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ORIGINAL ARTICLE

Non-sprouting angiogenesis in neurohypophysis after traumatic injury of the cerebral cortex.

Electron-microscopic studies

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Abstract

OBJECTIVES: Angiogenesis comprises two different mechanisms: endothelial sprouting and intussusceptive microvascular growth. The sprouting process is based on endothelial cell migration, proliferation and tube formation. Intussusceptive microvascular growth divides existing vessel lumens by formation and insertion of endothelial columns into the vessel lumen. The morphological features of microvessels of cerebral cortex and neurohypophysis were evaluated in a model of the cerebral traumatic injury.

METHOD: The observations were conducted seven days after induction of cortical trauma. Traumatic injury was induced in the fronto-temporal region of cerebral cortex in general anesthesia with 20mg/kg ketamine hydrochloride.

RESULTS: Seven days after traumatic brain injury in sections from cerebral cortex and neurohypophysis we can observe morphological features of angiogenesis. Endothelium of the cerebral cortex possesses high endotheliocytes tightly connected and enveloped by amorphous basement membrane-like material. Transcapillary pillars tightly connected with neighbouring endothelial cells split the newly formed vessels and branching takes place. In neurohypophysis we can observe all stages of non-sprouting angiogenesis: proliferation endothelial cells on the inside mother vessel, splitting newly formed blood vessels by transcapillary pillars directed into the vessel lumen, maturation of endothelium and network formation.

CONCLUSION: The mechanical injuries directly induced angiogenesis not only in cerebral cortex, but also in neurohypophysis. Our studies show that mechanism of angiogenesis is not the same as observed previously in neurohypophysis after focal cerebral ischemia (Neuroendocrinology Letters 2001; 22: 87–92). This study indicates that mechanism of angiogenesis can depend on kind of induction.

Introduction

Vessel formation can occur by a number of different processes [1]. Early in development, vessel formation takes place in a process referred as vasculogenesis, in which endothelial cells differentiate and proliferate in situ within a previously avascular tissue, and then coalesce to form a primitive tubular network. Angiogenic remodelling refers to the process by which this initial network is modified to form the interconnecting branching patterns characteristic of the mature vasculature. During this time, vessel walls also mature, as endothelial cells integrate tightly with supporting cells such as pericytes, smooth muscle cells and surrounding matrix [2].

A different process, referred to as angiogenic sprouting, involves the sprouting from existing vessels into a previously avascular tissue. In some cases, it seems as mature vessels must first be destabilized to allow for subsequent sprouting [3]. The vessels formed by sprouting are initially immature and must further develop. The recent explosion in identifying and characterizing physiological regulators of blood vessel growth demands reevaluation of therapeutic efforts aimed at regulating blood vessel growth-whether it be promoting vascular ingrowth to replenish ischemic tissue or repairing damaged and leaky vessels during inflammation or other pathological setting [4]. Therapeutic angiogenesis may ameliorate vascular insufficiency and may also provide direct beneficial effects on neural integrity, indicating a new paradigm for the treatment of neural disorders. An improved understanding of the mechanisms underlying the new vessel formation and participation of endothelial cells, basement membrane, extracellular matrix and perivascular cells as well fibrocytes and macrophages are required [5].

In our studies, morphological features underlying the formation of new vessels and their maturation in cerebral cortex and neurohypophysis induced by pathological conditions after traumatic injury of brain cerebral cortex were investigated. The mechanical injuries directly induced angiogenesis not only in cerebral cortex, but also in neurohypophysis. Our studies currently being conducted to determine if this mechanism of angiogenesis is the same as observed in neurohypophysis after focal fotochemically-induced cerebral ischemia [6].

Material and methods

Eight adult, male Wistar rats (200–250g) were used in this experiment. Six animals were utilized on 7 days after traumatic injury. Two animals served as a control group. Material for ultrastructural studies was sampled from cerebral cortex and neurohypophysis 7 days after procedure.

After general anesthesia with 20mg/kg ketamine hydrochloride the skin was incised and removed. Traumatic injury was induced in the fronto-temporal region of the cerebral cortex. Operated region was hemisected with a small scalpel and wound was closed. The wound was sutured and dressed under aseptic conditions. The rats remained at standard laboratory conditions for 7 days.

Seven days after the procedure the brains were perfused via the left heart ventricle with 2% paraformaldehyde, 2.5% glutaraldehyde in 0.1M cacodylate buffer, pH 7.4 at 20°C. Tissue sections were fixed in the same solution for 20 h and postfixed in 1% OsO_4 and 0.8% K_4FeCN_6. Then, material for microscopic studies was processed for transmission electron microscopy and analyzed in JEM-1200EX.

All surgical procedures and treatments were approved by the local animal Ethical Committee.

Results

Material from the control group, not traumatized was ultrastructurally unchanged and we did not observe development of the angiogenic phenothype.

