ALCOHOL–ALKALI DISSOCIATION OF AN EVERTED VESSEL: A SIMPLE METHOD OF ANALYSIS OF THE THREE-DIMENSIONAL ORGANIZATION OF THE ARTERIAL INTIMA

M. D. Rekhter, V. A. Kolpakov, and A. A. Mironov

UDC 611.13.018.74.086.3

KEY WORDS: vessels; methods of investigation.

The intima of arteries, including the endothelium, the subendothelial layer, and the inner elastic membrane, plays a key role in the development of atherosclerosis and of other pathological processes [2, 5, 6]. The surface of endothelial cells (EC) has been studied in detail by scanning electron microscopy (SEM) [1], but the structure of the elastic membrane has received a less detailed study [5]. No data could be found in the literature on spatial organization of the subendothelial layer, in which the principal processes leading to stenosis take place. We have developed a new method of investigating the three-dimensional structure of the different components of the intima.

The method is a version of alcohol–alkali dissociation of tissues [6]. The method is based on dissolving the extracellular matrix with potassium ethoxide, after which the tissue breaks up into separate cells. They are kept in the dissociating solution. The cell suspension is investigated later under the phase-contrast microscope.

In our method, the dissociation process is stopped at an early stage, and what is analyzed is not the cell suspension but a tissue fragment remaining after processing. Potassium ethoxide interacts most actively with the surface of the specimen. If the vessel is turned inside out this surface will consist of endothelium. The appearance of the first EC in the dissociating solution must signify destruction of their basement membrane. Consequently, if dissociation is stopped at the moment when the first EC appear in the solution, depending on the degree of development of the subendothelial connective-tissue fibers, either these fibers or subendothelial cells will be found on the surface of the residual specimen. The appearance of the first subendothelial cells in the solution, in turn, guarantees the possibility of SEM analysis of the main mass of subendothelial cells remaining on the surface of the specimen. Finally, the moment of complete disappearance of cells from the solution must signify complete dissociation of the subendothelium and, correspondingly, exposure of the inner elastic membrane.

The test objects were the aorta from a human fetus and adult, cow, pig, rabbit, and rat. The material was fixed with 2.5% glutaraldehyde ("Reanal," Hungary), dissolved in medium 199 or in buffered physiological saline (BPS), pH 7.4-7.6. Vessels fixed by both immersion and perfusion (under physiological pressure) methods were used. Whereas the method of fixation plays a key role in the study of EC [1], we did not find that the structure of the subendothelial intima is strictly dependent on this parameter. The fixation time was deliberately not restricted and varied from 1 day to 1 year.

The procedure is as follows. After fixation the segment of the blood vessel is washed with BPS, after which it is everted with the endothelium facing outward by means of forceps. The edge of the specimen is turned inward and compressed with tantalum or microsurgical clips, after which the specimen is placed in a solution of potassium ethoxide (a mixture of 30% KOH and 96% alcohol, 1:1). The specimen is incubated at 37°C with periodic vigorous shaking. Every 10 min a sample of solution is withdrawn and studied in the MBI-15 phase-contrast microscope (LOMO, USSR), and the specimen is transferred into a fresh portion of warm solution. After a certain time (which varies individually for each object) the first polygonal cells, identified as EC, are found in the solution (Fig. 1a). Later their number increases. Besides polygonal cells, stellate or Y-shaped cells subsequently appear (Fig. 1b), and are characteristic of subendothelial cells [6].

Central Research Laboratory and Department of Anatomy, Ivanova Medical Institute. (Presented by Academician of the Academy of Medical Sciences of the USSR V. V. Kupriyanov.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 112, No. 11, pp. 549-552, November, 1991. Original article submitted December 29, 1990.

