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New vessel formation after surgical brain injury in the rat's cerebral cortex
I. Formation of the blood vessels proximally to the surgical injury

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Abstract. Postnatal neovascularization has been previously considered synonymous with angiogenesis but it was found that circulating endothelial progenitor cells may home into sites of neovascularization and their differentiation into endothelial cells is consistent with vasculogenesis. In this study, we investigated neovascularization of the adult rat's cerebral cortex after surgical brain injury by electron microscopic ultrastructural and immunocytochemical studies. We found places with disrupted brain parenchyma. The blood vessels showed an incomplete endothelial lining. In the brain parenchyma we observed fibrin, likely derived from disrupted blood vessels. In the plasma there were cell aggregates characterized by endothelial-like features with fibrils in the cytoplasm, untypical for endothelial cells. These endothelial-like cells participated in the process of new vessel formation. We used the anti-α_V β_3 integrin antibody to visualize the different morphogenic stages of newly formed blood vessels. We demonstrated the relationship between α_V β_3 integrin localization and different stages of new vessel formation. Our data suggest that growth and development of new blood vessels due to neovascularization following trauma of the adult rat brain are not restricted to angiogenesis but encompass vasculogenesis as well.

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INTRODUCTION

The vascular system is one of the first systems to function during vertebrate development. The formation of a precisely patterned, seamless network of blood vessels is absolutely essential for embryonic survival. Blood vessels are formed *via* two processes: vasculogenesis and angiogenesis (Risau 1991). In the early embryogenesis, the vascular system develops by vasculogenesis in which angioblasts differentiate into endothelial cells to form a primitive capillary network, whereas angiogenesis, the sprouting of capillaries from preexisting blood vessels, is involved in the late stage of embryogenesis and in the adult (Risau and Flamme 1995). The endothelial cells of cerebral capillaries differ functionally and morphologically from those of non-cerebral capillaries (Risau 1998). During embryonic development, the cerebral vascular system originates from the perineural plexus when vascular system invades the proliferating neuroectoderm, indicating that the cerebral vascular system is primarily developed by angiogenesis and not by vasculogenesis (Pardanaud et al. 1989). Vascular branching is accompanied by vessel maturation characterized by pericyte recruitment and formation of contacts between vessels and astrocytic processes. In the rat brain, angiogenesis is completed around postnatal day 20 (Plate 1999).

Angiogenesis in adults involves sprouting from existing vessels into a previously avascular tissue or the splitting of preexisting vessels by transcapillary bridges or posts of the extracellular matrix (Carmeliet 2000). Blood vessels generated by both sprouting and non-sprouting angiogenesis are progressively pruned and remodeled into a functional circulatory system.

Postnatal neovascularization has been previously considered to result exclusively from the proliferation, migration and remodeling of fully differentiated endothelial cells derived from preexisting blood vessels i.e. angiogenesis (Folkman and Shing 1992). However, endothelial precursor cells have been identified in bone marrow and in peripheral blood in adults. Angioblast-like circulating endothelial progenitor cells are present in the peripheral blood and have been isolated from adult animals (Asahara et al. 1997, Weinstein 1999, Rafii 2000). These cells may contribute to neoangiogenesis in adults, consistent with vasculogenesis. In animal models of brain ischemia, endothelial cell progenitors were found to incorporate into sites of active neovascularization (Isner et al. 2000).

In adult species there are no morphological electron microscopic studies illustrating neoangiogenesis consistent with vasculogenesis. In our studies, we observed neovascularization in the cerebral cortex induced by pathological conditions after surgical injury of the brain cerebral cortex. The morphological features of new vessel formation and participation of endothelial cells, basement membrane compartment, perivascular cells and macrophages may allow to understand the mechanisms underlying the neovascularization in adult rat brain. These findings may have clinical importance and suggest new therapeutic strategies to enhance regeneration and recovery after brain trauma.

The new vessel formation requires a highly regulated interaction between endothelial cells and the surrounding extracellular matrix proteins. Endothelial cells express the proteins which are the members of the integrin family of receptors. Of the many integrin receptors, only $\alpha_V \beta_3$ integrin receptor is capable of recognizing all matrix proteins including integrin, fibrin, fibronectin and vitronectin (Charo et al. 1990). In support of the concept that $\alpha_V \beta_3$ integrins may be critical for new vessel formation during wound repair after traumatic brain injury, we demonstrate the relationship between $\alpha_V \beta_3$ integrin expression and different stages of new vessel formation.

METHODS

Eight adult, male Wistar rats (200-250 g) were anaesthetized with 20 mg/kg ketamine hydrochloride and immobilized in a stereotactic apparatus. After the skin was incised the frontal bone was trepaned 2 mm laterally from the bregma and 2 mm anteriorly to the coronar suture and the dura mater was incised. The operated cortex was hemisected with a small scalpel and the wound was closed. The skin was sutured and dressed under aseptic conditions. In two animals which served as controls the same procedure was applied except that no hemisection was done. The rats remained under standard laboratory conditions for 4 days with free access to food and water.