Cerebral cortex

Seven days after traumatic brain injury in sections from cerebral cortex we can observe morphological features of angiogenesis. In Fig. 1 the newly formed capillaries are visible. Endothelial cells of the young vessels are high and connected by junctions. The basement membrane of the newly formed capillaries is seen in the form of incomplete layers of amorphous material. It consists of the fine fibrillar material devoid of collagen fibrils. Probably, the immaturity of basement membrane by this way is manifested. In the perivascular space macrophages are present. The macrophages contain fragments of phagocytized cells and lysosomal vacuoles filled with membrane fragments and lipid globules.

At this time in sections from cerebral cortex we can observe branching of newly formed vessels (Fig.2). The young vessel consists of high abundant endothelial cells. Hypertrophic endothelial cells are characterized by numerous ribosomes, a well developed rough endoplasmic reticulum, a conspicuous lamellae of the Golgi apparatus. That endothelial cells are partly connected by junctions. The narrow lumen of vessel is split by transcapillary pillar tightly connected with neighbouring endothelial cell. The endothelial cells are enveloped by blurred and homogenous basement membrane material

Neurohypophysis

Many times the sections from neurohypophysis 7 days after traumatic brain damage, show capillaries with vigorous endothelial cells proliferating on the inside mother vessel to form a bridge (Fig. 3). In proliferating endothelium we note two nucleuses and centrosomes with tubules of mitotic spindle. On the luminal surface of newly created vessel there are numerous invaginations and long narrow microvilli directed into the lumen of the vessel. The basement membrane is unchanged on the ultrastructural level. The newly formed blood vessel is characterized by a very narrow lumen and hypertrophic endothelium rich

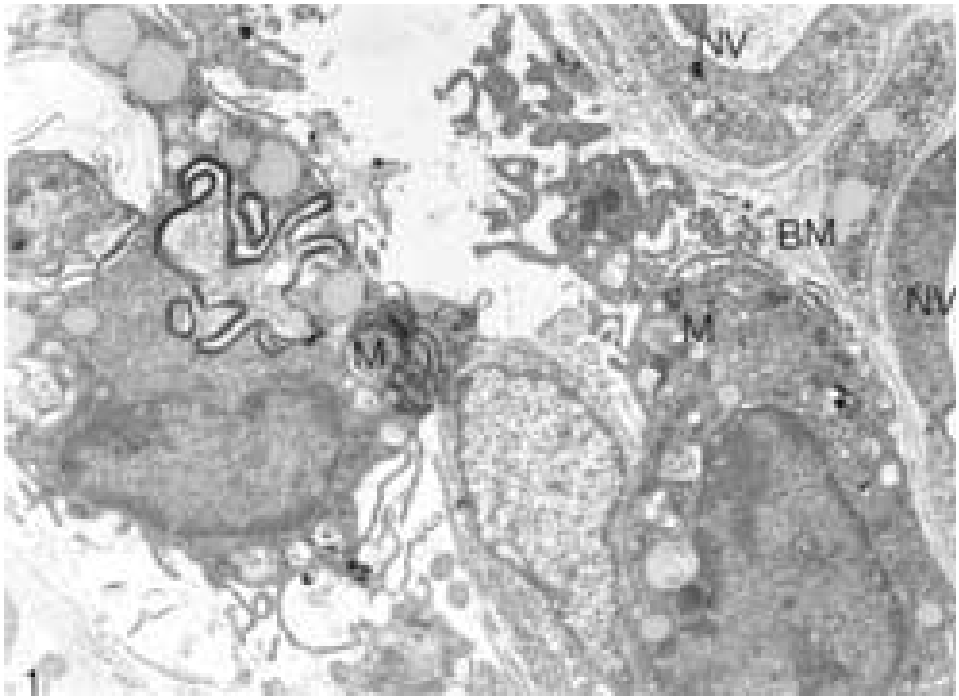

Fig. 1. Cerebral cortex. Newly formed blood vessels (NV) enveloped by basement membranes (BM) seen in the form of incomplete layers of amorphous material. In the perivascular space macrophages (M) with fragments of phagocytized cells are present. x 8000. (Photo 85% of original size. *Publisher's comment*)

