The immature endothelial cell in new vessel formation following surgical injury in rat brain

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Abstract

OBJECTIVES: We investigated neovascularization in the cerebral cortex of the adult rat after surgical brain injury by ultrastructural, immunocytochemical and immunochemical means. Previously we described endothelial-like cell that participates in new vessel formation on plasma proteins that served as a provisional matrix in the region immediately adjacent to the traumatic injury. In the present study we describe new vessel formation in the multistep process with the alterations in endothelial-like cell immunophenotype. **METHOD:** The observations were conducted from 2 to7 days after induction of cortical trauma. Traumatic injury was induced in the fronto-temporal region of cerebral cortex. **RESULTS:** We show that endothelial-like cell could not successfully terminate its development without the presence of pericyte and astrocyte. New formed blood vessels were accompanied by fibroblast and lipofibroblast cells differentiated probably from common progenitor. **MAIN FINDINGS:** Our result suggests that endothelial-like cell is committed endothelial cell between progenitor endothelial cell and terminally differentiated endothelium stage. Therefore, we propose that out of endothelial stem cells present in blood at different stages of morphogenetic differentiation and specifically arrested in "check points" of development trauma mobilize the group of the most differentiated progenitors. These may contribute to new vessel formation. **CONCLUSION:** Our model should be useful for the characterization of endothelial commitment and endothelial cell differentiation after brain injury.

ORIGINAL ARTICLE

Introduction

Vasculogenesis and angiogenesis are two different processes defining the formation of new blood vessels [21]. As angioblasts and hematopoietic precursors in the newly formed tubes share several surface markers, it has been suggested that they derive from a common precursor, the hemangioblast. Once the primary vascular plexus is formed, new capillaries can form by sprouting or by splitting from the existing vessels in a process called angiogenesis [22]. Vessels mature according to their specific functions: pericyte and smooth muscle cells are recruited and the vessel wall is modified by deposition of extracellular matrix. Whereas vasculogenesis is restricted to embryonic development, angiogenesis continues to operate throughout life when new vascularization is required. The notion that vasculogenesis is observed only during embryogenesis has been

changed since ample evidence has been shown that bone-marrow derived endothelial cells exist on the site of new blood vessels, indicating the participation of vasculogenesis in the postnatal neovascularization [1, 2, 26 ,19]. Thus, endothelial cells can differentiate from angioblasts in the embryo and from endothelial progenitor cells, mesangioblasts, and multipotent adult progenitor cells in adult [18, 20]. This in turn suggests that, as during development, new vessel formation in the adult may depend at least in part on a process of vasculogenesis.

Many studies have demonstrated also the presence of mature circulating endothelial cells in the peripheral circulation [26, 28]. Authors suggested that mature endothelial cells might appear in the circulation randomly by shedding from the vascular wall. Trauma induced by surgery may also result in the introduction of endothelial cells to the peripheral circulation because patients with sickle cell crisis have been shown to have increased numbers of activated circulating endothelial cells [28].

Meanwhile, endothelial precursor cells have properties similar to those of embryonic angioblast, which can be defined as migratory endothelial cells with the capacity to circulate, proliferate, and differentiate into mature endothelial cells, but which have not yet acquired characteristic markers and have not yet formed a lumen [25, 5]. Although there is an evidence for the existence of angioblasts during embryonic development, the existence of angioblast-like endothelial precursor cells in adult circulation has been hampered by the absence of specific phenotypic markers and functional assays to define this cell population.

Both endothelial precursor cells and mature endothelial cells may express similar endothelial-specific markers, including vascular endothelial growth factor receptor-2 (VEGFR-2/Flk-1).

Vascular endothelial growth factor (VEGF) is known as a specific endothelial mitogen associated with angiogenesis and expressed in the brain [8, 17]. Hypoxic induction of angiogenesis is thought to result largely from increased VEGF expression [27], which is associated with neovascularization in ischemic tissues. VEGF-mediated enhancement of capillary permeability leads to procoagulant activity, through the release of von Willebrand factor, and monocyte chemotaxis across the endothelium [8]. These effects suggest a role of VEGF in angiogenesis associated with physiological and pathological processes.

Recent studies have shown that Flk-1 is critical for the normal development of both hematopoietic and endothelial lineage [7].

Most studies on endothelial progenitor differentiation concentrated on the expression of endothelial markers. In our investigation the ultrastructural features and change of Flk-1 and VEGF expression in the course of differentiation of immature endothelial cells participating in new vessel formation was evaluated.

MATERIAL AND METHODS

Surgical procedure

Male adult Wistar rats (200–250g) were anaesthetized with 20mg/kg-ketamine hydrochloride. Traumatic brain injury was induced in the fronto-temporal region of the cerebral cortex after scull exposure as described earlier [9]. In sham operated controls the same procedure was applied except that no hemisection was done. After recovery from anesthesia, the rats remained under standard laboratory conditions for 2, 4 and 7 days. Each experimental group consisted of 6 animals (in ultrastructural, immunocytochemical and immunochemical studies): 6 sham operated and 6 unoperated rats that were used as controls.

Ultrastructural and immunocytochemical methods

Material for ultrastructural and post embedding immunocytochemical studies was sampled from the cerebral cortex region, immediately adjacent to the operated region. Two, four and seven days after the surgery animals were perfused via the left heart ventricle and brains were processed for transmission electron microscopy and analyzed in a JEM-1200EX as was described earlier [9].

