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

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Abstract. We investigated the features of newly formed blood vessels after surgical brain injury of the rat's cerebral cortex distal to the operated region. We document the process of split mature blood vessels by an endothelial bridge and morphological features of newly formed vessels. We did not observe a disruption of brain parenchyma. The endothelial lining in vessels was complete. The morphological features of the endothelial cells and basement membrane show that non-sprouting angiogenesis takes place distally to the surgical injury.

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INTRODUCTION

Vessel formation can occur by a number of different processes (Risau 1997). Early in development, vessel formation takes place in a process referred to 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 remodeling refers to the process by which this initial network is modified to form the interconnecting branching patterns characteristic of the mature vasculature.

A different process, referred to as angiogenic sprouting, involves the sprouting from existing vessels into a previously avascular tissue. In some cases, it seems that mature vessels must first be destabilized to allow for subsequent sprouting (Ware and Simons 1997). The vessels formed by sprouting are initially immature and must further develop.

Intussusceptive capillary growth represents a new principle for microvascular growth. According to this concept, the capillary network expands by the formation of transcapillary tissue pillars, which give rise to new vascular meshes (Frontczak-Baniewicz and Walski 2002).

The recent explosion in identifying and characterizing physiological regulators of blood vessel growth demands reevaluation of the therapeutic efforts aimed at regulating blood vessel growth. Namely, it has to be defined whether it will be promoting vascular ingrowths to replenish ischemic tissue or rather repairing damaged and leaky vessels during inflammation or other pathological conditions (Stockhammer et al. 2000). 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. To design a proper treatment an improved understanding of the mechanisms underlying new vessel formation and participation of endothelial cells, basement membrane, extracellular matrix and perivascular cells and macrophages are required.

In this study, morphological features underlying the formation of new vessels and their maturation in the cerebral cortex induced by pathological conditions after surgical injury of the rat brain cerebral cortex were investigated. We wanted to determine the scheme of angiogenesis in cerebral cortex distally to the surgical injury.

METHODS

Eight adult, male Wistar rats (200-250 g) used in this experiment, were anaesthetized with 20 mg/kg ketamine hydrochloride and immobilized in a stereotactic apparatus. 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 cut. The brain was hemisected with a small scalpel and the wound was closed. The skin was sutured and dressed under aseptic conditions. In two control animals (sham) the same procedure was applied except hemisection. Experimental and control rats remained under standard laboratory conditions for 4 days.

The animals were perfused *via* the left heart ventricle with 2% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 at 20^{o}C. Tissue sections were fixed and processed for transmission electron microscopy as described by Walski et al. (1995). Material for ultrastructural studies was sampled from the cerebral cortex of the operated animals 2-3 mm from the area immediately adjacent to the operated region. Material from control animals was sampled from strictly matching areas of the intact cerebral cortex.

The animals were handled according to the guidelines of the local ethic committee for experimentation on animals.

RESULTS

In the examined cortical tissue we did not observe disrupted brain parenchyma. The endothelial lining of the blood vessels was complete. However, the blood vessels were ultrastructurally different from those typically found in the intact rat cerebral cortex. The endothelial bridges split mature capillaries, forming the young capillary vessels characterized by hypertrophic endothelium rich in organelles (Fig. 1). The endothelial bridges were tightly connected with neighbouring endothelial cells. A very interesting feature was a connection that sometimes could be observed between a pericyte and the endothelial cell forming the bridge (Fig. 1a). The young capillary vessels formed distally to the injured region were observed, as shown in Fig. 2. The endothelial cells were ultrastructurally typical for brain capillaries. The cytoplasm was rich in organelles and contained a well-marked nucleus. In our material the vessel lumen has not yet been formed but a blurred thickened basement membrane showed the young capil-

