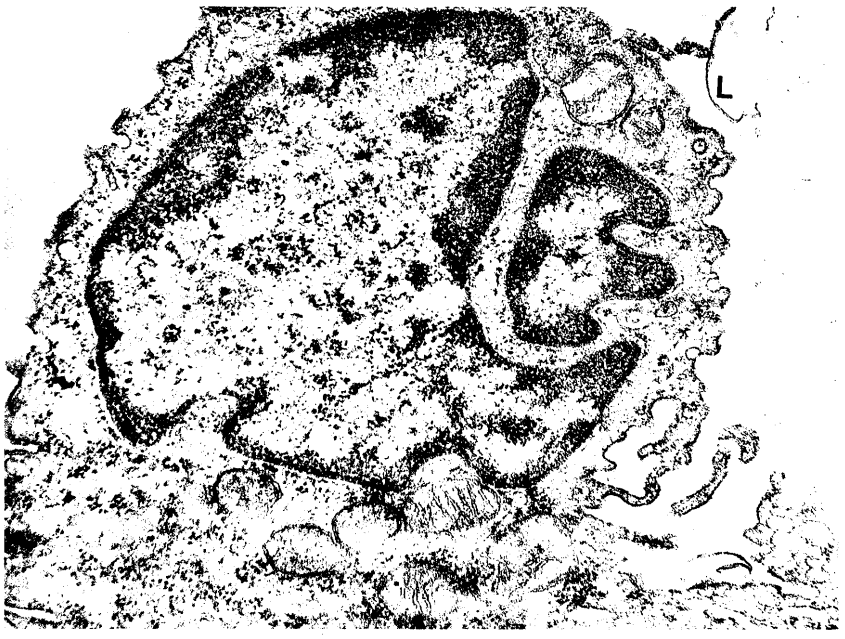


Fig. 9. TEM micrograph of a rat thoracic aorta three months after terminating the atherogenic diet showing a regenerating endothelial cell of cuboidal shape indenting into the vessel lumen (*L*); m-mitochondria. ×9,800

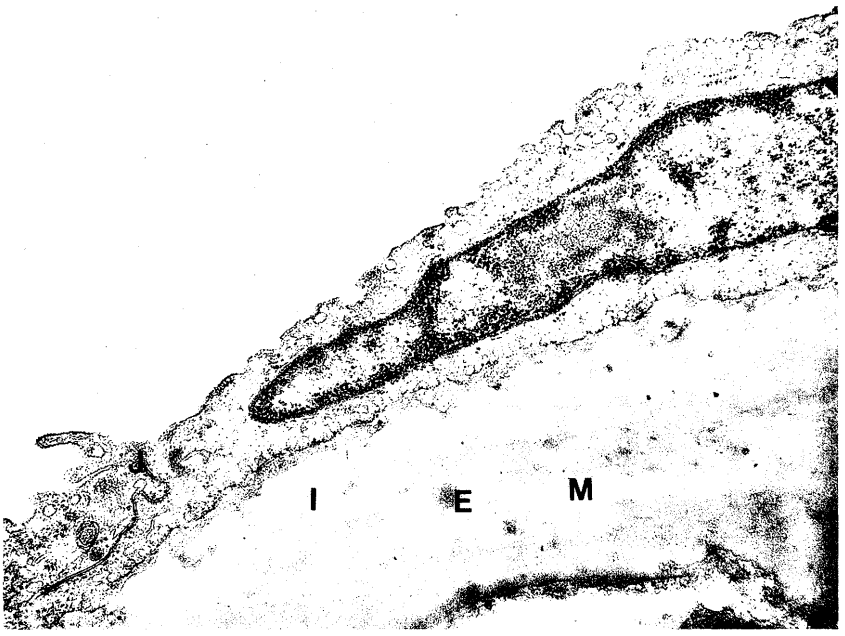


Fig. 10. TEM micrograph of a rat thoracic aorta three months after ceasing the atherogenic diet, showing a fragment of regenerated endothelial cell; interendothelial junction J, morphologically normal; very thin subendothelial space. IEM; internal elastic membrane. ×9,800

neighbouring healthy endothelial cell. A beginning process of the regeneration also concerned small defects in the transverse intercellular bridges. Usually dispersed platelets and red blood cells could be seen near the regenerating cellular defects. White blood cells were observed more rarely in comparison to the previous group. Parietal microthrombi and microgranular rough material were seen very rarely.

Sometimes small areas lined with endothelial cells which seemed to be completely regenerated could be found. The configuration of the lining containing regenerated cells of the endothelium was markedly disturbed, as excessively elevated and rounded areas were accompanied by the flattening of some areas of the endothelial surface (Fig. 6). It should be added that extensive, deep defects in the arterial endothelium did not in general show morphologic indices of regeneration.