 ©1992 Plenum Publishing Corporation

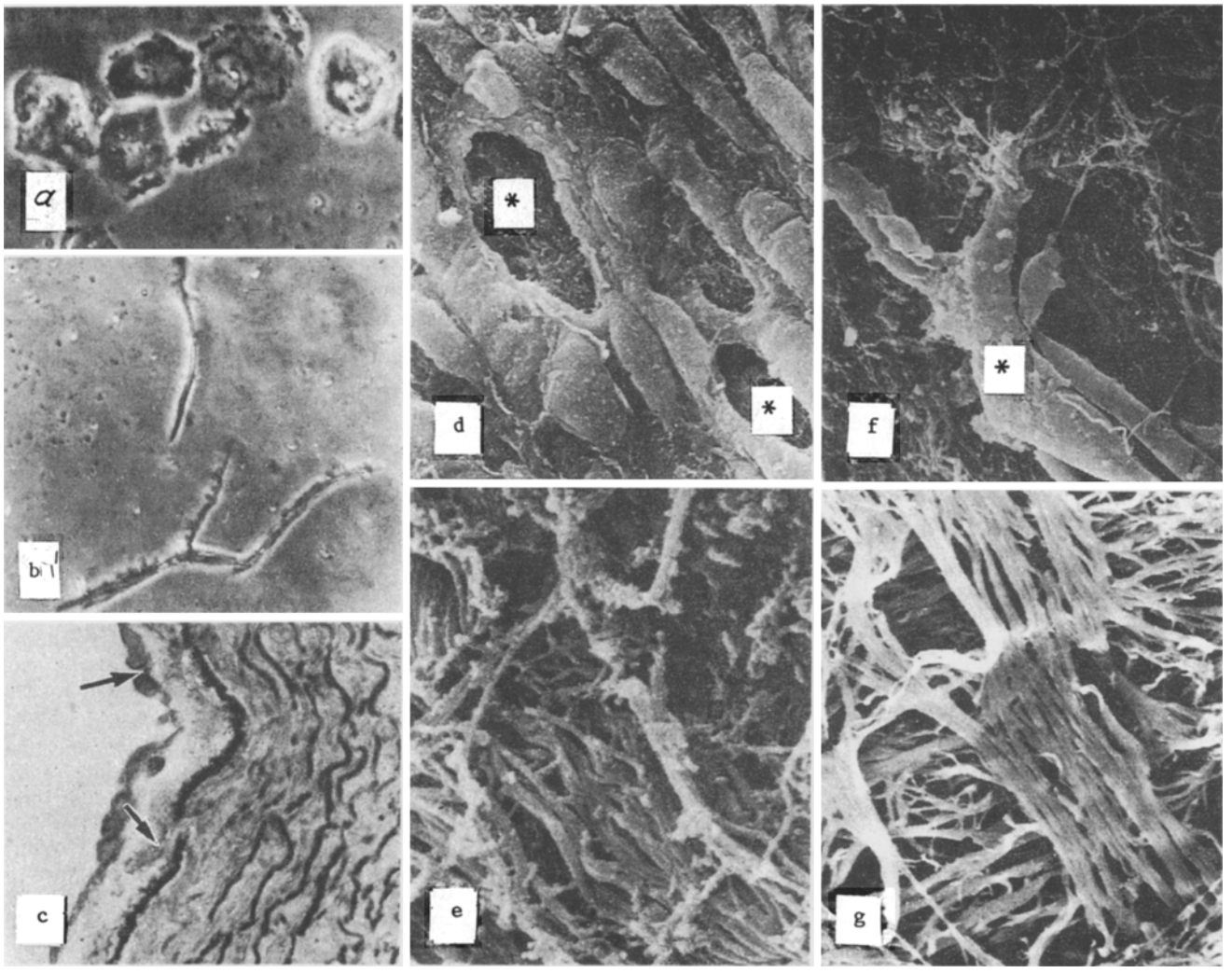

Fig. 1. Three-dimensional organization of inner elastic membrane of human fetal aorta: a) EC in suspension (phase-contrast microscopy, 440×); b) subendothelial smooth-muscle cells in suspension (phase-contrast microscopy, 440×); c) semithin section: arrows indicate subendothelial smooth-muscle cells (stained with methylene blue, 620×); d-g) alcohol–alkali dissociation: d) defects in endothelial layer the size of a cell (*, SEM, 1800×); e) subendothelial cells (SEM, 750×); f) subendothelial smooth-muscle cells (*, SEM, 3000×); g) inner elastic membrane (SEM, 1500×).

No polygonal cells are found in the next portions, but mainly branched and fusiform cells are present. Finally, the solution becomes generally free from cells. After a short time the specimen breaks up completely. Under these circumstances many fusiform cells appear in the solution. This sequence of events is observed only during the analysis of vessels with a well-developed subendothelial intima (aorta of man, cow, and pig). In vessels not containing subendothelial cells (aorta of rabbit and rat) only the time of appearance and disappearance of EC can be distinguished before disintegration.