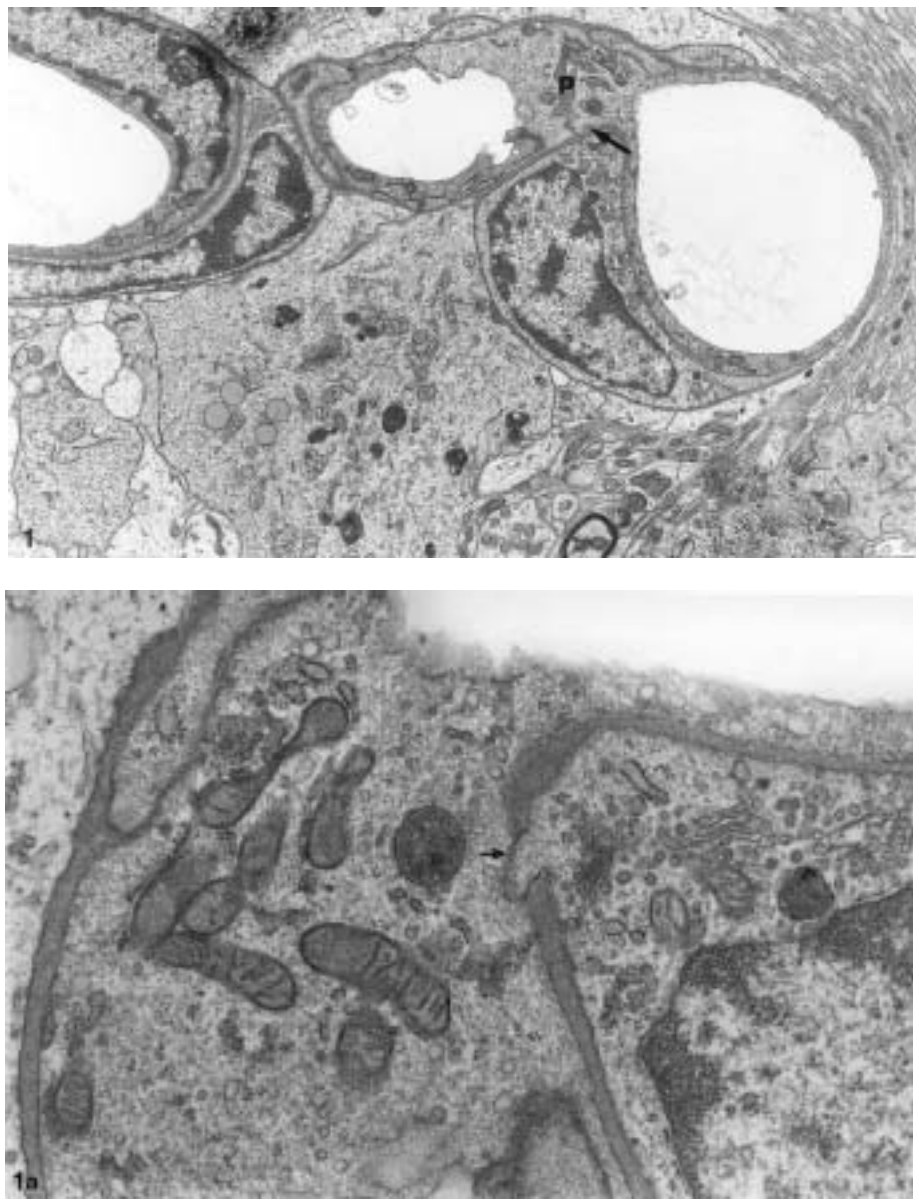

Fig. 1. The section of the cerebral cortex distal to the surgical injury. A new capillary vessel is formed by bridging. The endothelial pillar (p) is tightly connected with neighbouring endothelial cells. The basement membrane is ultrastructurally unchanged. Magnification, x 10,000; a, high magnification of the junction between the endothelial pillar and the pericyte (arrow). Magnification, x 35,000.

lary vessels. In the basement membrane, single collagen fibrils were present. As shown on Fig. 2 the same ultrastructural characteristics was featured by the basement membrane that envelops the macrophage present in the vicinity of the young capillary vessel. The basement membrane that envelops the precapillary vessel presented beyond the young blood vessel possesses ultrastructurally similar features, is blurred and thickened. The ultrastructural similarity of the basement membrane of the precapillary vessel and of the young capillary vessel lets us propose that the precapillary vessel is the "mother" vessel for the young one. The young capillaries formed during non-sprouting angiogenesis often participated in recurrent neovascularization exemplified on Fig. 3. A tangential section shows immature endothelial cells forming the bridge and apparently two luminal cavities. One of the luminal cavities contains fluid material. The basement membrane possesses a thickened and blurred structure.