For immunocytochemical studies the following antibodies were used at dilutions indicated: the goat polyclonal VEGF (A-20-G) (1:50) Santa Cruz Biotechnology, USA, Nº sc-152-G; the mouse monoclonal IgG_1 Flk-1 (A-3) (1:100) Santa Cruz Biotechnology, USA, Nº sc-6251; mouse antibody against $\alpha_v \beta_3$ integrin complex (1:50) (sc-7312), Santa Cruz Biotechnology, USA); goat anti mouse (H+L) conjugated with colloidal gold particles of 12 nm in diameter (Jackson ImmunoResearch, USA) (1:50); rabbit anti goat IgG immunogold conjugate 20 nm (1:100) (British Biocell International, Cardiff, UK).

Immunochemistry

VEGF and Flk-1 immunoreactivity was determined by Western blotting. Briefly, aliquots of brain tissue homogenate containing approximately 40 μg protein were electrophoresed on linear SDS-PAGE gels (5% or 10% polyacrylamide) for Flk-1 and VEGF detection [16]. Following electrophoresis, proteins were transferred to nitrocellulose membrane and treated with anti VEGF and Flk-1 antibodies raised against synthetic peptides followed by incubation with an appropriate secondary antibody. ECL Western Blotting Detection Reagent Kit (Amersham, UK) and Alkaline Phosphatase Conjugate Substrate Kit (Bio-Rad, USA) were used to visualise primary antibody complexes with VEGF and Flk-1. Immunoreactivity quantitation was performed by densitometric scanning with a model Ultra-Scan XL laser densitometer (Pharmacia LKB Biotechnology, Uppsala, Sweden).

Antibody raised against peptides corresponding to the $\alpha_v \beta_3$ integrin complex was not investigate in this study.

The local animal Ethical Committee (No. 241/2003) approved all surgical procedures and treatment.

RESULTS

Ultrastructural and immunocytochemical studies

Control animals and sham operated animals did not show any morphological signs of pathology in blood vessels.

Rats subjected to surgical brain injury demonstrated extensive disruption of brain parenchyma, characterized by incomplete endothelial lining and extravasations of blood elements to the surrounding tissue. A strong angiogenic response was observed 2 days after surgical injury when new capillary vessels appeared. They were characterized by cells revealing endothelial-like features but possessing fibrils in the cytoplasm, atypical for endothelial cells. The vessels enveloped only by basement membrane-like material. Pericytes or astrocytes did not accompany them. Some of endothelial-like cells looked vacuolised (Fig.1).

Screening the material we found that new capillaries formed by endothelial-like cells were frequently at different stages characterized by altered ultrastructural features and different for VEGF and Flk-1-like immunoreactivity.

Young capillary vessels with high hypertrophic endothelium and narrow vessel lumen possessed high of Flk-1–like and VEGF-like immunoreactivity in endothelial-like cells. Marked immunoreactivity of Flk-1 and VEGF in endothelial-like cells was observed at day 2 as well as day 4 in capillaries characterized by the above-presented features (Fig.2, Fig.3).

A general decrease in the Flk-1 and VEGF immunoreactivity of endothelial-like cells was detected repeatedly at day 2 and 4 in those capillaries, which contained endothelial-like, cells migrating from mother vessel and trespassing to perivascular space. At that stage Flk-1-like immunoreactivity was dispersed in the cytoplasm of endothelial-like cell and less intense than in the earlier stage (Fig.4, Fig.5). Single gold particles of VEGF-like immunoreactivity were present too. The endothelial-like cell that turn away from primary capillary vessel were characterized by an unaltered ultrastructural features and weak Flk-1-like immunoreactivity (Fig.6).

To discern between immature and proliferative phenotype of endothelium we used $\alpha_v \beta_3$ integrin. The high $\alpha_v \beta_3$ integrin-like immunoreactivity was present only on proliferating blood vessels formed by endothelial-like cells (Fig. 7). No marked $\alpha_v \beta_3$ integrin-like immunoreactivity was present on quiescent blood vessels.

Four and seven days after surgical injury in the neighbourhood of blood vessels that were formed immediately adjacent to the lesion, fibroblasts (Fig.2) and lipofibroblasts (Fig. 8) were present.

Seven days after brain injury we can observe fibroblasts and lipofibroblasts near the morphologically altered blood vessels (Fig. 9). They were characterized by hyperplasia of endothelial-like cells that were still in the process of proliferation (Fig.10).

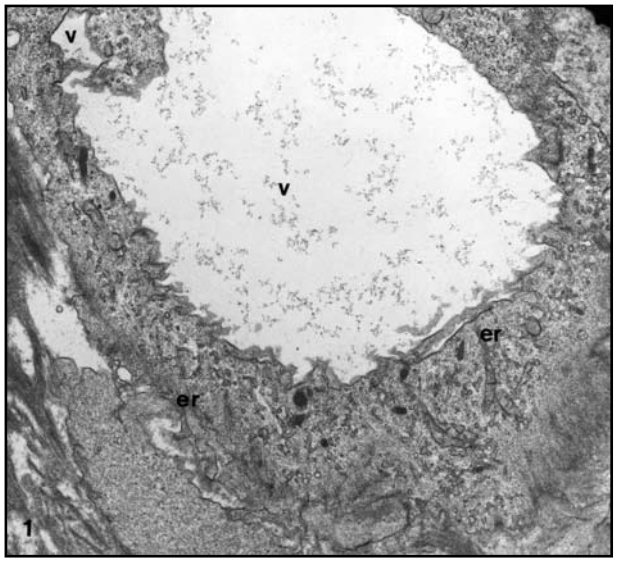

Figure 1. Two days after surgical brain injury. The endothelial-like cell rich in endoplasmic reticulum (er) with features of vacuolisation (v). The basement membrane-like material surrounds the endothelial-like cell. x 10000.
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The hypothetical way of generation of the mature endothelial cell, fibroblast and lipofibroblast from angioblast is showed in Fig. 11.