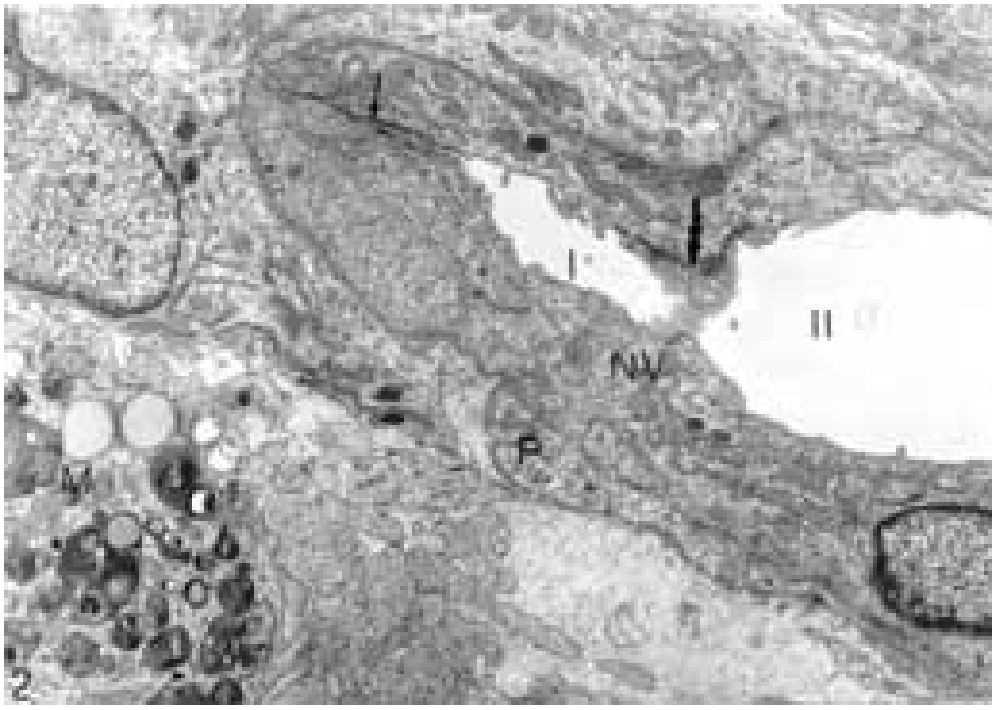

Fig. 2. Cerebral cortex. The newly formed blood vessels NV (I, II) with hypertrophic endothelial cells partly connected by junctions (arrow). The narrow lumen of vessel is split by transcapillary pillar tightly connected with neighbouring endothelial cell (double arrow). Pericyte (P) with numerous fibrills is enveloped by blurred basement membrane. In the perivascular space macrophage (M) is present. x 8000. (Photo 85% of original size. *Publishers comment*)

in organelles, and basement membrane seen as the layers of amorphous material (Fig.4).

Next, transcapillary pillars turned towards the vessel lumen split the newly formed blood vessels (Fig.5, Fig.6). The transcapillary pillars are tightly connected with neighbouring, immature (without fenestrations) endothelium. Endothelial pillars are enveloped by newly synthesized fine fibrils of basement membrane-like material (Fig.6, Fig.7). Formation of two new vessels is accompanied by maturation of the endothelium. On the luminal surface of endothelium we can see fenestrations. The fenestrations are typical for endothelium in neurohypophysis. We can recognize morphological features of splitting in newly formed blood vessels that possess narrow lumen and high endothelium (Fig.8). The transcapillary pillars can be formed in several

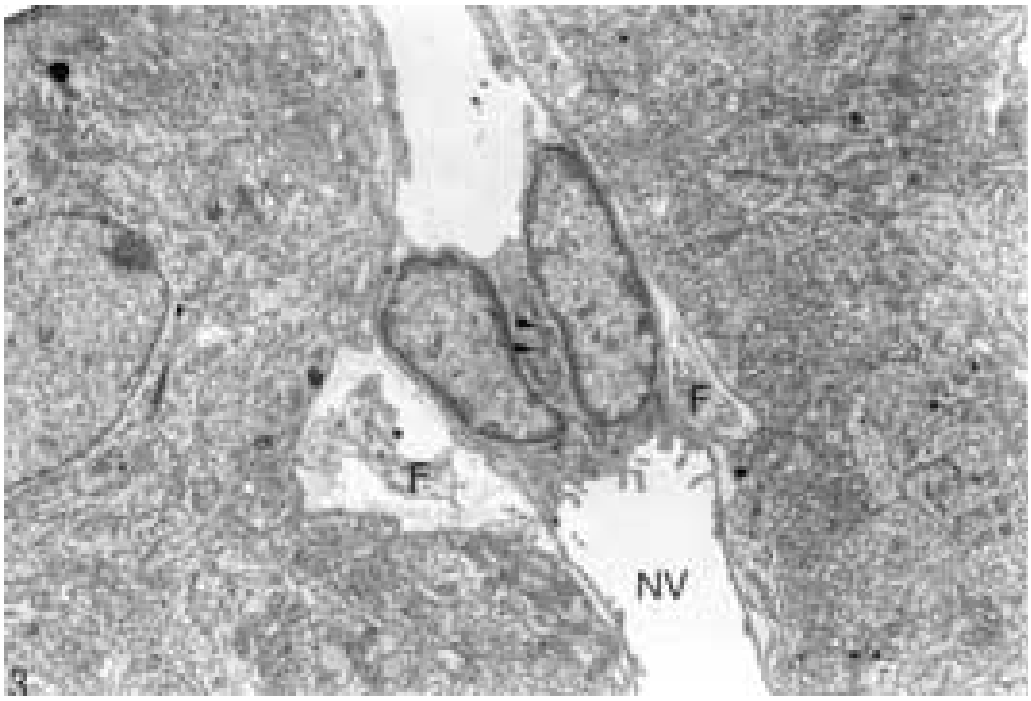

Fig. 3. Neurohypophysis. Longitudinal section of blood vessel shows the bridge created from proliferating endothelial cells. In the cytoplasm of endothelial cells centrosomes are visible (arrowheads). Luminal surface of newly created vessel (NV) possesses numerous invaginations and long narrow microvilli. In the perivascular space fibroblasts are present (F). x 5000. (Photo 85% of original size. *Publisher's comment*)