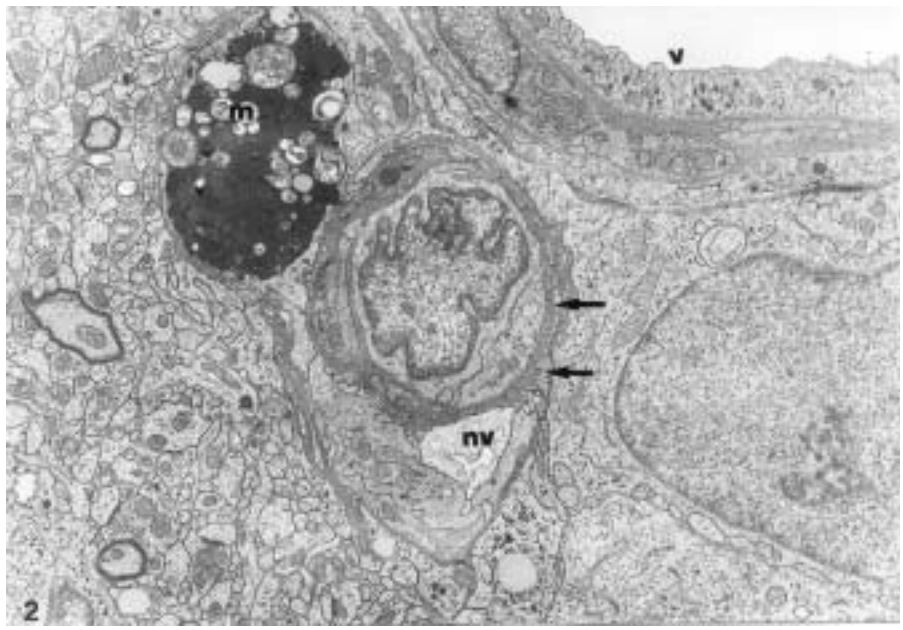

Fig. 2. The young capillary vessel (nv) is formed in the neighbourhood of the old blood vessel (v). The young capillary vessel has not yet formed a lumen. The basement membrane with cross sectioned collagen fibrils (arrows) envelops the young capillary vessel. Similar features of the basement membrane are observed in the old blood vessel. Part of macrophage (m) is seen in the vicinity of the vessels. Magnification, x 8,000.

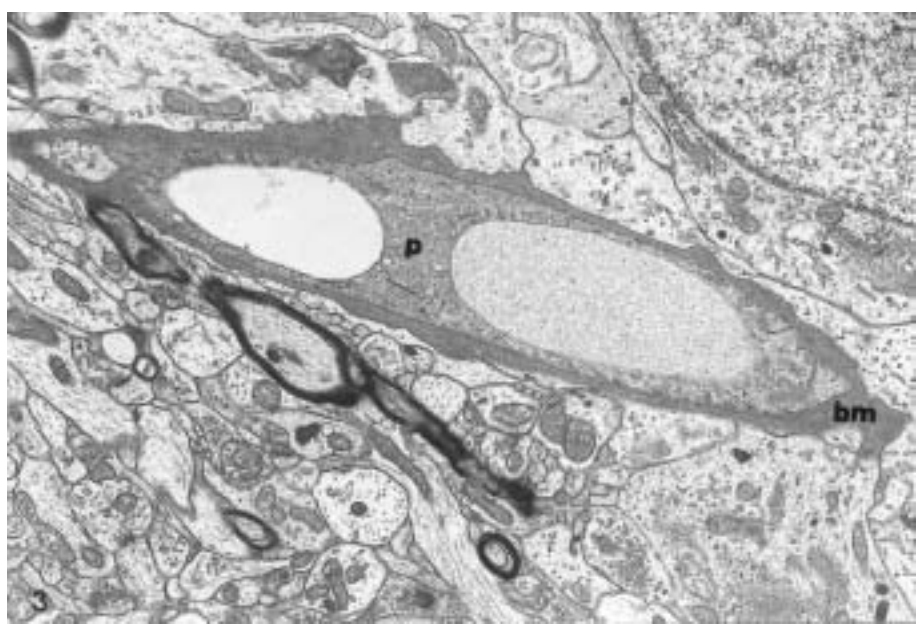

Fig. 3. The newly formed blood vessel is again split by an endothelial bridge (p) during secondary non-sprouting angiogenesis. The basement membrane (bm) is thickened and blurred. Magnification x 8,000.

