Morphological Analysis of Newly Formed Vessels and Cellular Microenvironment

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 148, No. 11, pp. 593-596, November, 2009
Original article submitted July 23, 2009

Newly formed microvessels were studied by the topical method (combination of light and electron microscopy with preliminary visualization of the isolated new vessel with horseradish peroxidase) on day 8 of growth. Five different zones with different transport characteristics of the vascular wall and cellular microenvironment were detected over the vascular length. A direct correlation between cellular microenvironment and formation of transporting canals in the endothelial lining of the newly formed vessels was detected.

Key Words: *new microvessel; transporting characteristics; cellular microenvironment; topical method; endothelial lining*

Problems of tissue regeneration and neoangiogenesis, associated with it, attract special attention of scientists in recent years. Problems of vasculogenesis regulation in adult organism are studied with the analysis of the effects of inductors and inhibitors on adjacent cells [1] or vessels under conditions of their exposure to physical factors [2]; experimental studies are carried out on endothelial tissue culture [4]. The formation of new vessels with special emphasis on the development of their transporting characteristics and the impact of adjacent tissue cells on these processes received little attention.

We studied morphofunctional characteristics of different connective tissue cells during neoangiogenesis over the entire length of new vessels and evaluated their role in the development of transport characteristics of the new microvessels.

MATERIALS AND METHODS

Experiments were carried out on male Chinchilla rabbits (1.5-2.0 kg; *n*=15) in accordance with ethical standards (Decision of the Local Ethical Committee of N. I. Pirogov Russian State Medical University). The vessels of the perilimbic area and new vessels (NV) growing into the ocular corneal stroma after burn inflicted by silver nitrate under rometar narcosis were examined. The material for the study was collected as follows. Rabbits narcotized with rometar (2 mg/kg) were intravenously injected with plant peroxidase (mol. weight 40,000 Da, 4-5-nm molecules; 25 mg/100 g) for NV visualization 10 min before collection of the material. Before removal of the eye and separation of the cornea, the organ was *in situ* fixed by instillation of cold 2.5% glutaraldehyde in 0.1 M phosphate buffer. Enucleation was carried out after sacrifice by anesthetic overdosage. The isolated cornea was left in the fixative for 1.5 h, after which it was dissected (in a droplet of the fixative) into 16 segments, the central part of the cornea and the sclera were removed, with 2-mm fragments left on the side of the sclera and 3-4-mm fragments on the side of the cornea. The surface and posterior epithelium of the cornea were then removed on a cryotome, each fragment was dissected into 2 parts, and further manipulations were carried out by routine methods. Preincubation (30 min at ambient temperature) in a mixture of Tris-HCl-diaminobenzidene tetrachloride with 1% H_2O_2 in buffer solution (pH 7.4) and incubation in a thermostat at 37°C (1 h) were carried out for histochemical detection

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0007-4888/09/1485-0838

of the reaction product for plant peroxidase, visualizing the limbic capillary network and new vessels grown in the rabbit eye corneal stroma. One NV was distinguished in each preparation under a magnifying glass (Fig. 1, *a*) and the preparation was embedded in araldite mixture. A pyramid was grinded from selected blocks on an LRB-3 pyramitome (under semithin section control) and slices for electron microscopy were prepared starting from the corneal limbus side; 20-25 serial semithin (1 μ) sections were then sliced, after which the pyramid was again grinded and sections for ultramicroscopic study were sliced. These manipulations were repeated until the NV was over. A total of 5-6 section levels were obtained. Ultrathin sections contrasted with uranyl acetate and lead citrate were examined under a Hitachi12A HU-12 electron microscope at ascending voltage of 75 kV.

RESULTS

The study of NV on day 8 of growth along its entire length clearly showed sites with different structure of vascular wall endotheliocytes, levels and degree of formation of transporting canals and the adjacent cellular microenvironment. These results are in line with other reports [6] and, we think, despite great variety of factors influencing neovasculogenesis, they are explained by organ specificity of the cornea.