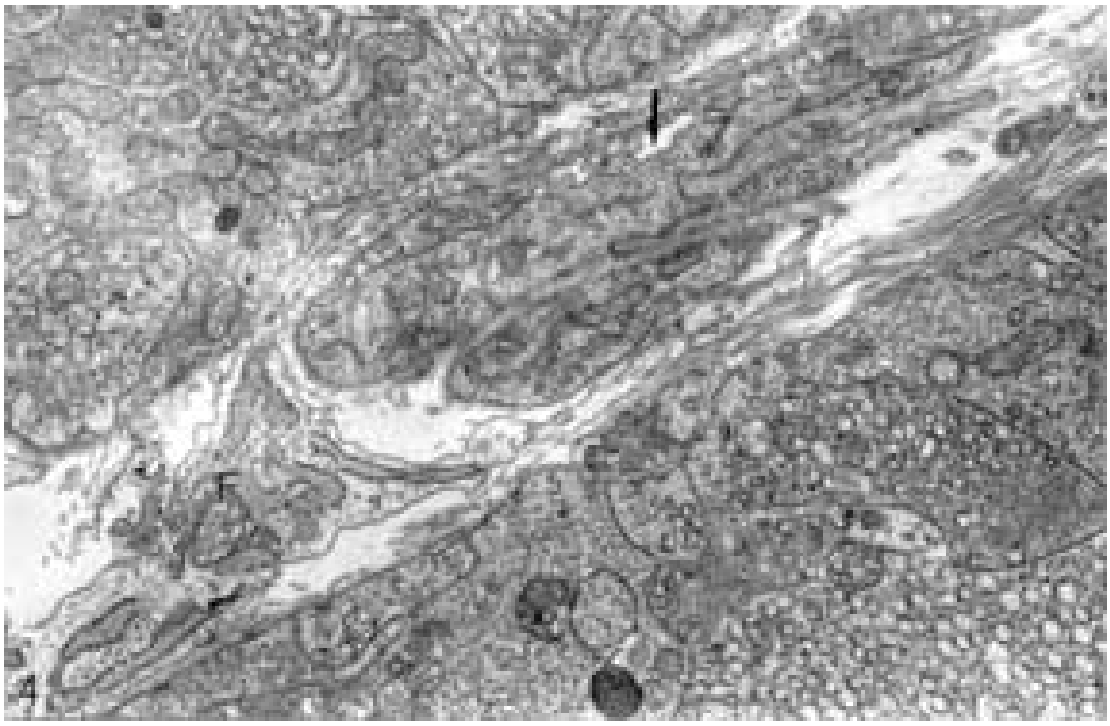

Fig. 4. Neurohypophysis. New capillary vessel with narrow lumen (arrow) and hypertrophic endothelium. Fibroblast in the vicinity of newly formed vessel is seen (F) x 15000. (Photo 85% of original size. *Publisher's comment*)

places of the same blood vessel. In the Fig.8 we present splitting of the new vessel in two places and simultaneously branching the same vessel (network formation). In the same vessel we can see tight connection between endothelial cells. One of them there is in the place where branching or fusion of two vessels into larger one takes place. Ultrastructural features of this vessel do not indicate which process is presented. Perivascular cells (pericytes) are present during pruning and organization of capillaries into a structural network of branching vessels.