For ultrastructural morphological studies, four animals were perfused *via* the left heart ventricle with 2% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 for 20 min at 20^{o}C. The tissue for ultrastructural studies was sampled from the cerebral cortex, from the area immediately adjacent to the operated region. The tissue from control animals was sampled from the matching region of the intact cerebral cortex.

The material was further fixed in the same solution for 20 h and postfixed in a mixture of 1% osmium tetroxide (OsO_4) and 0.8% potassium ferricyanide $K_4[Fe(CN)_6]$. Then, the material for microscopic studies was processed for transmission electron microscopy and analyzed in a JEM-1200EX.

For immunocytochemical studies, four animals were perfused *via* the left heart ventricle with 3.5% paraformaldehyde, 0.1% glutaraldehyde in 0.1 M sodium phosphate buffer (PBS) at pH 7.4 (30 min). Cortical tissue was postfixed in the same mixture for 2 hours at +4°C. Subsequently they were rinsed for 2 h in PBS, treated with 1% OsO_4 for 1 hour, dehydrated in increasing concentrations of ethanol and finally embedded in Epon. Ultrathin sections were processed according to the post-embedding immunogold procedure. Briefly, the sections were mounted on the formwar-coated nickel grids, placed in 10% hydrogen peroxide for 10 min, rinsed in PBS for 30 min and exposed for 10 min to 5% bovine serum albumin in PBS. The monoclonal mouse antibody against α_V β_3 integrin complex (sc-7312, Santa Cruz Biotechnology, USA) was diluted 1:20 in PBS and applied to the slices for 24 h at +4°C. Then, the grids were washed in PBS for 30 min and exposed to goat anti mouse IgG (H+L) conjugated to colloidal gold particles of 18 nm in diameter (Jackson ImmunoResearch, USA) diluted 1:50 in PBS. After incubation for 1 hour in darkness, the grids were washed with PBS for 15 min, followed by washes with distilled water for 15 min. Ultrathin sections were air-dried, stained for 10 min with 4.7% uranyl acetate and for 2 min with lead citrate. Control ultrathin sections were prepared using normal murine serum instead of anti- α_V β_3 integrin complex antibody. The sections were examined and photographed using a JEOL 1200EX electron microscope.

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

RESULTS

In the sections obtained from the area immediately adjacent to the lesion we have found the regions with disrupted brain parenchyma. In a few cases, vessel walls appeared mechanically disrupted and the endothelial lining was incomplete. Macrophages were detected in the perivascular space. These cells contained vacuoles with cellular debris, likely originating from damaged cells which were phagocytized (Fig. 1). The cytoplasm of these macrophages contained also lipid droplets. In the brain parenchyma itself, we could also observe what appeared to be deposits of fibrin, which likely originated from disrupted blood vessels. Screening the material we found often in the homogenous plasma proteins

