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Endothelial basement membrane and seamless-type endothelium in the repair process of cerebral infarction in rats*

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Summary. Ultrastructural analysis of capillary changes during the repair process of experimental cerebral infarction induced in rats was carried out with special reference to the endothelial basement membrane (BM) and seamless-type endothelial cells. Following degeneration of endothelial cells and pericytes, their BMs, without any interruption or fragmentation, were left in the lesion. Newly formed capillaries grew from vessels in the surrounding brain tissues into the reactive zone of infarcts. While the capillaries in cross-section possessed multilayered BMs, these membranes in tangential section comprised an outer BM with extremely wavy profile and an inner one showing a normal trilayered structure, uniformly enveloping the endothelial surface. It is therefore suggested that the sprouting of regenerating capillaries might invade the remaining cavities of BM, resulting from endothelial degeneration. In these new vessels, seamless-type endothelial cells lacking interendothelial contacts were observed frequently. These two different and previously unobserved findings appear to be at the heart of the regeneration mechanism of reactive capillary proliferation.

Key words: Basement membrane – Capillary growth – Seamless endothelial cell – Regenerating capillary – Experimental cerebral infarction

Introduction

In the repair process of cerebral injuries some cell reactions such as macrophage infiltration, astrocyte proliferation and reactive capillary regeneration play important roles in the accumulation of oedema fluid and elimination of necrotic cells (Klatzo 1967; Blakemore 1971; Garcia and Kamijo 1974; Mitchell et al. 1978; Ikuta et al. 1983). As experimental models for studying these pathological mechanisms, cerebral infarction produced by middle cerebral artery (MCA) occlusion or cold lesions are most commonly used (Klatzo 1967; Garcia et al. 1971; Tamura et al. 1981).

In many reports dealing with reactively regenerating capillaries in such lesions, most attention has been directed to the altered blood-brain barrier (BBB) and intractable oedema. Ultrastructural and tracer studies have revealed that regenerating endothelial cells show increased permeability to various substances via increased numbers of pinocytotic vesicles, transendothelial diffusion and transjunctional permeation (Reese and Karnovsky 1967; Persson and Hansson 1976; Mitchell et al. 1978; Beggs and Waggener 1978). However, studies on the mechanisms of degeneration and subsequent regeneration of endothelial cells have been rare (Mitchell et al. 1978; Brierley and Brown 1981), and these aspects seem to be not well understood.

Therefore, the aim of the present study was to reveal some of the ultrastructural characteristics of these vessels appearing in experimental cerebral infarcts in rats. We describe here the morphology of the basement membrane (BM) during the processes of endothelial degeneration and regeneration, and the seamless-type endothelial cells (SEs), both of which are considered to be closely related to the regeneration mechanism of endothelial cells.

Materials and methods

A total of 25 adult Wistar white rats, weighing between 250 and 300 g, were used. The rats were housed two per wire-bottomed cage in a room lighted between 5 am and 5 pm, are fed standard rat chow and water ad libitum.

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The operation technique for producing cerebral infarction by means of MCA occlusion was carried out according to the method described by Tamura et al. (1981). Under Nembutal anesthesia (50 mg/kg body weight, i.p.), the heads of the animals were fixed in the lateral position, and a round craniectomy, 2.5 mm in diameter, in the middle temporal bone was made in front of the temporo-mandibular joint using a dental drill after skin incision and lateral retraction of the temporal muscle.

The translucent dura mater covering the brain was opened along the MCA, which ran vertically in the subarachnoid space beneath the burr hole. The trunk of the MCA and its several branches were occluded for a length of approximately 2 mm using the forceps tip of an electrical-coagulator. The veins running parallel to the artery were carefully avoided. After the bone defect had been covered with the temporal muscle, the skin incision was sutured. These procedures were carried out under sterile conditions using a dissecting microscope.