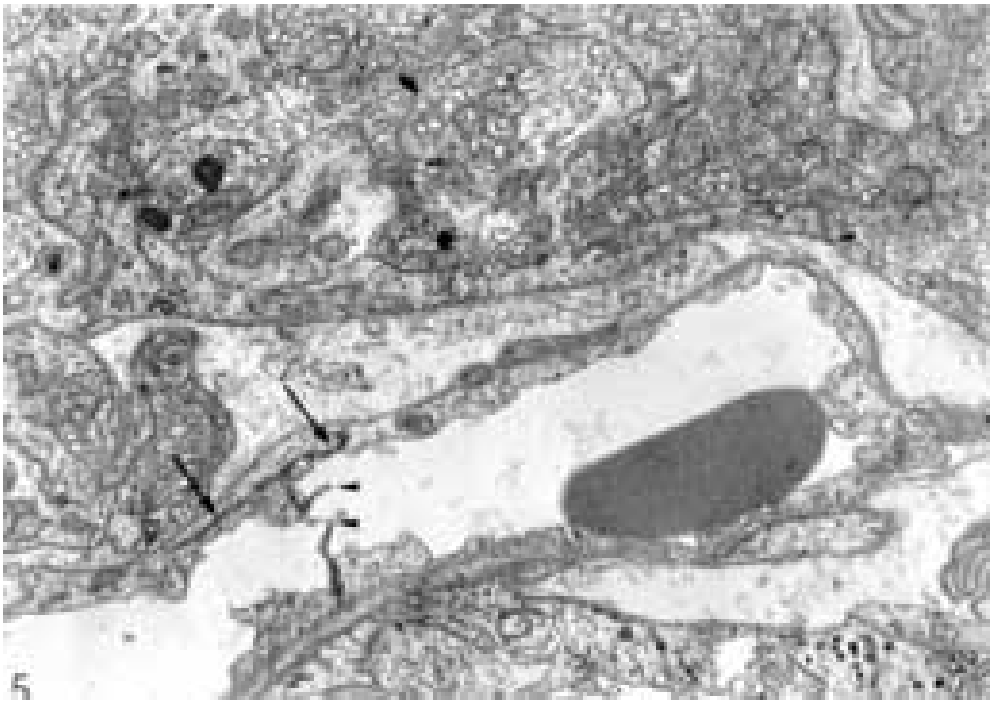

Fig. 5. Neurohypophysis. Transcapillary pillars (arrowheads) directed into the vessel lumen split newly formed blood vessel. Transcapillary pillars are tightly connected with neighbouring, immature endothelial cells (arrows). x 8000. (Photo 85% of original size. *Publisher's comment*)

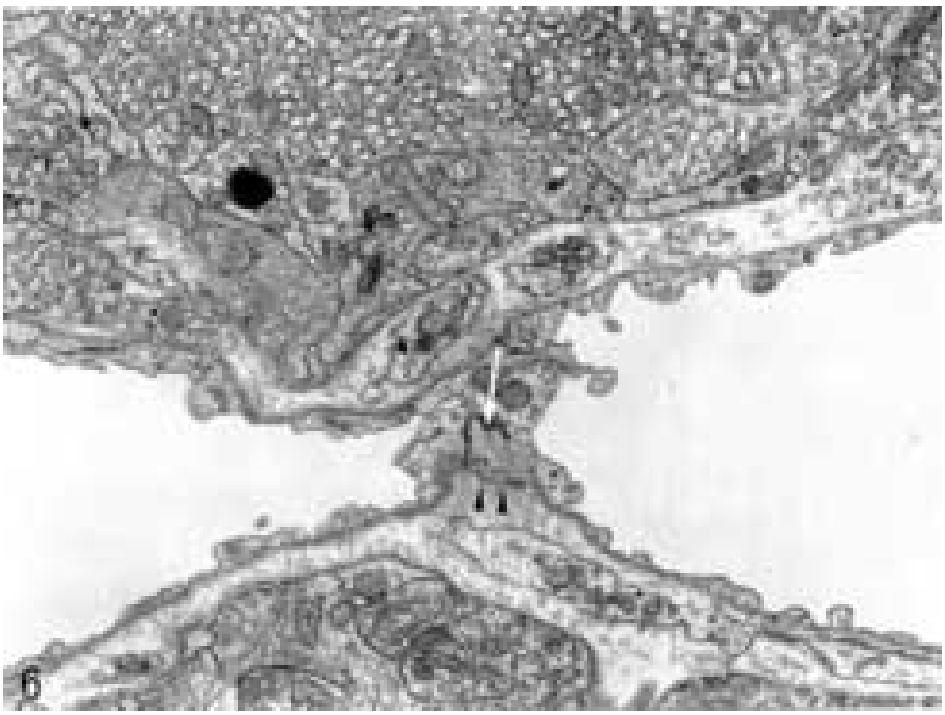

Fig. 6. Neurohypophysis. Transcapillary pillars are enveloped by newly synthetized fine fibrillar basement membrane-like material (arrowheads). The tight connection between transcapillary pillar and neighbour endothelial cell is seen (arrow). x 10000. (Photo 85% of original size. *Publishers comment*)

Discussion

When the mechanical injury is made several interrelated processes takes place. Angiogenesis, reactive gliosis, migration of inflammatory cells in the central nervous system are triggered by brain trauma [7,8]. Numerous studies have demonstrated the critical role of angiogenesis and increased blood flow for successful wound repair [9]. Vascular disruption from tissue injury due to trauma or surgery leads to a hypoxic zone, where the oxygen tension approaches zero in the early stages of the healing wound [10]. In this dynamic process, angiogenesis is vital for the delivery of oxygen, nutrients and growth factors necessary to initiate the processes of wound healing. Adair *et al*. [11] and D'Amore and Shima [12] found that both an inad-