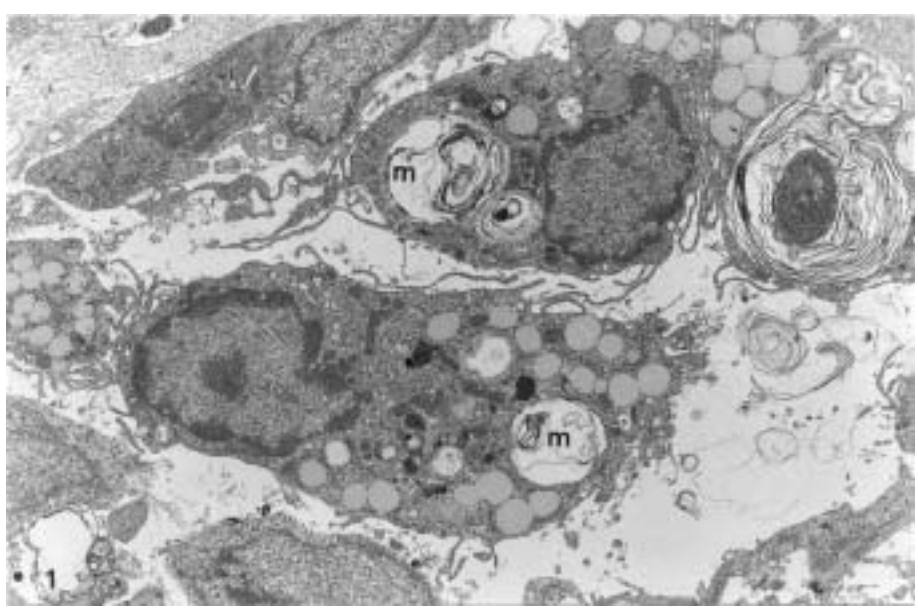
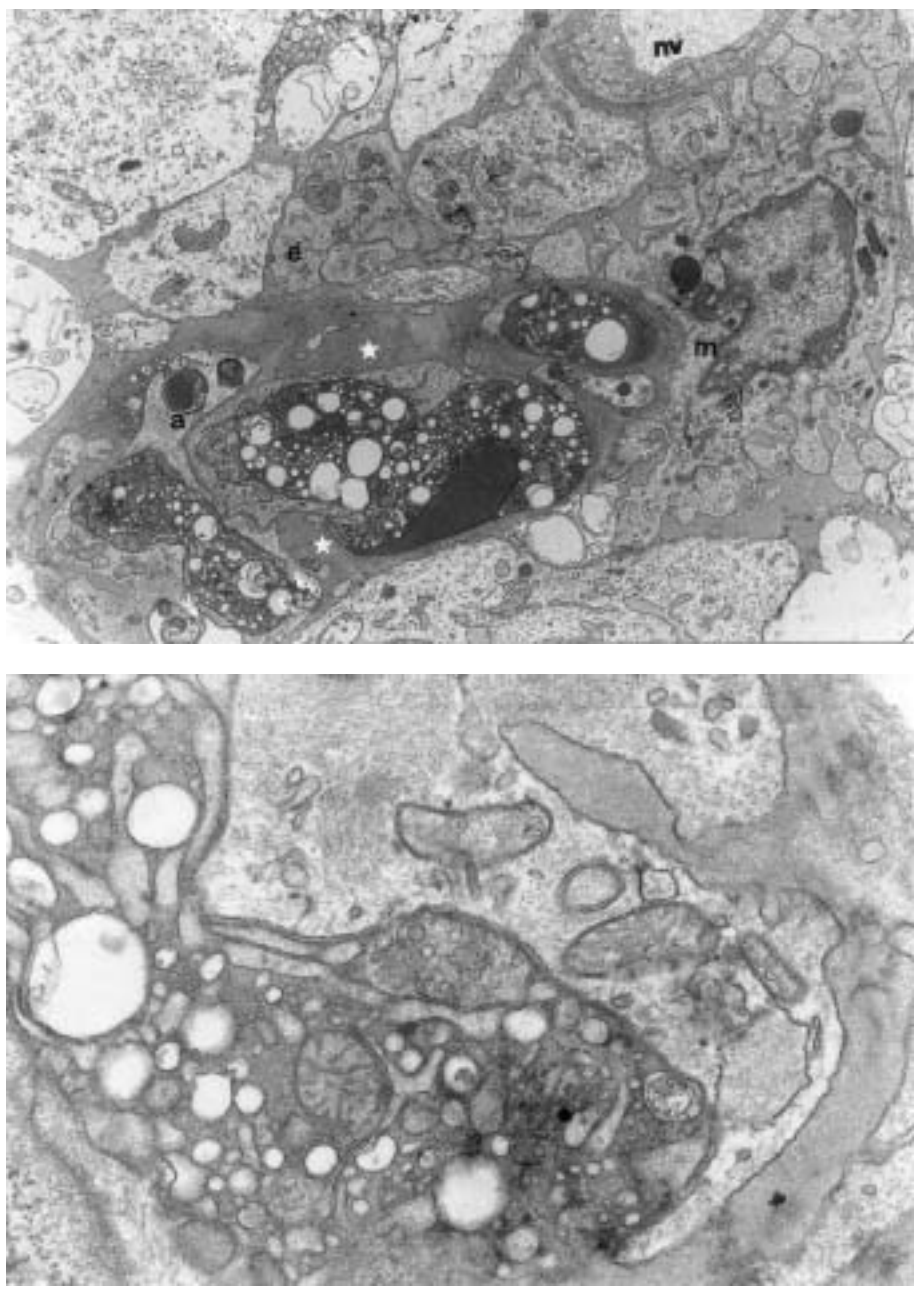


Fig. 1. Section of the cerebral cortex immediately adjacent to lesion. The perivasular space with macrophages (m) is seen. The macrophage contains vacuoles with cellular debris, likely originating from damaged cells which were phagocytized. Magnification, x 8,000.

immersed the atypical cell aggregates (Fig. 2A). These cells revealed endothelial-like features but possessed fibrils in the cytoplasm, atypical for endothelial cells (Fig. 2B). The tight junctions between endothelial-like cells are not present. Some cells could not be distinguished as they were not enveloped by basement membrane material. In the diluted fibrin there was a young capillary vessel enveloped by blurred and homogenous basement membrane-like material. The endothelial cells are not connected by tight junctions. The



2A

2B

Fig. 2. A, the area in the vicinity of the blood vessel with plasma fibrin proteins (white asterisks) likely from damaged vessels. The aggregates of the endothelial-like cells (e) with fibrils in the cytoplasm are immersed in plasma fibrin proteins. They are accompanied by macrophages (m) and apoptotic endothelial-like cells (a). The young capillary vessel (nv) surrounded by thickened and blurred basement membrane without tight junctions between endothelial cells is seen above the endothelial-like cells aggregates. Magnification, x 8,000; B, high magnification of the endothelial-like cell with fibrils in the cytoplasm. Magnification, x 50,000.