New vessels in the immediate vicinity of a postcapillary or a venule (maternal vessels; MV) and at a distance of 140-150 μ from it in the zone of NV separation from MV are largely similar by their characteristics to the initial mature MV. Their endotheliocytes are differentiated and have *maculae et zonulae occludentes* in sites of cell-cell contact formation (Fig. 2, *a*), microvesicles are located mainly in the transport and pericontact zones of the cytoplasm with pericytes around them. In contrast to MV, pericytes of NV in this zone form virtually no contacts with endotheliocytes. Endotheliocytes of NV wall at a distance of 150-250 μ from MV (in the transitional zone) form open cell-cell contacts, the wall is starting to thin, and some transendothelial transport channels (TETC) are formed by two, rarely by three microvesicles. The endotheliocyte cytoplasm has large bundles of microfilaments (Fig. 2, *b*), which not only provide motor reactions of cells and form cell-cell contacts and growing vessel contour, but also regulate the barrier and transporting properties of the vascular wall [5]. Fibroblasts are seen in the immediate vicinity of the NV wall in the adjacent tissue; mast cells are sometimes seen. The next NV zone is the zone of active development of all transport communications, the longest zone, located at a distance of 250-600 μ from MV. Its endothelium is sharply thinned and forms fenestrae (Fig. 2, *c*); cell-cell contacts are open, the vesicles are united in TETC, these channels are usually presented by two or three microvesicles (Fig. 2, *d*). The adjacent tissue contains mast cells; numerous platelets are seen in the vascular lumen. At a greater distance from MV (600-700 μ) the NV endotheliocytes are much thicker; high mobility of cell membranes of the luminal and basal surfaces is worthy of note; the fenestrae disappear, cell-cell contacts acquire maculae occludentes; open cell-cell contact are still sometimes seen, though rarely; the content of microvesicles in the cell cytoplasm sharply decreases. The adjacent tissue contains fibroblasts and cells resembling microphages. Further from MV (750-800 μ) the thick endothelium of the NV apex is completely undifferentiated; the NV wall is separated by two poorly differentiated endotheliocytes connected by dense cell-to-cell contacts with *maculae et zonulae occludentes*; the vascular lumen is narrow (Fig. 1, *b*). The adjacent tissue contains cells which can be referred to endothelioblasts and to fibroblasts or pericyte-like cells. Macrophages are identified at some distance from the NV apex. It is obvious that the vector, direction, and rate of NV growth depend on

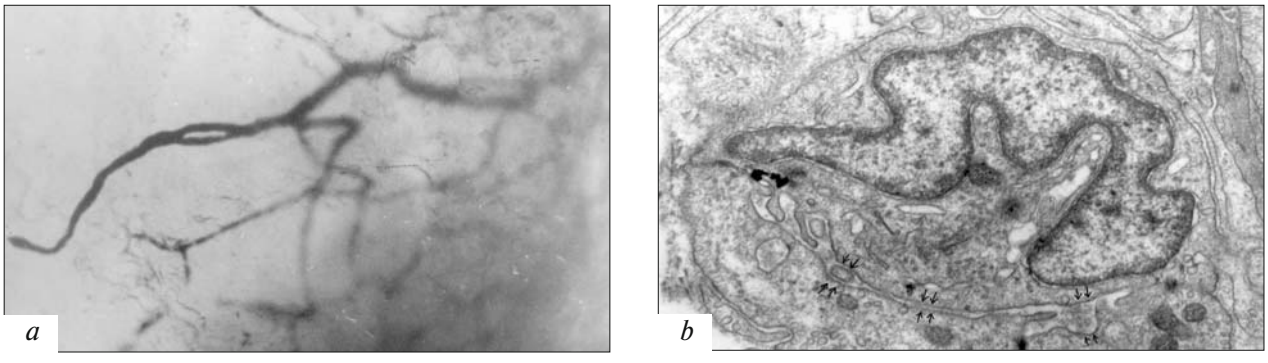

Fig. 1. Structure of NV. *a*) NV on day 8 of growth; horseradish peroxidase in the lumen. Total preparation of rabbit eye cornea, ×350. *b*) cross-section of the apical part of NV. Narrow lumen (shown with an arrow), protrusions on the basal and luminal surfaces of the endothelium. Cell-cell contacts have maculae occludentes, ×12,000.