Immunochemical studies

Tissue expression of VEGF and Flk-1 was examined in frontal brain cortex (the region surrounding surgical injury) by Western blotting. As illustrated in Fig. 12 the immunoreaction with antibodies to VEGF was observed in all experimental groups, however a peak of immunoreactivity was seen four days after injury, where the immunoreactivity was enhanced two fold ($p<0.01$) compared with two days after injury and three fold ($p<0.05$) compared with sham control. With Flk-1 antibody (Fig.13) significant increase of immunoreaction was observed two days after injury and dropped significantly four days after injury.

DISCUSSION

Previously, we studied by electron microscopic ultrastructural and immunocytochemical methods neovascularization of the adult rat cerebral cortex after surgical brain injury. The border zone of the injured region is a viable parenchyma with characteristic in this process edema, hypercoagulability, leukocyte invasion and angiogenesis [9]. The blood vessels showed an incomplete endothelial lining. In the brain parenchyma, we observed fibrin diluted from disrupted blood vessels and complexes of cells characterized by endothelial-like features with fibrils in the cytoplasm (untypical for endothelial cells) that aggregate in the plasma. These endothelial-like cells

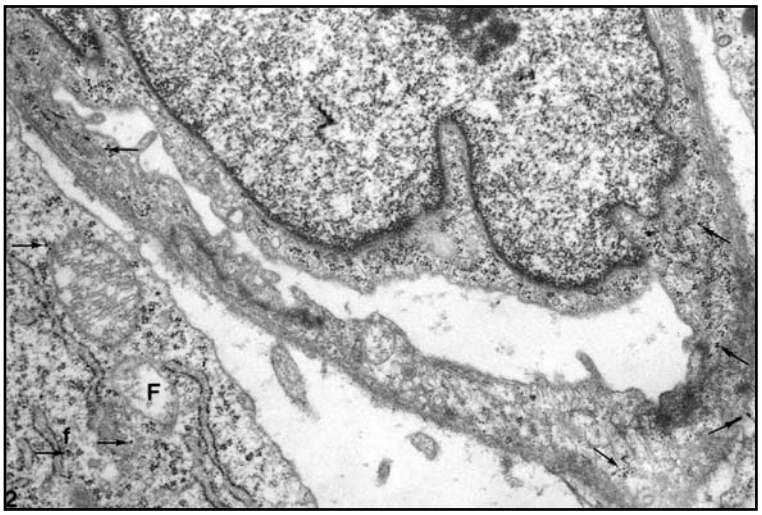

Figure 2. Four days after surgical injury. Capillary vessel with high hypertrophic endothelium containing fibrils in the cytoplasm is seen. In the vicinity of a fibroblast (F) with fine fibrillar material (f) in the cytoplasm is found. Flk-1-like immunoreactivity is seen (arrows). x 15000.

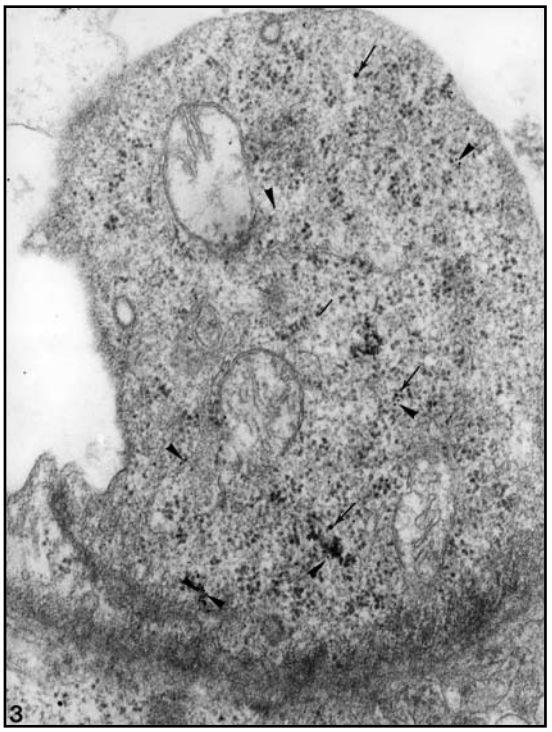

Figure 3. The high magnification of the endothelial-like cell two days after surgical injury. In the cytoplasm the high Flk-1-like (arrow heads) and VEGF-like (arrows) immunoreactivity is present. x 40000.

Figure 4. The young blood vessel two days after surgical brain injury. The endothelial-like cell (e) seem to leave the blood vessel. x15000.

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participated in the process of new vessel formation. Plasma proteins leaked from these nascent vessels served as a provisional matrix.

Our present observations are connected with new vessels, which are formed immediately adjacent to the operated region without plasma proteins that would serve as a matrix. Participation of characteristic endothelial-like cells in this process was investigated by measurement of the expression of VEGF and its Flk-1 receptor, which are the earliest marker of progenitor endothelial cell. Flk-1 characterizes the hemangioblast, a bipotent stem cell. Whether hemangioblasts persist in adult life is not known, and only hemopoietic stem cells and endothelial progenitors have been identified in adult life. Like hemangioblasts, endothelial progenitors express Flk-1 [19]. Current concepts about vasculogenesis and regeneration hold that in circulating blood co-exist different types of cells, which participate in and regulate formation of new vessels. These are: hemangioblasts, angioblasts, multipotent adult progenitor cell and circulating mature endothelial cell. More recently several observations have provided direct evidence for the existence of endothelial progenitor cell. These cells may contribute to neoangiogenesis in adults, which is consistent with vasculogenesis. In animal models of brain ischemia, endothelial cell progenitors were found to incorporate into sites of active neovascularization [1, 31, 23, 13, 9].