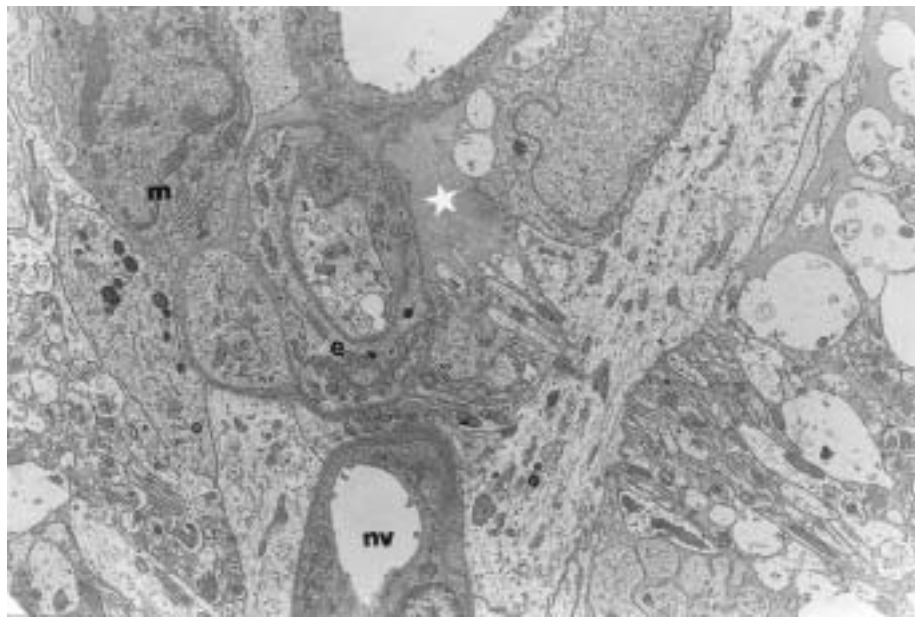


Fig. 3. The area in the vicinity of the blood vessel with diluted plasma fibrin proteins (asterisk) and endothelial-like cells (e) surrounded by basement membrane and accompanied by macrophage (m). The young capillary vessel (nv) is present below. Magnification, x 5,000.

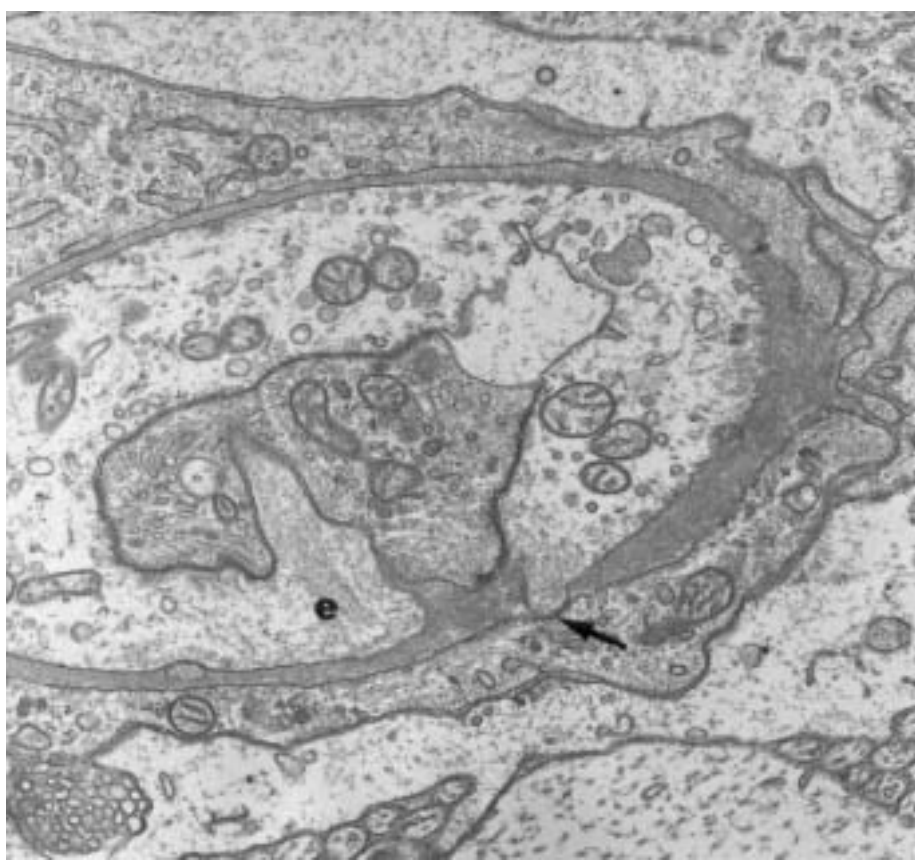
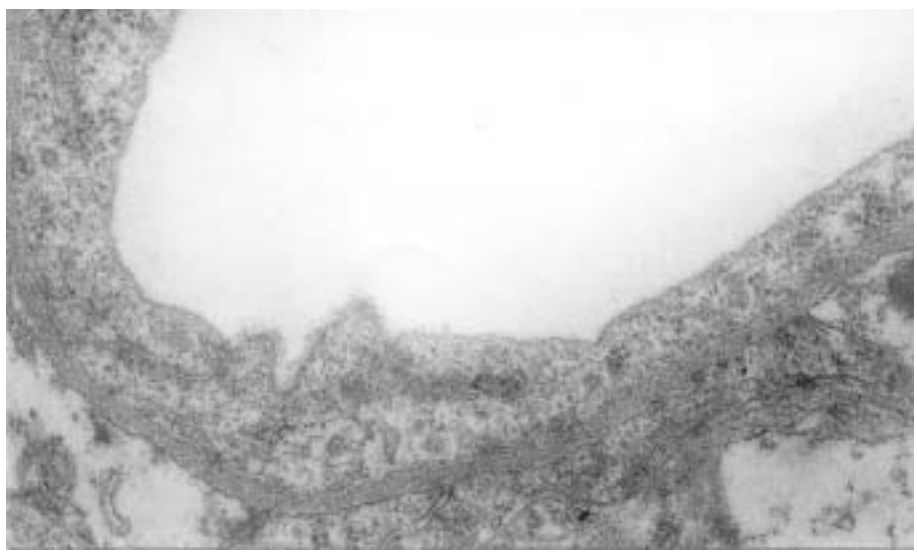