At each stage of processing dissociation can be stopped by thoroughly washing the specimen in several changes of BPS or distilled water. We stopped the processing at the following moments: 1) on the appearance of the first EC in the solution, 2) on the appearance of the first subendothelial cells in the solution, and 3) at the moment of disappearance of the cells from the solution.

The vessels were prepared for SEM by the traditional method: postfixation in osmium tetroxide (1 h) and 1% tannic acid (30 min), dehydration, drying by passage through the critical point from liquid carbon dioxide in an HCP-2 apparatus, and spraying with platinum in a JFC-1100 apparatus. The objects were analyzed in the S-570 microscope with accelerating voltage of 20 kV. All apparatus was from "Hitachi" (Japan). Additionally, before processing, semithin sections

were cut from each specimen and stained with methylene blue. We would emphasize the importance of this simple operation because data on the thickness and structure of the intima enable the tactics of dissociation to be properly planned.

Let us briefly examine the results of SEM analysis of the structure of the intima of the arteries after alcohol–alkali dissociation, using as an illustration the human fetal aorta. The adult human aorta had the thickest intima, whereas that from the aorta of the pig and cow is somewhat thinner. In all these cases, several rows of intimal cells were arranged between the endothelium and the inner elastic membrane. In the rat and rabbit the intima was very thin and contained no subendothelial cells. The human fetal aorta occupied an intermediate position. In some areas the subendothelium consisted of a connective-tissue matrix only, whereas in other zones groups of subendothelial cells were found (Fig. 1c).

On SEM analysis of preparations processed before the appearance of the first EC in solution, defects were found in the endothelial monolayer the size of one or a few cells (Fig. 1d). In some cases the endothelium had desquamated in a continuous sheet. In this case thin fibers, lightly packed together, were exposed. The superficial fibers formed a thin network. Deeper fibers formed bundles arranged parallel to the long axis of the vessel (Fig. 1e). The first layer evidently consisted of remnants of the basement membrane of EC, the second layer subendothelial collagen fibers. If the intimal cells were located in the immediate vicinity of the endothelium, they could be discovered at this stage of the procedure (Fig. 1f).

Processing before the appearance of the first subendothelial cells was used in only a few cases, when a well-developed intima was present in the arteries. In the aorta of man, cow, and pig the subendothelial cells appeared to be about equal in size. They were stellate in shape and established contact with each other through processes, forming a thin network. If the specimens were incubated before disappearance of the cells from solution a structure consisting of thick fibers, collected into bundles, was exposed (Fig. 1g). In most objects studied (except the human fetal aorta) the fibers were so tightly packed together that they looked like homogeneous laminae. Between the laminae "windows" were visible. The picture described above identifies these structures as the inner elastic membrane.

Detection of acellular connective-tissue structures and of the concealed surfaces of cells of other organs and tissues by SEM is impossible because of the technique used to prepare the specimens, and separate investigations are needed [3, 4, 7]. By the method described in this paper, it is possible to visualize reproducibly the subendothelial fibers, cellular assemblages, and the inner elastic membrane after the ordinary fixation procedure.

To detect cells, the most popular method is to "etch" the extracellular matrix with hydrochloric acid and collagenase [3, 4]. We attempted to use this approach to study the three-dimensional organization of the intima of the human aorta under normal conditions and in atherosclerosis [2], but we were compelled to make an empirical choice of the conditions for processing each specimen, thereby reducing the reproducibility of the results. By using potassium ethoxide, it is possible to individualize the conditions of processing, by introducing a simple informative parameter (determination of the times of appearance and disappearance of cells of a particular type in consecutive samples of dissociating solution).

LITERATURE CITED

1. Ya. L. Karaganov, A. A. Mironov, and V. A. Mironov, Arkh. Anat., **90**, No. 1, 93 (1986).
2. M. D. Rekhter, E. R. Andreeva, G. A. Alimov, et al., Tsitologiya, **32**, No. 11, 1102 (1990).
3. P. Baluk and G. Gabella, Cell Tissue Res., **250**, 551 (1987).
4. A. P. Evan, Biomed. Res., **2**, 119 (1981).
5. J. Nakatake and T. Yamamoto, Atherosclerosis, **64**, 191 (1987).
6. A. N. Orekhov, E. R. Andreeva, V. V. Tertov, and A. V. Krushinsky, Acta Anat. (Basel), **119**, 99 (1984).
7. T. Sugimoto and T. Ogata, Arch. Histol. Cytol., **52**, 257 (1989).