DISCUSSION

The morphological examination of the cerebral cortex distal to the surgical injury showed angiogenic processes proceeding by non-sprouting angiogenesis. The new capillary vessels created during this process were formed by typical ultrastructurally unchanged endothelial cells enveloped by a blurred and thickened basement

membrane with visible collagen fibrils. This basement membrane ultrastructurally corresponded to the basement membrane of the "mother" vessel presented in the proximity of the new capillary.

Among the inducers or modulators of angiogenesis, macrophages are considered to be the major protagonists (Sunderkotter et al. 1994). Walski and Gajkowska (2001) showed the passage of inflammatory cells from blood to the brain parenchyma four days after cortical injury. These observations indicated that opening of tight junctions may constitute a venues for the passage of leukocytes from the blood to the perivascular space where the blood-borne monocytes transformed to brain macrophages. Different subpopulations of macrophages were observed in the region of traumatic brain damage (Walski and Gajkowska 2001). In the present study we document the presence of macrophages in the vicinity of newly formed vessels in the cerebral cortex.

In our material tight junctions did not connect the endothelial cells of newly formed capillary vessels in the vicinity of the operated region. The tight junction is the specific feature of the brain endothelial cells which differs them from those present in most peripheral tissues. However, during development, when blood vessels first enter the brain they are also discontinuous (Kniesel et al. 1996).

In the young capillaries formed during non-sprouting angiogenesis, endothelial cells were accompanied by pericytes. The junctions between the endothelium and pericytes were a well-marked attribute of those vessels. The endothelial cells in capillaries of the central nervous system are closely associated with pericytes. Early in embryonic development, invasion of the intraneuronal tissue by the perineural mesenchymal cells is accompanied by pericytes (Bauer et al. 1993). The subsequent transformations of endothelial cell junctional complexes into tight junctions are also associated with pericyte coverage. The maturation of the microvasculature is dependent on the establishment of endothelial cell-pericyte gap junctions (Fujimoto 1995). The endothelial cell-pericyte bridge and gap junctions may also be involved in the initiation of new vessel growth (Diaz-Flores et al. 1994). Namely, pericytes are thought to provide structural support for the capillary wall, acting as a scaffold along which endothelial cells migrate during sprouting (Nehls et al. 1992). Endothelial cells secrete elements of the extracellular matrix to stimulate pericyte proliferation (Newcomb and Hermann 1993). Pericytes themselves synthesize many components of the extracellular matrix that change during maturation. These similarities between the two cell types revealed during angiogenesis suggest that both may participate in the formation of capillary sprouts (Schor et al. 1992). During wound healing, pericytes from preexisting microvessels, newly derived pericytes, come into contact with endothelial cells that form new vessels and exert an inhibitory effect on endothelial cell proliferation (Hirschi and D'Amore 1996).

In summary, after cerebral mechanical injury, there is neovascularization with formation of new vessels in the area distally to the lesion. Our data strongly indicate, that neovascularization in that area occurs *via* angiogenesis to consist of sprouts that originate as the result of proliferation and migration of differentiated endothelial cells from parent vessels.

CONCLUSION

This study, in connection with the accompanying paper, raises the problem of different responses of brain capillaries in the areas adjacent to the operated region and distal to the surgical injury. Zonal analysis of vascular morphogenic processes is relevant in the context of therapeutic angiogenesis. Our observations indicate that the angiogenic cascade in the brain is far from being understood. A thorough understanding of the mechanisms of vascular morphogenesis in the brain will be a requisite for the rational translation of this knowledge into clinical applications.

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