At intervals of 3, 5, 7, 10, 14, and 21 days after the operation, groups of 3 animals were killed by perfusion through the left cardiac ventricle with phosphate-buffered 1% glutaraldehyde-3% paraformaldehyde, pH 7.3, for 10 min at room temperature and further fixed in the same fixative for 24 h. After the brains had been removed and coronally sliced, tissue samples including the lesion were immersed in 1% osmium oxide, dehydrated with an ethanol series, and embedded in epoxy resin. Semithin sections (~ 1 μm) were stained with toluidine blue and safranin, and used for selection of appropriate areas for ultrathin sectioning. These sections were finally contrasted with 1% uranyl acetate and 1% lead citrate, and viewed with a transmission electron microscope (TEM; Hitachi 11B, 75 kV).

Precise statistical analysis for evaluating the chronological and locational frequency of seamless endothelial cells (SEs) in the present study was impossible because of the small number of animals used and the various sizes of infarcts induced (Chen et al. 1986). However, an estimate of the preliminary number of SEs in lesions seemed to be important in order to clarify their gross frequency.

By electron microscopy, cross-sectioned regenerating capillaries from 84 to 120 in each group were carefully observed in the reactive zone, and SEs that satisfied the criteria described by Bär et al. (1984) were counted.

Results

The cerebral infarcts induced by MCA occlusion were distributed in the fronto-parietal region, and varied in size from 2.0 to 10 mm in diameter (3.0 mm in most cases). At coronal cut surfaces, each lesion at the center involved all neuronal layers of the cortex and to a lesser extent the underlying white matter. Ammon's horn and basal ganglia were spared. Tissue samples taken from the lesion through the center were subjected to the following studies.

The histology of infarcts was subdivided into three zones by Garcia and Kamijo (1974); the central, reactive and marginal zones. Three days after MCA occlusion, the lesions are composed of a central zone with coagulation necrosis and a marginal zone with a spongy neuropil as well as perivascular and perineuronal vacuoles. In the marginal zone, a few granulocytes and macrophages and some small vessels with a diameter larger than that of capillaries in the surrounding cortex are noticed.