Disruptions of brain parenchyma allow endothelial progenitors to penetrate through vessel wall. Here we show that a class of endothelial-like cells contributes to new vessel formation in the region immediately adjacent to the trauma.

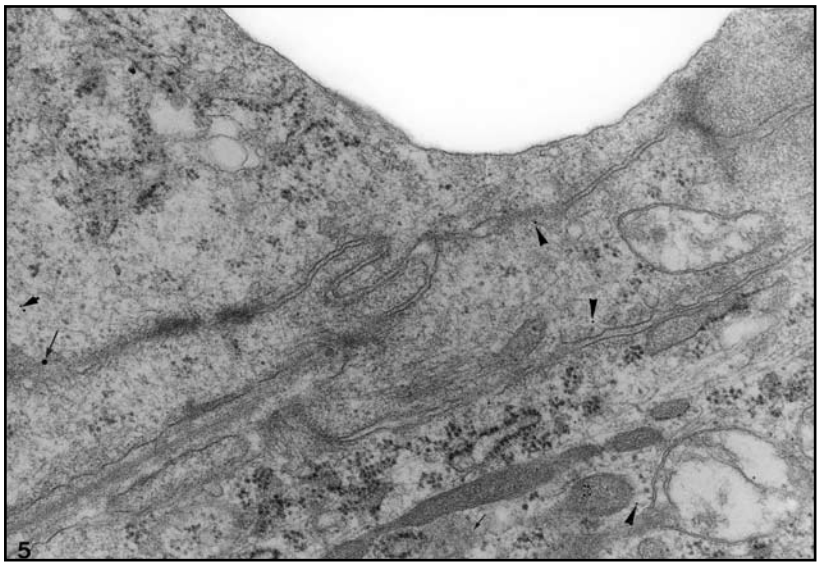

Figure 5. The high magnification of the above-described blood vessel with Flk-1-like immunoreactivity (arrow heads) dispersed in the cytoplasm of endothelial-like cell and single gold particles of VEGF-like immunoreactivity (arrows). x 45000.

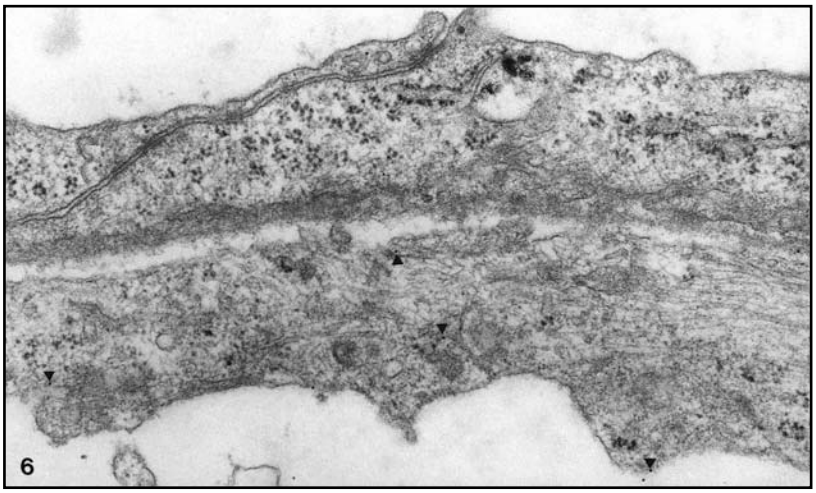

Figure 6. The part of the endothelial-like cell that turn away from primary blood vessel four days after surgical brain injury. The weak Flk-1-like immunoreactivity is seen (arrow heads). x 40000.

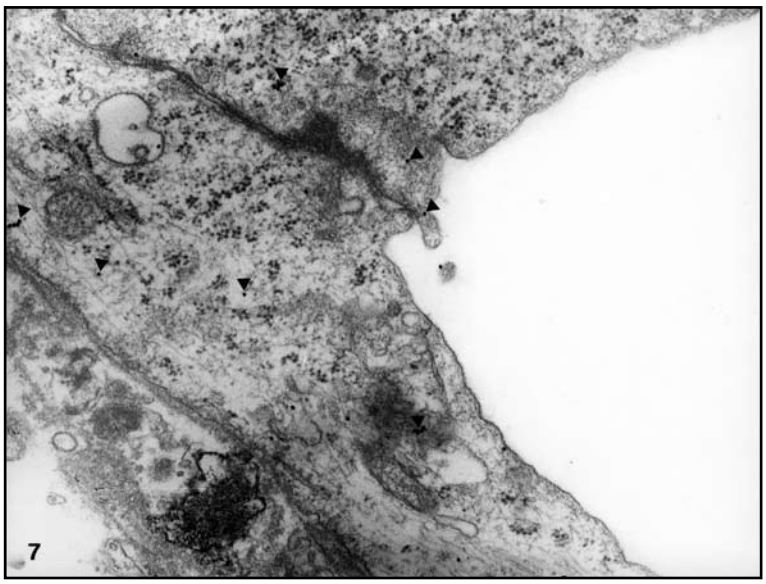

Figure 7. Two endothelial-like cells with features of proliferation and high $\alpha_v \beta_3$ integrin-like immunoreactivity (arrow heads) four days after surgical brain injury. x 40000.