Fig. 4. The endothelial-like cells (e) surrounded by basement membrane. The junction between endothelial-like cell and the pericyte is visible (arrow). Magnification, x 15,000.

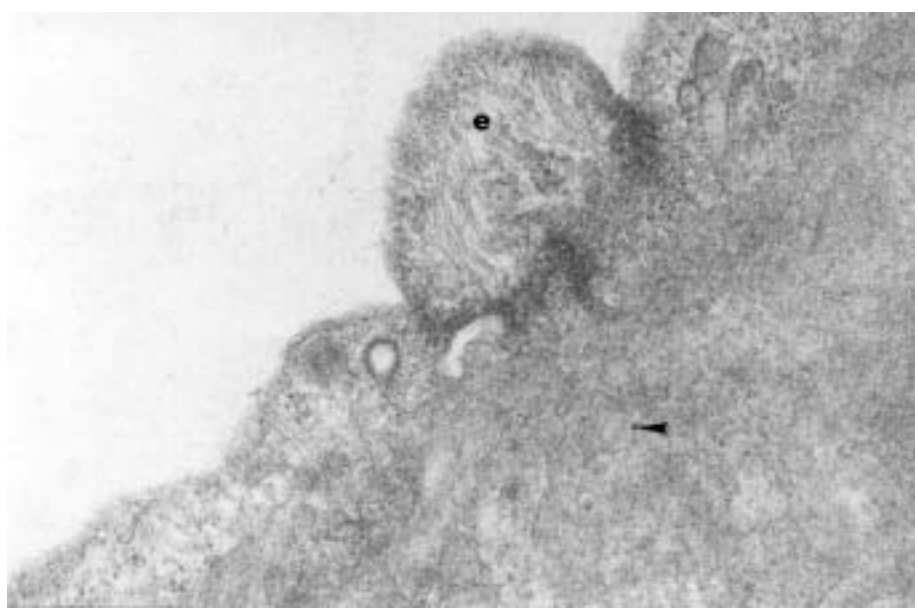
perivascular phagocyte accompanies the young capillary vessel. Some of endothelial-like cells seemed to be in the process of disintegration. We observed that whereas the mitochondria were ultrastructurally unchanged, there were fragmented nuclei with condensed chromatin present in the cytoplasm. These features point to the process of apoptosis in these cells.

In Fig. 3 we show the area in the vicinity of the old mature blood vessel of the cerebral cortex. In this area we could see again in plasma proteins, likely from damaged blood vessel, endothelial-like cells characterized by fibrils and enveloped by a blurred, homogenous basement membrane. The vessel lumen is subsequently formed. The young capillary vessel with high hypertrophic endothelium is present beyond the area of diluted fibrin.

The morphological features of the young capillary formed by endothelial-like cells detected repeatedly in material obtained from the area immediately adjacent to