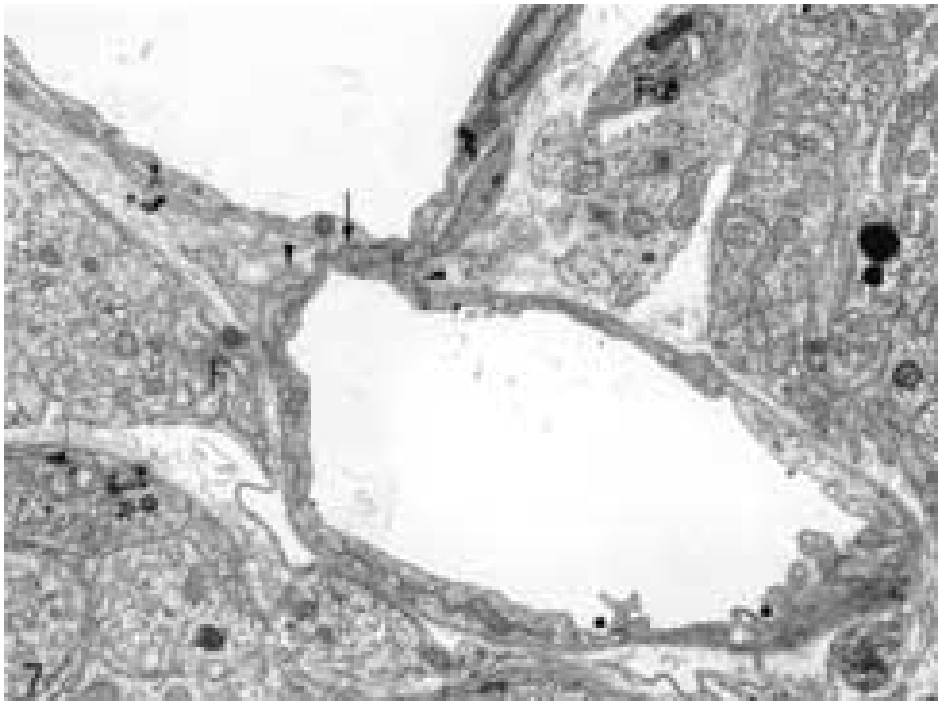

Fig. 7. Neurohypophysis. Two capillaries interconnected by tight junction (arrow). Newly synthetized fine fibrillar basement membrane-like material (arrowheads) envelope transcapillary pillars. On the luminal surface of endothelial cells fenestrations are formed (stars). In the perivascular space fibroblasts are present (F). x 8000. (Photo 85% of original size. *Publisher's comment*)

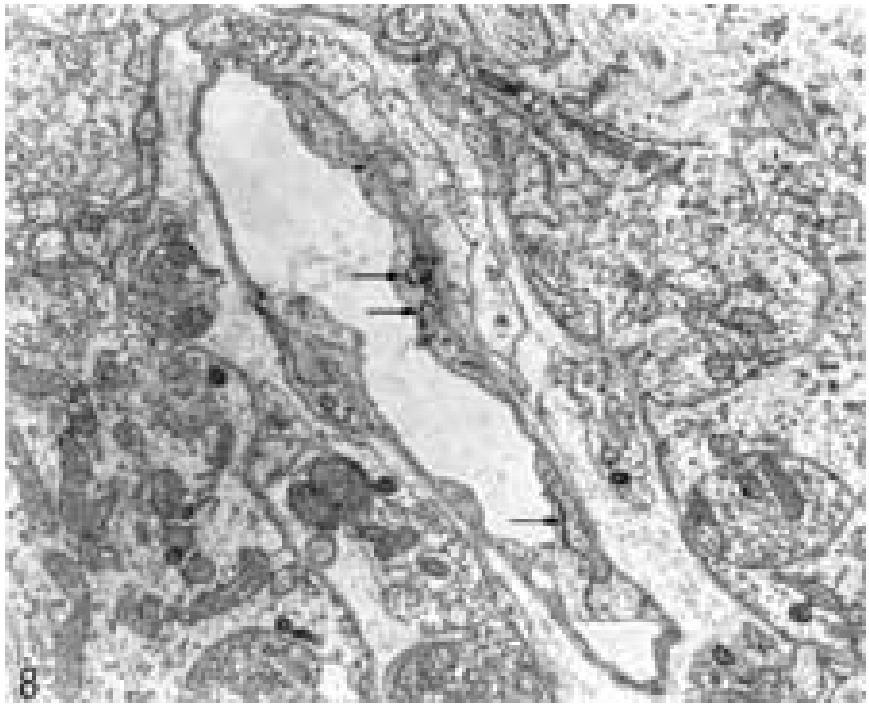

Fig. 8. Neurohypophysis. Capillary vessel with transcapillary pillars formed in several places. The tight junctions between endothelial cells and transcapillary pillars are seen (arrows). x 12000. (Photo 85% of original size. *Publishers comment*)

equate vascular supply and the resulting reduction of oxygen tension at the wound site lead to compensatory angiogenesis to satisfy the metabolic demands of the tissue. Induction of angiogenesis is a well-known phenomenon after cerebral hypoxia/ischemia [6, 12,13,14].