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We investigate changes of endothelial-like cells in time up to injury to the end of the process of new capillaries formation. We noted that neovascularization is a continuous phenomenon, that leads to establishment of the mature endothelial phenotype. The earliest stadium was primary capillary vessel formed from endothelial-like cell with high immonoreactivity of VEGF and Flk-1. In the embryonic development vasculogenesis begins when VEGF binds to the first of its two receptors-Flk-1 [22]. This signal causes the differentiation of mesodermal cells into endothelial cell as well as their proliferation. This is some very important stadium to the next steps of live endothelial-like cell. Developmentally Flk-1+ endothelial-like cells proliferate and blood vessel formed becomes the "mother vessel" in the process of sprouting angiogenesis [30].

Our morphological and immunocytochemical studies indicated different stages of new vessel formation with different expression of Flk-1 and VEGF. The high immunochemical reactivity seen on Western blots confirmed our morphological and immunocytochemical analyses. The majority of cells with hematopoietic potential were shown to be Flk-1 $^{+}$ during the early stages of differentiation and Flk-1 $^{-}$ at later stages [15].

Thus, we need to discuss what kind of cell is endothelial-like cell. Formation of mature endothelium from circulating endothelial progenitor cells requires at least 2 weeks, in contrast to the growth pattern of circulating

Figure 8. The young blood vessel with features of hyperplasia enveloped by basement membrane-like material connected with lipofibroblast (L) seven days after experiment. In that cell the fine fibrillar material is seen. x 15000.

Figure 9. Seven days after surgical brain injury. The blood vessel formed by endothelial-like cells with features of hyperplasia and vacuolisation (v). In the vicinity we can see the fibroblasts (F) with fine fibrillar material in the cytoplasm. x 8000.

Figure 10. The part of the blood vessel with features of hyperplasia and mitotic apparatus (m) in the cytoplasm. x 15000.

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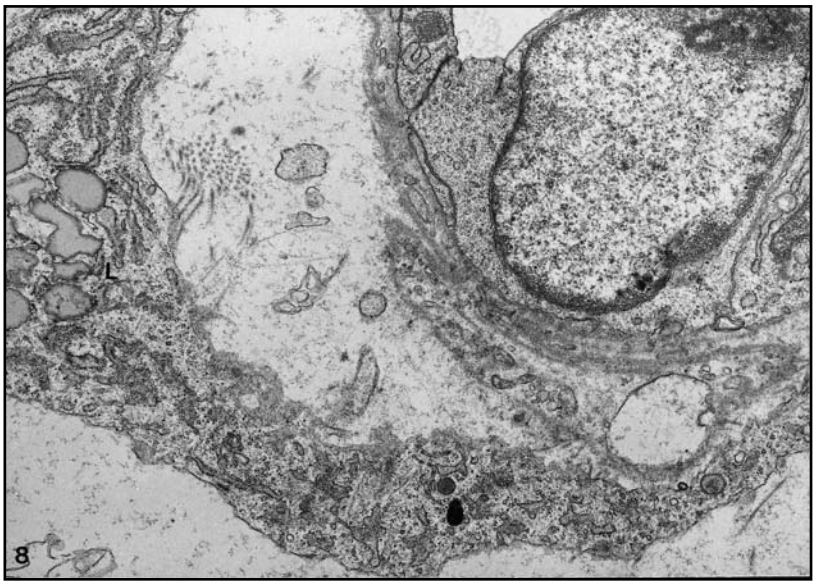