5A

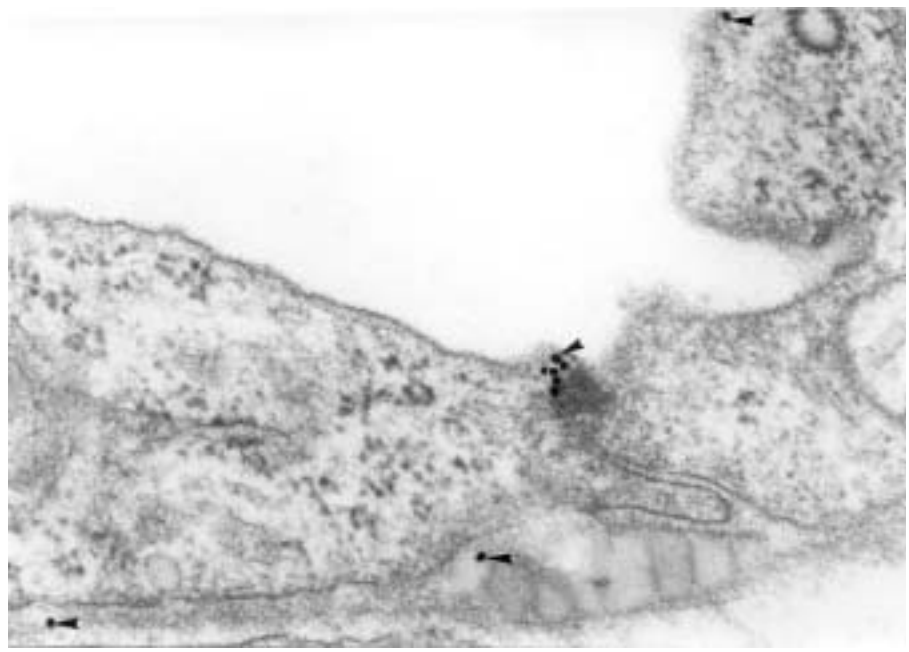


5B

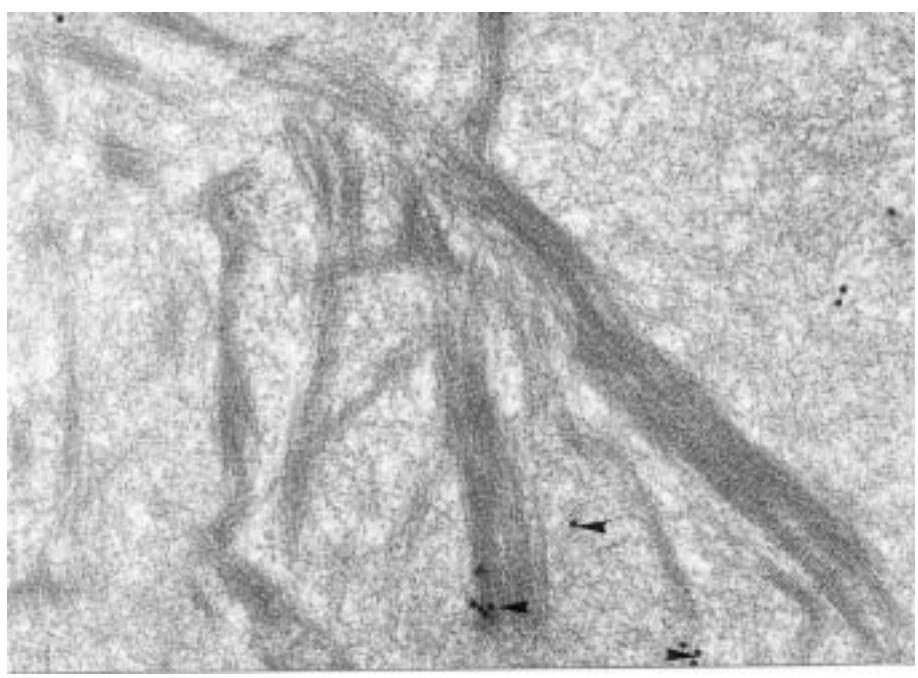
Fig. 5. Localization of $\alpha_V \beta_3$ integrin in capillary vessels of the cerebral cortex proximal to the surgical trauma. A, capillary vessel from control animal. $\alpha_V \beta_3$ integrin immunolabeling is not present. Magnification, x 40,000; B, The newly formed blood vessel. The endothelial-like cell (e) is seen between ultrastructurally unchanged endothelial cells. A single gold particle is seen in the basement-membrane-like material (arrowhead). Magnification, x 45,000.

the lesion are shown in Fig. 4. The atypical endothelial cells, enveloped by a homogenous blurred basement membrane, do not display tight junctions. The vessel lumen has not formed yet. In the vicinity of that vessel, a pericyte and a perivascular astrocytic processes are present. We note the junction between the endothelial-like cell and the pericyte.

New capillaries formed by endothelial-like cells or by ultrastructurally unchanged endothelium accompanied by endothelial-like cells were observed frequently



5C



5D

Fig. 5. Localization of $\alpha_V \beta_3$ integrin in capillary vessels of the cerebral cortex proximal to the surgical trauma. C, $\alpha_V \beta_3$ integrin immunolabeling in the newly formed blood vessel. High immunoreactivity of the $\alpha_V \beta_3$ integrin is observed in the junction formed between endothelial cells, the endothelial cell cytoplasm and in the subendothelial space (arrowheads). The gold particles are also present in the basement membrane. Magnification, x 70,000; D, plasma proteins likely originating from a disrupted blood vessel. Note the gold particles present in the vicinity of the fibrillar material (arrowheads). Magnification, x 60,000.

within the homogenous plasma proteins in the area immediately adjacent to the lesion.