The method described in this report produces a traumatic focal lesion in the rat's cerebral cortex. The mechanical injuries directly induced angiogenesis not only in cerebral cortex, but also in neurohypophysis. Our studies currently being conducted to determine if this mechanism of angiogenesis is the same as observed in neurohypophysis after focal cerebral ischemia [6].

Blood vessels are generated by both sprouting and non-sprouting angiogenesis and progressively pruned and remodeled into a functional circulatory system. Non-sprouting angiogenesis is a process of splitting pre-existing vessels by transcapillary bridges or posts of extracellular matrix [15].

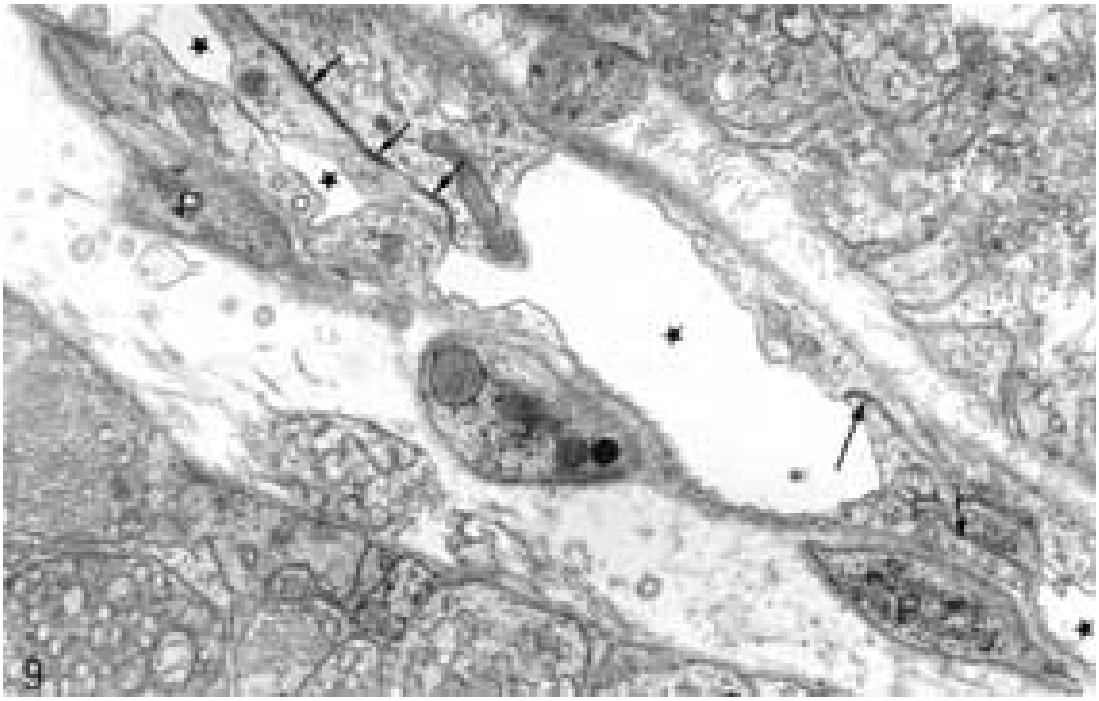

Fig. 9. Neurohypophysis. We show splitting of the new vessel in two places and simultaneously branching the same vessel (network formation). We can see tight junctions between endothelium and transcapillary pillars (arrows) in newly formed blood vessels (stars). One of them there is in the place where branching takes place. In the wall of the vessel pericytes (P) are present. x 12000. (Photo 85% of original size. *Publisher's comment*)

In our studies we observed non-sprouting angiogenesis which occurred by proliferation of endothelial cells inside a vessel (bridging), producing a wide lumen that can be split by transcapillary pillars tightly connected with neighbouring endothelial cells. Maturation of the endothelial network involves remodeling and pruning capillary-like vessels with uniform size, and irregular organization into a structured network of branching vessels. The morphological features of maturation are presented here. During maturation of capillary network the vessel walls also mature because endothelial cells integrate tightly with perivascular cells (for example pericyte) and surrounding extracellular matrix. Dynamic remodeling of the extracellular matrix, involving both matrix degradation and deposition of new matrix components, extracellular environment takes place to facilitate the different phases of angiogenesis.

We observed the correlation between capillary proliferation in neurohypophysis and traumatic injury in cerebral cortex. Neurohypophysial angiogenesis is probably induced by release of the some angiogenic factors from the traumatic focus in the cerebral cortex. Nitric oxide and cytokines have been implicated as regulators of the central nervous system injury response [16,17]. These factors enter the area of injury from blood or are released by damaged or activated cells [18]. Nitric oxide may act as a crucial signal to promote endothelial cell differentiation into vascular tube [19]. Thinking over the reason of angiogenic activity after traumatic cortex injury we have also taken another possibility into consideration. From the many cells and cell products as inducers or modulators of angiogenesis, macrophages are have emerged as a major protagonists [20,21]. Macrophages are present in all tissues and in case of inflammation are recruited from the blood-borne monocytes. The different subpopulations of macrophages were observed in the region of traumatic brain damage [22]. Now we show macrophages in the vicinity of newly formed vessels in cerebral cortex.