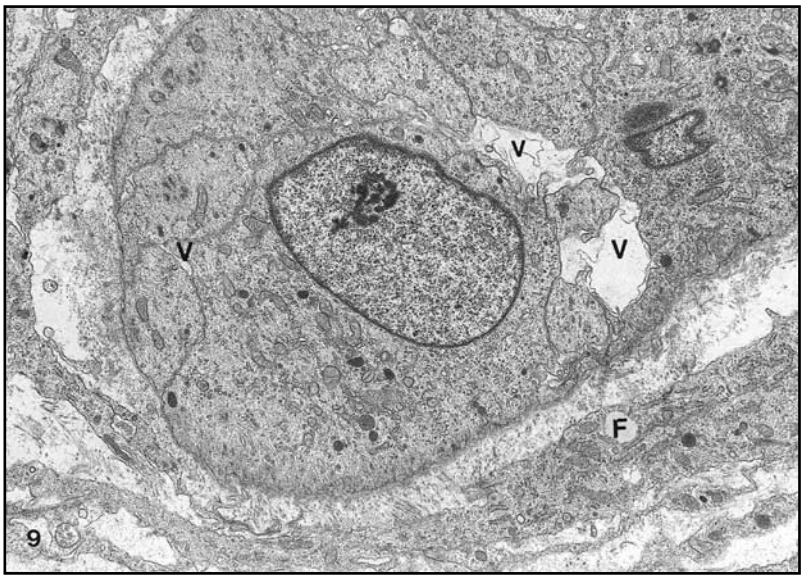

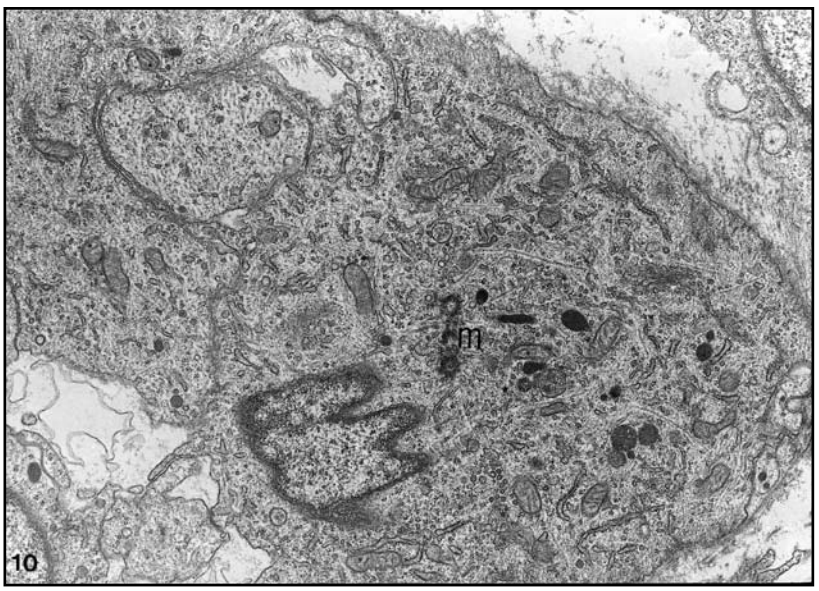

mature endothelial cells, which attach and proliferate immediately after placement in culture medium [19]. Multistep process of microvascular formation and alterations in endothelial-like cell immunophenotype found in our study let us to suppose that endothelial-like cell is committed endothelial cell between progenitor endothelial cell and terminally differentiated endothelium. Participation of endothelial-like cell in new vessel formation 2 days after brain injury point to presence of those cells in blood and their morphogenetic readiness. Therefore, we suppose that in blood there are endothelial stem cells in different stages of morphogenetic differentiation and specifically arrested in "check points" of development. Brain trauma may contribute to extravasation of endothelial-like cells and initiation of the next steps in their development.

Our immunocytochemical studies revealed $\alpha_v \beta_3$ integrin-like immunoreactivity in capillary vessels formed by endothelial-like cells. The integrin $\alpha_v \beta_3$ is the marker of proliferative phenotype of endothelial cell, and it is only minimally expressed on quiescent blood vessels but is significantly upregulated during angiogenesis [4]. That results indicate that endothelial-like cell may possess the proliferative phenotype before they terminate in development.

Terminally differentiated brain capillaries are constituted of endothelial cells surrounded by basement membrane and of pericytes embedded

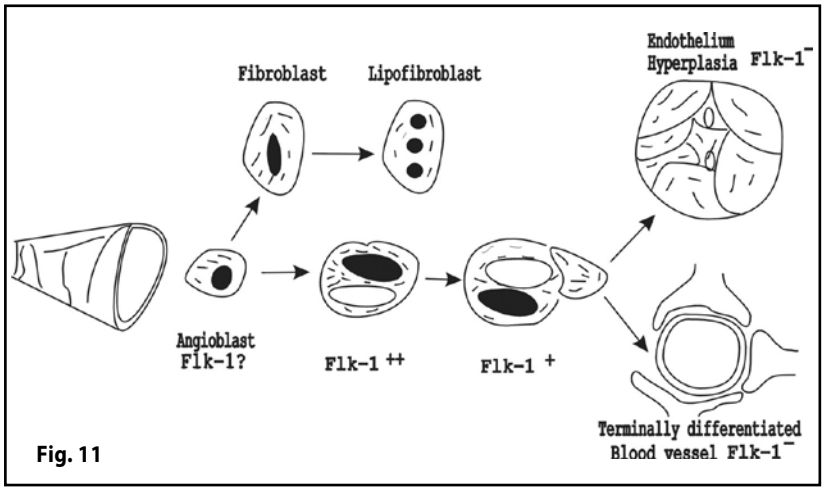

Fig. 11

Figure 11. Postsurgical origin of capillary vessel in brain parenchyma. Hypothetical way of generation of mature endothelial cell, fibroblast and lipofibroblast from angioblast. Endothelial-like cell differentiates to adult and hyperplastic endothelium with alterations in its immunophenotype. The second way of differentiation from the angioblast tends towards the fibroblast and lipofibroblast formation.

Figure 12. Western blot analysis (A) and optical density (B) of the rat brain cortex homogenates after surgical injury stained with pAb VEGF. Lane: 1,3-sham control; 4,5-two days after surgical injury; 2-four days after surgical injury.

Figure 13. Western blot (A) and optical density (B) of Flk-1 in homogenates of rat brain cortex after surgical injury. Immunoreaction with mAb against Flk-1. Lane: 1-sham control; 2-two days after surgical injury; 3,4-four days after surgical injury.