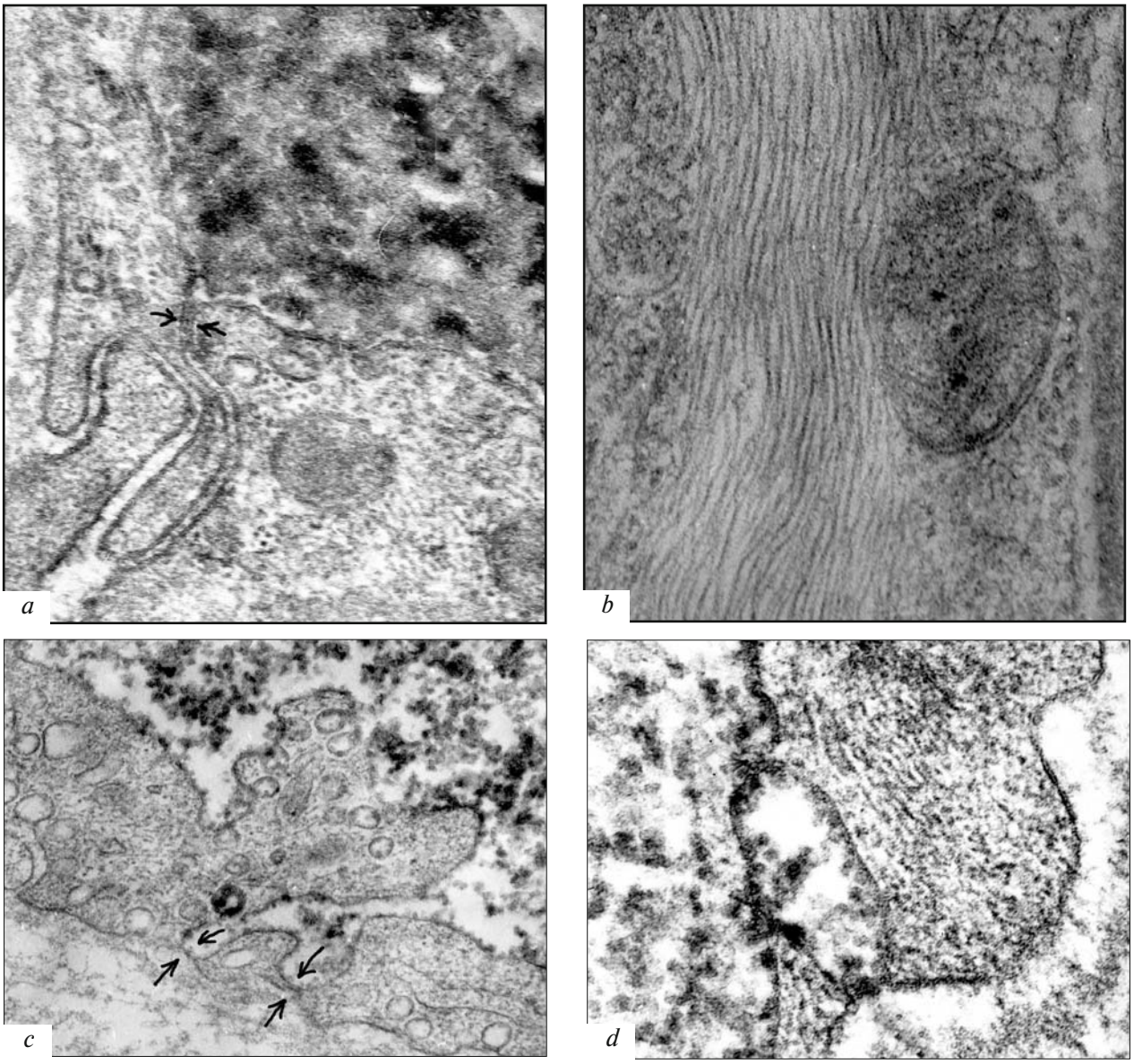

Fig. 2. Ultrastructure of NV endotheliocytes in different growth zones. *a*) cell-cell contact in zone of NV separation from MV. Contains an obliteration zone (arrows), ×90,000. *b*) microfilament bundles in NV cytoplasm in the zone of high mobility of cell membrane, ×60,000. *c*) fenestrae (arrows) into endotheliocytes in the zone of active transport of substances, ×90,000. *d*) TETC (arrows) in an NV endotheliocyte in the zone of active transport of substances is filled with horseradish peroxidase, ×90,000.

the structure of the sublying substrate [3], substances released by the adjacent corneal epithelium [7], and are determined by the cellular microenvironment.

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