Angiogenic activity of macrophages is associated with their secretory activity. In cerebrovascular ischemia, brain macrophages that express VEGFR-1 are attracted to the site of injury by secreted VEGF in order to stimulate angiogenesis [23,24]. Attracted or resident macrophages can in turn release dilators of blood vessels and activators of endothelial adhesion molecules. Platelets sticking to the vessel wall can for a short time release TGF-β, PDGF, IGF-I, EGF or PDEGF. These factors induce angiogenic effects and act as chemoattractants for monocytes [17]. Macrophages are able to promote all phases of angiogenic process by virtue to their secretory products [25]. Thus we suppose that angiogenic activity in cerebral cortex may be induced by factors generated by macrophages. We do not know if factors generated in macrophages of the injured cerebral cortex can be inducers of angiogenic activity in neurohypophysis.

The increased blood flow that occurs for successful wound repair [9] may be essential for angiogenic activity in neurohypophysis. Increased delivery of vasoactive substances transported by increased blood flow in cerebral cortex could promote vessel proliferation in neurohypophysis. Angiogenesis as adaptative response of microvasculature in neurohypophysis to shear stress induced by increased blood flow in cerebral cortex may be a real explanation of this phenomenon. Wall shear stress increased blood flow for successful wound repair [9] is archeological force that shears the luminal sur-

face of the blood vessel when blood flows over the endothelial wall [26]. Shear stress plays a significant role in vascular remodeling inducing endothelial cell membrane deformation and cytoskeletal rearrangement which transfers stress to different regions of the endothelial cell where mechanotransduction can occur. The transmitted stress leads to morphological and functional response [27]. These include rapid second messenger activation, NO production, cytokine growth factor production, and expression of multiple genes. These genes could provide for a wide range of cellular activities, including mitogenic activity in neighbouring cells, monocyte recruitment, and trafficking of inflammatory cells [28].

Angiogenesis also involves differential recruitment of associated supporting cells, such as pericytes. Pericytes are thought to provide structural support for the capillary wall, acting as a scaffold along which endothelial cells migrate during sprouting [29]. Endothelial cells secrete elements of the extracellular matrix to stimulate pericyte proliferation [30]. Pericytes themselves synthesize many components of the extracellular matrix that change during maturation. The many similarities between the two cell types during angiogenesis suggest that both may participate in formation of capillary sprouts [31]. During wound healing pericytes from preexisting microvessels, newly derived pericytes, come into contact with endothelial cells that are forming new vessels and exert an inhibitory effect on endothelial cell proliferation [32].

In the vicinity of newly formed blood vessels in neurohypophysis there are fibroblasts. Fibroblasts invade the wound in the first few days of healing and have multiple functions important to wound repair, such as collagen synthesis, extracellular matrix reorganization [33,34].

Angiogenesis is a multistep process for which changes in the extracellular matrix have to be combined with the coordinated supply of appropriate angiogenic factors. In these processes many classes of agents may promote necessary degradation of extracellular matrix [35,36]. Photochemically-induced angiogenesis [6] in neurohypophysis was accompanied by visible changes in basement membrane and extracellular matrix. During separation of endothelial cell from each other in a mother vessel in sprouting angiogenesis the basement membrane was blurred and extracellular matrix surrounding it was homogenous and consisted of the fine fibrillar material devoid collagen fibrils. Migration of endothelial cells takes place in homogenous, altered extracellular matrix. The traumatic-induced non-sprouting angiogenesis in neurohypophysis proceeds without well-marked morphological changes in extracellular matrix. We can observe only fine fibrillar basement membrane-like material penetrating among interconnected endothelial cells and pillars. Newly formed blood vessels are enveloped by immature basement membrane. Incomplete layers of amorphous material round blood vessels manifest immaturation of the basement membrane. An amorphism of basement membrane results probably from intensive synthesis of basement membrane material in order to form a scaffold newly created vessels [5].

Maturation of the endothelial network involves remodeling and pruning capillary vessels with uniform size, and irregular organization into a structural network of branching vessels. The newly formed capillaries in our studies show significant morphological features of remodeling and branching.

Remodeling appears to be initiated by circulating factors and other signals such as shear stress. Shear stress strongly affects endothelial cells, inducing modification of cell-cell as well as cell-extracellular matrix junctions and upregulating growth factors such as PDGF [37,38]. Increased shear stress increases PDGF secretion from endothelial cells, attracting neighbouring perivascular cells expressing the PDGF receptor and leading to their attachment to endothelium. This in turn activates TGF-β, which may induce alterations in the extracellular matrix that stabilize the phenotype of endothelial cell, as well as inhibits their proliferation [39].

We should pay attention to coexistence of different stages of maturation in the same vessel. We think, that the maturation process follows a gradient from the proximal parts to the terminal parts of the vascular system, like it takes place during cerebral vascularization [40].

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