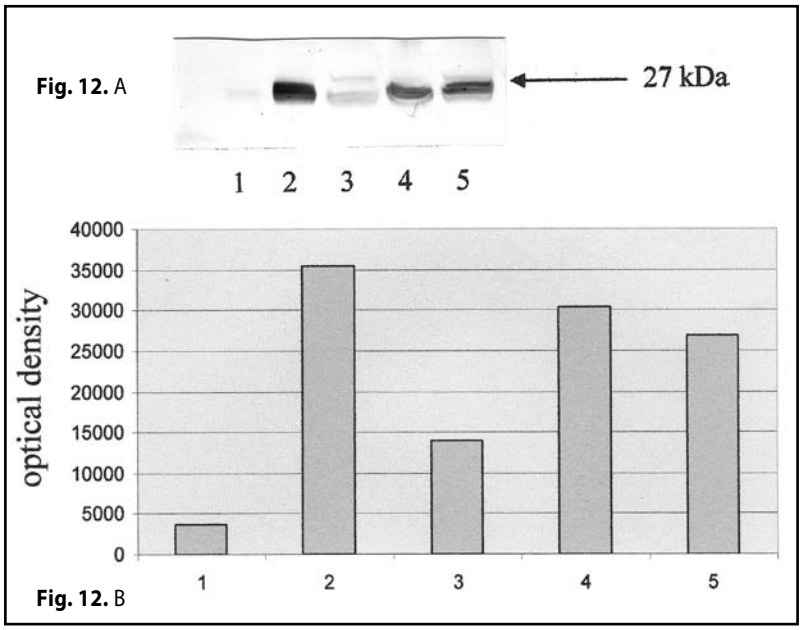

Fig. 12. A

Fig. 12. B

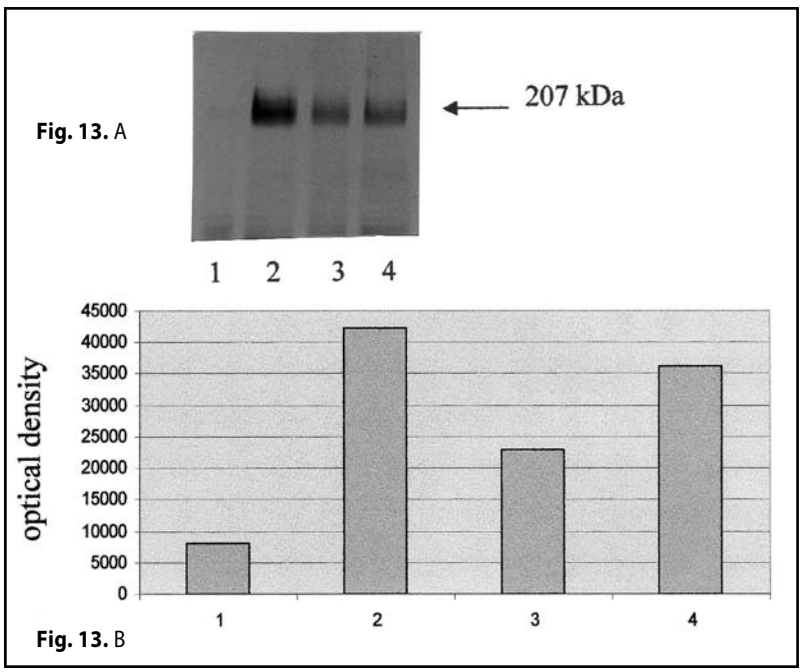

Fig. 13. A

Fig. 13. B

within the basement membrane. New vessels formed in the area immediately adjacent to the operated region were surrounded by basement membrane-like material only without pericytes and astrocytic processes in their neighborhood. Establishment of a functional vascular network further requires that nascent vessels mature into durable vessels. The association of pericytes and smooth muscle cells with newly formed vessels regulates endothelial cell proliferation, survival, migration, differentiation, vascular branching, blood flow and vascular permeability [6]. Hellstrom et al [10] described in vivo models of brain angiogenesis in the absence of pericytes, using PDGF receptor-β (PDGF-B) deficient mice. Another experiments have suggested that pericyte-endothelial cell contact inhibit both endothelial and pericyte proliferation indicating a negative correlation between the presence of pericyte and the proliferation of endothelium [11, 3]. In our material we observed hyperplasia of endothelium that might promote micro aneurysm formation as suggested by Hellstrom et al [10]. In hyperplastic vessels endothelium is still characterized by fibrils phenotype. So, our results indicate that the endothelial-like cell could not successfully terminate development without presence of pericyte or astrocyte.

Our very interesting finding was the presence of the lipofibroblasts in our material (lipofibroblast was not

described in brain parenchyma earlier). Mural cells originate from multiple sources during development [12]. Common progenitors (Flk-1+) differentiate into endothelial cells, fibroblasts or vascular smooth muscle cells in the presence of VEGF [32, 14]. Finally, it is possible that fibroblast can differentiate into lipofibroblast as was described in lung [29].

The majority of the vascular precursor populations identified to date in adult blood and bone marrow are heterogeneous. Thus the true vascular and other progenitors remain to be defined.

In fact circulating progenitors and different immunophenotypes of circulating immature endothelial cells may exist in blood in different stages of maturation and may result from different stages of differentiation. Trauma or vascular injury may mobilize the group of the most differentiated progenitors that may contribute to new vessel formation and cells unconnected with blood vessels, as fibroblasts and lipofibroblasts. Thus, endothelial-like cells with filaments in cytoplasm may be less mature than the endothelial cells and our model should be useful for the characterization of endothelial commitment and of the endothelial cells differentiation after brain injury.