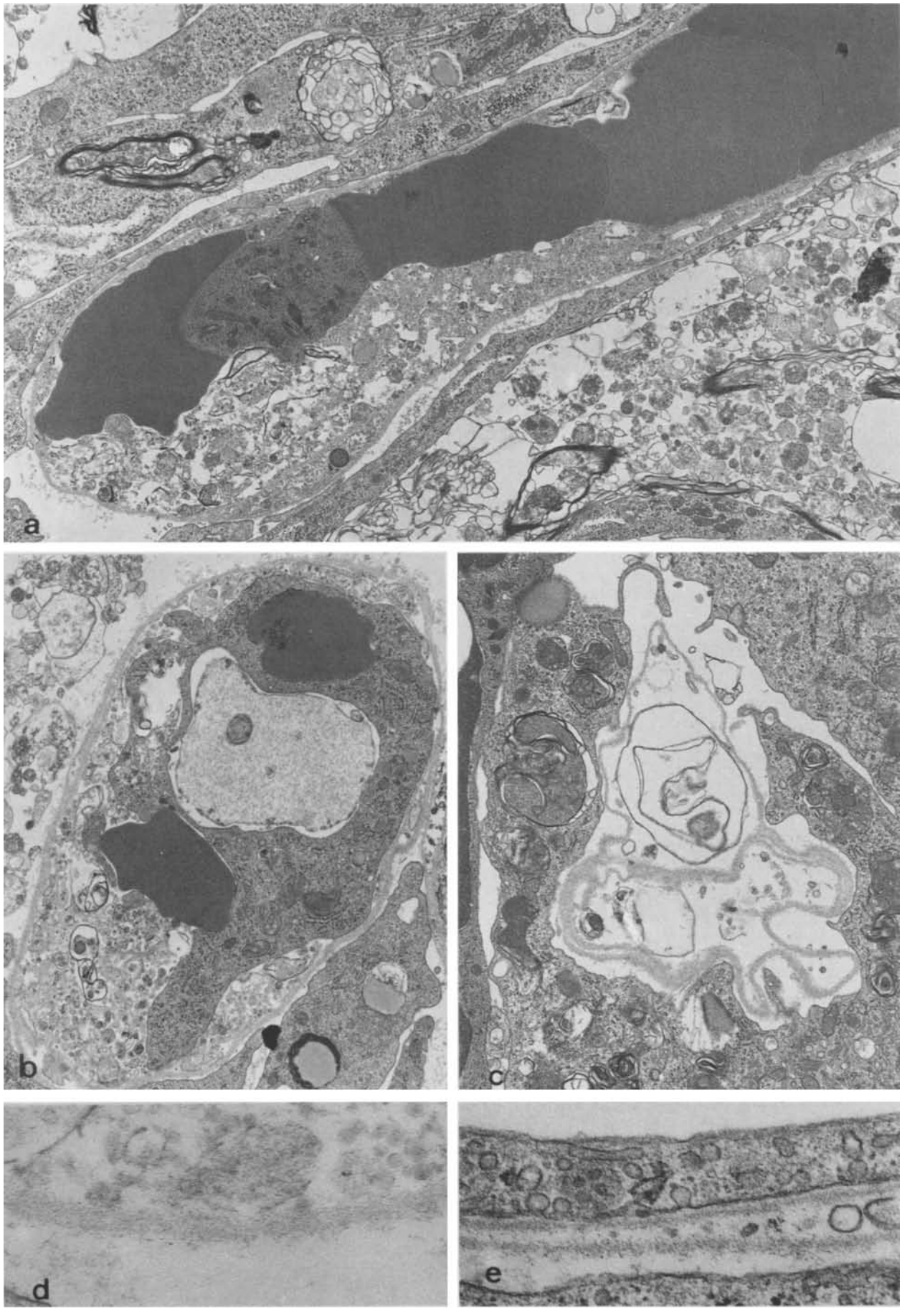
On the fifth and seventh days after MCA occlusion, the marginal zone becomes ill-defined, and a reactive zone located between the central zone and surrounding brain tissue becomes recognizable filled with numerous macrophages and proliferated blood vessels. These blood vessels have irregular vascular lumina, many arborizations and hypertrophic endothelial cells with prominent nuclei, indicative of reactively regenerating capillaries. Some of them show definite connection with surrounding brain capillaries or with leptomeningeal venules. The highest density of these vessels in the reactive zone occurs between 7 and 10 days after MCA occlusion.

On the 14th and 21th postoperative days, most lesions show atrophy and a loose texture with astrocytic gliosis. The number of macrophages decreases, and the vascular density is also reduced in the reactive zone. Reactive capillaries have a smaller caliber than vessels previously observed, and show disappearance of their tortuosity, indicating the process of vessel regression.

On electron microscopy, within 3 to 5 days after MCA occlusion, endothelial cells in the marginal or reactive zone show ultrastructural signs of degenerative and regenerative alteration.

The former type of change is recognizable by the presence of pyknotic nuclei, disintegrated cell organelles and interrupted cytoplasmic membranes (Fig. 1 a), together with some macrophages phagocytosing the cell debris (Fig. 1 b). These changes are also evident in pericytes. The normal trilayered structure of the endothelial BM, consisting of an inner lamina rara, a lamina densa and an external lamina rara (Bär and Wolff 1972; Abrahamson 1986), becomes obscure and changes to a homogeneous band (50–100 nm thick) of electron-dense