Expression of α_V β_3 integrin on blood vessels of the cerebral cortex proximally to the injury

In the morphologically unchanged capillary vessels, α_V β_3 integrin-like immunoreactivity was not detected (Fig. 5A). Some newly formed blood vessels observed in that region present endothelial cells without tight junctions in between them as well as endothelial-like cells (Fig. 5B). In endothelial-like cells, as well as in endothelial cells, no marked α_V β_3 integrin-like immunoreactivity was present. We did not observe α_V β_3 integrin-like immunoreactivity in the basement membrane of that vessel either. The young capillaries show aggregates of gold particles in newly formed junctions between endothelial cells and in the cytoplasm of endothelial cells (Fig. 5C). The gold particles are also scarcely present in the basement membrane of those vessels.

The α_V β_3 integrin-like immunoreactivity was also observed in the proximity of the plasma proteins originating from disrupted blood vessels (Fig. 5D).

DISCUSSION

The impact of traumatic brain injury on the ultrastructure of blood vessels was investigated in a trauma model. In this study, our attention concentrated on the ultrastructural features of the vessels in the area immediately adjacent to the lesion. Our studies indicated that the ultrastructural characteristics of the tissue is dependent on both traumatic severity and the distance from the operated region. Direct traumatic effects on cerebral vessels result in blood brain barrier breakdown and acute inflammatory response with entry of macrophages and blood elements into injured zone. It was indicated that different subpopulations of brain macrophages emerge after traumatic brain injury and the migration of cells from the blood to the perivascular space occurs (Walski and Gajkowska 2001). Inflammation may be a double–edged sword, in a sense that inflammatory cells and blood elements may also be involved in reparative and restorative processes.

Morphological analysis of the cerebral cortex adjacent to the operated region showed ultrastructural features atypical for this region. There were endothelial-like cell aggregates in plasma proteins that originated presumably from damaged vessels. The ultrastructural features of this area strongly suggest that new vessel formation occurs. Cells that were participating in neovascularization had the characteristic attributes of endothelium but were additionally rich in fibrils atypical for endothelial cells. We observed the "islets" of endothelial-like forms immersed in plasma proteins. We could not separate individual vessels among the endothelial-like cells. They were accompanied by macrophages. We could observe also "the primary vessel" immersed in plasma proteins, characterized by endothelial-like cells, enveloped by basement membrane, that had not formed the lumen yet. We use the term "the primary vessel" because ultrastructural features of those vessels are not permanent. The different populations of endothelial-like cells are worth notice in the area immediately adjacent to the lesion. It seems that the newly formed primary vessels coexisted at different stages of maturation and that endothelial-like cells loose untypical morphological features after subsequent divisions.

The ultrastructural features of the newly formed vessels were different from those presented earlier (Frontczak-Baniewicz and Gajkowska 2001, Frontczak-Baniewicz and Walski 2002). We suggest that formation of new vessels in the cerebral cortex proximally to injury may take place in the process of postnatal vasculogenesis. The disruption of blood vessels made the migration of endothelial progenitor cells from blood to the perivascular space possible. Recent studies demonstrated that postnatal circulating bone marrow-derived endothelial progenitor cells may home to sites of neovascularization and differentiate into endothelial cells *in situ* (Asahara et al. 1999, Isner and Asahara 1999). This population of circulating endothelial progenitor cells was reported to be mobilized by regional ischemia or administration of vascular endothelial growth factor (VEGF) (Asahara et al. 1999, Takahashi et al. 1999). The process of incorporation of these circulating endothelial progenitor cells into sites of neovascularization appears to obligate yet undefined targeting machinery to recruit participation of the circulating cells (Daniel and Abrahamson 2000).

The characteristic morphological feature of the endothelial-like cells is the presence of fibrils in the cytoplasm. Although different composition and structural organization of the extracellular matrix may have important effects on endothelial phenotype and capillary morphogenesis (Madri 1997), little is known on the impact of altered extracellular matrix and, in particular, on the endothelial cell ultrastructure during vasculogenesis.

Distinction between angioblast-like circulating endothelial precursor cells and endothelial cells is difficult at the ultrastructural level and further observations with endothelial progenitor cell determinants, including Flk-1, Tie-2, Sca-1 and CD34 may confirm or infirm our suggestions. Despite this limitation, the atypical features of the endothelial-like cells found in this material prompt us to suggest that they are a consequence of their origin rather than of the altered extracellular matrix. The precursor endothelial cell may probably reflect the phenotype of embryonic angioblasts which are migratory endothelial cells with the capacity to circulate, proliferate and differentiate into mature endothelial cells, but which have neither acquired the characteristic markers of mature endothelium nor formed lumina (Rafii 2000).