It was noticed in TEM examinations that the regenerating cells often showed features of stimulation characterized by: (1) Increased electron density of cytoplasma (so celled "dark cells of the endothelium"); (2) The presence of numerous cytoplasmic processes; and (3) The relative increase of the number of some cellular organelles (Figs. 7 and 8). This mainly concerned micropinocyte vesicles, Weibel-Palade bodies, the Golgi apparatus, rough endoplasmic reticulum and sometimes mitochondria. Pinocyte vesicles (of diameters 700-900A) quite often included an electron dense amorphic material which made the cell hyaloplasma dark (Fig. 8). Weibel-Palade bodies of bacilliform and rounded cross-sections including a characteristic electron dense matrix were mainly localized near the cell nucleus (Fig. 7). Their numbers sometimes surpassed 30 (norm 5-10). Cholesterol clefts were not found in the regenerated endothelial cells; occasionally lipid drops were seen. Some regenerating cells had a cuboid shape and indentations into the vessel lumen (Fig. 9). Their basal membrane was not often completely regenerated. The subendothelial space below regenerating cells was usually widened but uninuclear phagocytes were not observed in it. A microfibrillar substance was still observed in this space (Fig. 8), from which the basal membrane of the regenerating cells may have been reconstracted.[10,35]

Sometimes completely regenerated cells of the endothelium were seen in the examined pictures. Their basal membrane was continuous. They had morphologically normal cell junctions, usually adhering tightly to each other. The subendothelial space was not widened (Fig. 10).

DISCUSSION

Two and three months after terminating the applied diet (rich in animal fat, cholesterol and methyl thiouracil) in 2/3 of examined rats, a regression of the atherosclerotic changes connected with the reconstruction of small and shallow defects in the endothelial lining of the aorta and reduced thrombotic changes was observed.

We would like to underscore that the characteristic morphological features indicating the stage of the reconstruction of the endothelium in the course of our experiment, similar to that demonstrated by other authors,[8,10,18,37,40] concerned mainly the following: (1) The presence of stimulating endothelial cells, or so-called "dark cells of endothelium" including an increased electron density of the cytoplasm, a relatively increased amount of some cellular organelles (especially micropinocytotic vesicles, Weibel-Palade bodies, Golgi apparatus, rough endoplasmic reticulum) as well as numerous delicate cytoplasmic processes; (2) The appearance of endothelial cells having a cuboid rounded shape, which indented into the vessel lumen; (3) Decreased thrombotic changes in the healing endothelial lining; (4) The presence in the subendothelial space below regenerating cells of a microfibrillar substance from which the basal membrane of these cells may have been reconstructed during the process of endothelial repair.

Moreover, lipid droplets and cholesterol clefts within the cytoplasm of regenerating endothelial cells as well as uninuclear phagocytes in the subendothelial space were absent.

The process presented here of the healing of the endothelial lining characterized by a "creeping on" of cytoplasmic processes from the preserved endothelium towards neighbouring defects resulting finally in their regeneration is also defined as a process of reendothelialization,[11,26,28,35] a phenomenon of endothelial regeneration,[10,24,25,26] a restitution of endothelial continuity, (in Latin: "restitutio ad integrum"[7,10]) or endothelial repair.[23,24] The reendothelialization time of the internal membrane of large arterial vessels in experimental conditions was different.[10,15,19,34,35,38,40] The difference mainly depended on the type of trauma, the extent and depth of the defect and the immune power of the organism. If the defect is shallow and of small diameter (e.g. evoked by a mechanical injury), the process of the endothelial cell regeneration lasts from several to several dozen hours, most often 72-96 h, which is the phase of quick healing.[7,11,25,26] But if the defect of the endothelial lining is deep and extensive, the process of the endothelial regeneration lasts markedly longer, usually one to sixty weeks, or the phase of slow healing.[3,10,11,23,26,35,37]

The present description of the endothelium and subendothelial space of the rat aorta after terminating the applied diet is similar to that described in other experimental models, e.g. after ceasing the atherogenic diet of a different chemical composition;[15,21,40] after stopping a mechanical trauma (an injury of the vessel internal membrane with a catheter); applying pressure on the vessel wall from the inside,[2,7,10,11,28,37]

after surgically terminating atherosclerotic changes using laser;[8] under the influence of medicines inhibiting platelet aggregation (dipiridamole, aspirin, ticlopidine, haparin,[12] drugs reducing inflammation (dexamethasone[9]), antioxidants (selenium, vitamin E[39]), intravenous injections of endotoxin E. colli;[22] or in the course of hyperoxia.[36]

On the basis of the examinations performed, it possibly supposed that the process of the healing of the endothelium and subendothelial space ("restitutio ad integrum") of the rat aorta beginning two or three months after stopping the atherogenic diet is slow, but does progress.

CONCLUSIONS

(1) One month after terminating the atherogenic diet, there were no signs of regeneration of the atherosclerotic changes in the endothelial cells, though an increased adherence of the white blood cells to the impaired endothelium was noted.

(2) Two and three months after stopping the atherogenic diet, 2/3 of the examined animals showed morphologic signs of the early stage of endothelial and subendothelial regeneration connected with the regression of the thrombotic changes.

(3) Extensive and deep defects in the endothelial lining did not show distinct signs of regeneration.

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