Fig. 1. Degenerating endothelial cells in the reactive zone of a cerebral infarct 5 days after MCA occlusion. (**a**) Inside the basement membrane (BM), blood cells and disintegrated cell debris of endothelial cells and pericytes are present without any extracellular space. TEM ×9400. (**b**) Cell processes of a haematogenous cell (probably a macrophage) engulfing cell debris, a disorganized nucleus and red blood cells. Note absence of evidence of fragmentation or interruption of the BM. TEM ×12000. (**c**) Remnant of endothelial BMs with a wavy appearance and multilayered profiles in the extracellular space of the lesion. A macrophage is partly enveloping the BM. TEM ×13000. (**d**) The BM covering the disorganized endothelial cells, showing a homogeneous band of electron-dense materials which is obscure the trilayered structure. TEM ×60000. (**e**) The BM enveloping regenerated endothelial cells. Note the inner BM with the trilayered structure. TEM ×60000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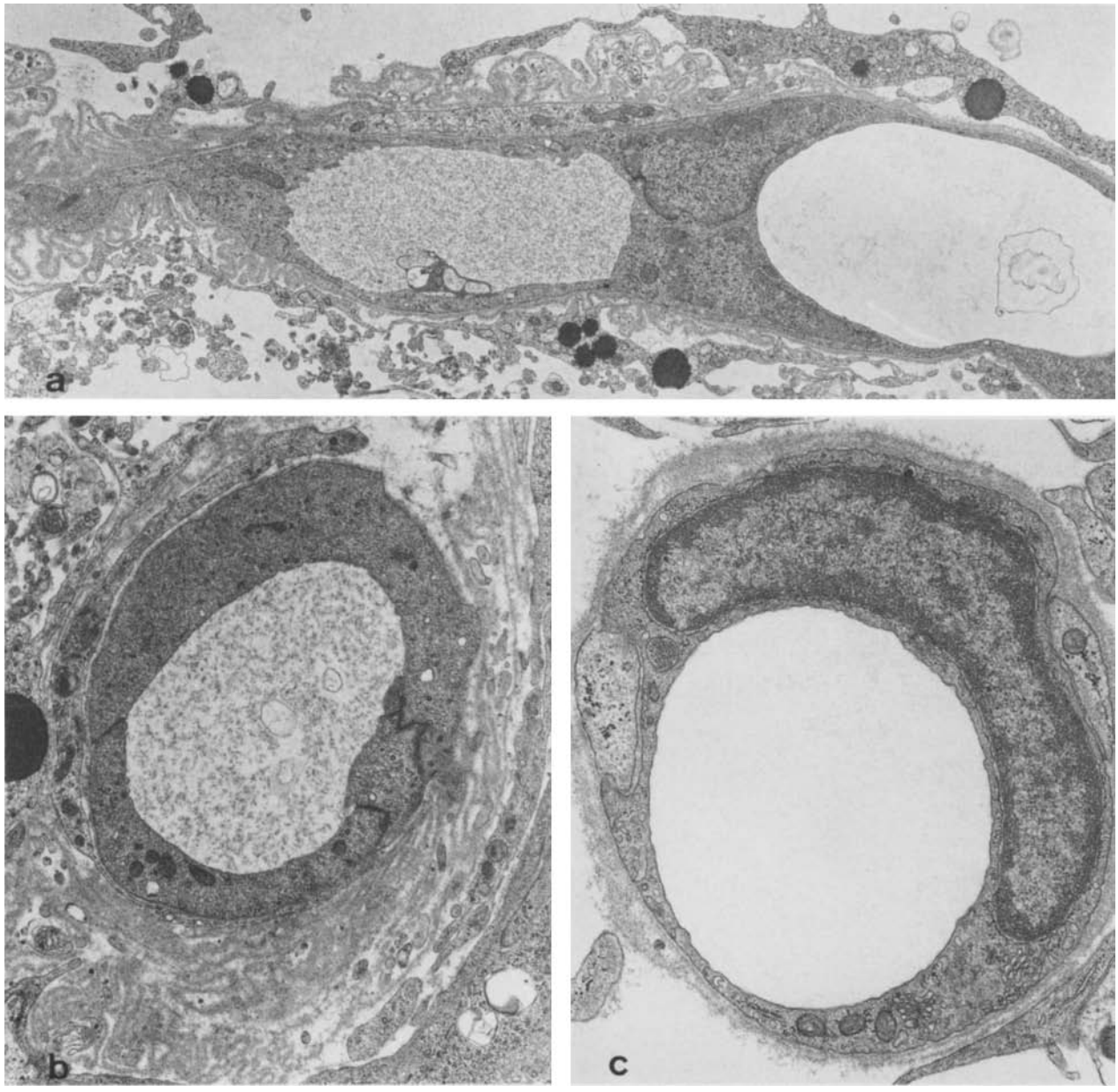