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REFERENCES

1 Asahara T, Murohara T, Sullivan A, van der Zee R, Li T, Witzenbichler B, Schatteman G, Isner JM. Isolation of putative progenitor endothelial cells for angiogenesis. Science 1997; **275**: 964–967.
2 Asahara T, Takahashi T, Masuda H, Kalka C, Chen D. VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. EMBO J 1999; **18**: 3964–3972.
3 Benjamin LE, Golijanin D, Itin A, Pode D, Keshet E. Selective ablation of immature blood vessels in established human tumors follows vascular endothelial growth factor withdrawal. J Clin Invest 1999; **103**: 159–165.
4 Brooks PC, Clark RA, Cheresh DA. Requirement of vascular integrin $\alpha_v \beta_3$ for angiogenesis. Science 1994; **264**: 569–571.
5 Caprioli A, Jaffredo T, Gautier R, Dubourg C, Dieterlen-Lievre F. Blood-borne seeding by hematopoietic and endothelial precursors from the allantois. Proc Natl Acad Sci USA 1998; **95**: 1641–1646.
6 Carmeliet P. Angiogenesis in health and disease. Nat Med 2003; **9(6)**: 653–660.
7 Cho SK, Bourdeau A, Letarte M, Zuniga-Pflucker JC. Expression and function of CD105 during the onset of hematopoiesis from Flk-1+ precursors. Blood 2001; **98(13)**:3635–3642.
8 Cobbs CS, Chen J, Greenberg DA, Graham SH. Vascular endothelial growth factor expression in transient cerebral ischemia in the rat. Neurosci Lett 1998; **249**: 79–82.
9 Frontczak-Baniewicz M, Walski M. New vessel formation after surgical brain injury in the rat's cerebral cortex. I. Formation of the blood vessels proximally to the surgical injury. Acta Neurobiol Exp 2003; **63**: 65–75.
10 Hellstrom M, Gerhardt H, Kalen M, Li X, Eriksson U, Wolburg H, Betsholtz C. Lack of pericytes leads to endothelial hyperplasia and abnormal vascular morphogenesis. J Cell Biol 2001; **153(3)**: 543–554.
11 Hirschi KK, Rohovsky SA, Beck LH, Smith SR, D'Amore PA. Endothelial cells modulate the proliferation of mural cell precursors via platelet-derived growth factor-BB and heterotypic cell contact. Circ Res 1999; **84**: 298–305.
12 Hungerford JE, Little CD. Developmental biology of the vascular smooth muscle cell: building a multilayered vessel wall. J Vasc Res 1999; **36(1)**: 2–27.
13 Isner JM. Angiogenesis: a "breakthrough" technology in cardiovascular medicine. J Invasive Cardiol 2000; 12 Suppl A: 14A–17A.
14 Jain RK. Molecular regulation of vessel maturation. Nat Med 2003; **9(6)**: 685–693.
15 Kabrun N, Buhring HJ, Choi K, Ullrich A, Risau W, Keller G. Flk-1 expression defines a population of early embryonic hematopoietic precursors. Development 1997; **124**: 2039–2048.
16 Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; **227**: 680–685.
17 Lennmyr F, Ata KA, Funa K, Olsson Y, Terent A. Expression of vascular endothelial growth factor (VEGF) and its receptors (Flt-1 and Flk-1) following permanent and transient occlusion of the middle cerebral artery in the rat. J Neuropathol Exp Neurol 1998; **57**: 874–882.
18 Luttun A, Carmeliet G, Carmeliet P. Vascular progenitors: from biology to treatment. Trends Cardiovasc Med 2002; **12**: 88–96.
19 Peichev M, Naiyer AJ, Pereira D, Zhu Z, Lane WJ, Williams M, Oz MC, Hicklin DJ, Witte L, Moore MAS, Rafii S. Expression of VEGFR-2 and AC133 by circulating human CD34+ cells identifies a population of functional endothelial precursors. Blood 2000; **95**: 952–958.
20 Reyes M, Dudek A, Jahagirdar B, Koodie L, Marker PH, Verfaillie CM. Origin of endothelial progenitors in human postnatal bone marrow. J Clin Invest 2002; **109**: 337–346.
21 Risau W, Flamme I. Vasculogenesis. Ann Rev Cell Dev Biol 1995; **11**: 73–91.
22 Risau W. Mechanisms of angiogenesis. Nature 1997; **386**: 671–674.
23 Rafii S. Circulating endothelial precursors: mystery, reality, and promise. J Clin Invest 2000; **105**: 17–19.
24 Rafii S. Circulating endothelial precursors: mystery, reality, and promise. J Clin Invest 2000; **105**: 17–19.
25 Robert B, St. John PL, Hyink DP, Abrahamson Dr. Evidence that embryonic kidney cells expressing flk-1 are intrinsic, vasculogenic angioblasts. Am J Physiol 1996; **271**(3pt2): F744–F753.
26 Shi Q, Rafii S, Wu HD, Wijelath ES, Yu C, Ishida A, Fujita Y, Kothari S, Mohle R, Sauvage LR, Moore MAS, Storb RF, Hammond WP. Evidence for circulating bone marrow-derived endothelial cells. Blood 1998; **92**: 362–367.
27 Shweiki D, Itin A, Soffer D, Keshet E. Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature 1992; **359**: 843–845..
28 Solovey A, Lin Y, Browne P, Choong S, Wayner E, Hebbel RP. Circulating activated endothelial cells in sickle cell anemia. N Engl J Med 1997; **337**: 1584–1590.
29 Torday JS, Torres E, Rehan VK. The role of fibroblast transdifferentiation in lung epithelial cell proliferation, differentiation, and repair in vitro. Pediatr Pathol Mol Med 2003; **22(3)**: 189–207.
30 Walski M, Frontczak-Baniewicz M. New vessel formation after surgical brain injury in the rat's cerebral cortex. II. Formation of the blood vessels distal to the surgical injury. Acta Neurobiol Exp 2003; **63**: 77–82.
31 Weinstein BM. What guides early embryonic blood vessel formation? Dev Dyn 1999; **215**: 2–11.
32 Yamashita J, Itch H, Hirashima M, Minetaro O, Nishikawa S, Yurugi T, Naito M, Kazuwa N, Nishikawa SI. Flk-1 positive cells derived from embryonic stem cells serve as vascular progenitors. Nature 2000; **408**: 92–96.