In the area of neovascularization, we observed apoptotic cells near the endothelial-like cells, what indicated that the process of apoptosis occurs in this region. During ontogeny, segments of circulation undergo involution in concert with changes in organogenesis (Flamme et al. 1995). Similarly, in the pathological context such as wound healing, there is a dynamic process of angiogenesis and network regression (Desmouliere et al. 1995). Some studies indicate that cell proliferation and cell death may occur in parallel as new vessels form and undergo remodeling (Pollman et al. 1999) and the apoptosis is associated with the involution of capillary networks (Gobe et al. 1997). During angiogenesis, cell death may be induced in those endothelial cells that do not attach to appropriate components of the extracellular matrix. This selection may regulate morphogenesis and differentiation of blood vessels (Eliceiri and Cheresh 1999).

The role of plasma proteins released from disrupted blood vessels has to be discussed. After injury or other inflammatory stimuli, distension of local capillaries and extrusion of fibrinogen are the first events commonly observed (Sunderkotter et al. 1994). Fibrinogen can be converted into fibrin by the procoagulant activities of endothelial cells and activated resident macrophages, thereby providing a migratory matrix and chemotactic stimuli for endothelial and inflammatory cells. Other blood components derived from plasma include fibronectin and vitronectin. These deposited molecules bind to integrins that anchor the cell cytoskeleton to the surrounding extracellular matrix and provide to the endothelial cell signals about the matrix environment. Therefore, it seems that immersion of the endothelial--like cells in the plasma may promote vessel formation.

The appearances of macrophages in the vicinity of the newly formed vessels in this region is meaningful. From the many cells, macrophages have emerged as major protagonists of angiogenesis (Folkman and Shing 1992, Sunderkotter et al. 1994). The angiogenic role of macrophages is associated with their secretory activity. In the cerebrovascular ischemia, brain macrophages that express vascular endothelial growth factor receptor-1 (VEGFR-1) are attracted to the site of injury by secreted VEGF in order to stimulate angiogenesis (Barleon et al. 1996, Berse et al. 1992, Gonzales-Scarno and Baltuch 1999). Attracted or resident macrophages can, in turn, release dilators of blood vessels and activators of endothelial adhesion molecules. Macrophages are able to promote all phases of the angiogenic process by virtue of their secretory products (Knighton et al. 1983). Thus, we suppose that angiogenic activity in the cerebral cortex may be induced by factors generated by macrophages.

In our study endothelial-like cells surrounded by morphologically differentiated basement membrane characterized the newly formed capillary-like vessels. Those vessels were accompanied by pericytes connected with endothelial-like cells. The endothelial cells in capillaries of the central nervous system are closely associated with pericytes. The maturation of the microvasculature is dependent on the establishment of endothelial cell-pericyte gap junctions (Fujimoto 1995). Endothelial cell-pericyte bridges and gap junctions may also be involved in the initiation of new vessel growth (Diaz-Flores et al. 1994).

During angiogenesis and vascular remodeling, the integrin $\alpha_V \beta_3$ has been identified as having an expression pattern linked to vasculature. The vitronectin receptor ($\alpha_V \beta_3$ integrin) is a member of the integrin family of adhesion protein receptors that binds a broad spectrum of ligands including fibronectin and fibrinogen in addition to vitronectin. The integrin $\alpha_V \beta_3$ is only minimally expressed on quiescent blood vessels but is significantly upregulated during angiogenesis *in vivo* (Brooks et al. 1994). Therefore, we used $\alpha_V \beta_3$ integrin immunocytochemistry to visualize of the newly formed blood vessels. We have found that $\alpha_V \beta_3$ integrin was specifically expressed on the newly formed capillary vessels with differentiated endothelial cells and constituted a basement membrane as well as was connected with plasma proteins.

Both results find explanation in the events resulting from brain injury: (1) the new vessel formation in the

area adjacent to the operated region that involves the migration of endothelial progenitor cells into the wound space and (2) accumulation of the plasma proteins originating from disrupted blood vessels. These proteins are associated with integrin substrates, fibrin, fibronectin and vitronectin and probably serve as a provisional extracellular matrix scaffold for the migration of endothelial precursor cells. In the young capillary vessels with resting endothelial-like cells, expression of α_V β_3 integrin was not observed, suggesting disconnection between the cells and the basement membrane. These findings suggest the important nature of the interaction of α_V β_3 cell surface receptors with matrix molecules during vasculogenesis.

CONCLUSION

Our ultrastructural studies indicate that, after cortical hemisection, new vessel formation takes place proximally to the transected region. We hypothesize that mechanical brain injury and disruption of brain parenchyma may provide an environment that supports the differentiation of endothelial progenitor cells into endothelial cells. The differentiated expression of α_V β_3 integrin in a course of new vessel formation indicates the differences in connections between endothelial-like cells and the basement membrane, and between endothelial cells and the basement membrane. Further studies are needed to quantify this neovascularization and examine its effects on traumatized brain. These findings may open up novel therapeutic strategies in the treatment of brain trauma.

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