Fig. 2. Regenerating capillaries in the reactive zone of infarcts 7 days (**a** and **b**) and 10 days (**c**) after MCA occlusion. (**a**) A tangential section of the capillary shows an immature endothelial cell with electron-dense cytoplasm, a sprouting bud and apparently two luminal cavities, one of which on the left side contains some fluid material, probably indicating lack of established circulation. The BM with a wavy appearance at the sprouting portion and with a thickened structure at the perikaryon can be seen. TEM ×6800. (**b**) Cross-section of a capillary, showing irregularly waved and multilayered BM. The inner layer seems to have a trilayered structure, like the newly formed BM of the regenerated endothelial cell. TEM ×12000. (**c**) A regenerating endothelial cell with rich cytoplasm and an irregular BM band. This cell is seamless. TEM ×19600

material. However, it does not show any interruption or fragmentation, and remains free in the extracellular spaces that are filled with oedema fluid (Fig. 1c and d). In many cases, the wavy or circular BM contains a small amount of cell debris or forms an empty cavity. The outside of the BM is partly enveloped by the processes of macrophages. During this immediate postoperative period, the reactive astrocytes do not possess a BM. The wavy BMs are clearly different in their location and wavy profile from the astrocyte BM, which appears in 7- to 10-day-old lesions and which envelops the vascular surface of astrocyte processes with a trilayered structure.

In the latter, endothelial cells are evident with prominent nuclei (sometimes two per cell) and thick cytoplasm containing rich organelles such as plasmalemmal vesicles and ribosomes. In tangentially sectioned vessels, some newly formed vascular lumina are located near the sprouting portion of the cytoplasm, and are not perfused by the fixative, the wall showing no intercellular contacts (Fig. 2a). The BM shows complex features. In the sprouting portion, at least, two layers are visible. The outer layer has a peculiar wavy pattern and sometimes shows no attachment to the endothelial cell. Inside the lamina, a small amount of cell debris can be seen, but no collagen fibrils. The inner BM usually exists along the endothelial surface and shows a trilayered appearance like that of a normal BM (Fig. 1e). The outer and inner BMs often fuse, consequently forming a thick homogeneous band near the perikaryon.

In cross-section, the endothelial BM shows a more complex profile consisting of several layers (Fig. 2b). However, the innermost layer appears to be a newly formed endothelial BM on the basis of its presence along the cell surface and its trilayered structure. The other layers are wavy and often show elongation to distant areas, corresponding to the outer BM observed in tangential sections. These regenerating capillaries frequently show SEs on cross-section. The frequency of SEs examined ranges from 23% to 42% among the various groups (Table 1).

Table 1. Frequency of seamless endothelial cells of regenerating capillaries in the reactive zone

	5	7	10	14	21 days*
Total no. of regenerating capillaries	84	120	92	106	116
No. of seamless endothelial cells	19	34	30	38	49
%	23	28	33	35	42

* After MCA occlusion

Within 7 to 10 days after the operation, many regenerating capillaries are enveloped by the processes of reactive astrocytes, which also extend into the extracellular spaces, dividing them into many compartments. Many endothelial cells are still hypertrophic, and degenerated cells are rarely observed in the reactive zone. Endothelial BMs show multiple layers in cross-section, and the remnant BM is not noticeable.

On the 14th to 21st postoperative days, endothelial cells in the reactive zone with a loose texture become thinner and have cytoplasm with an almost normal appearance, although plasmalemmal vesicles are still abundant at the periphery of the perikaryon. Endothelial fenestrae are evident in the cy-

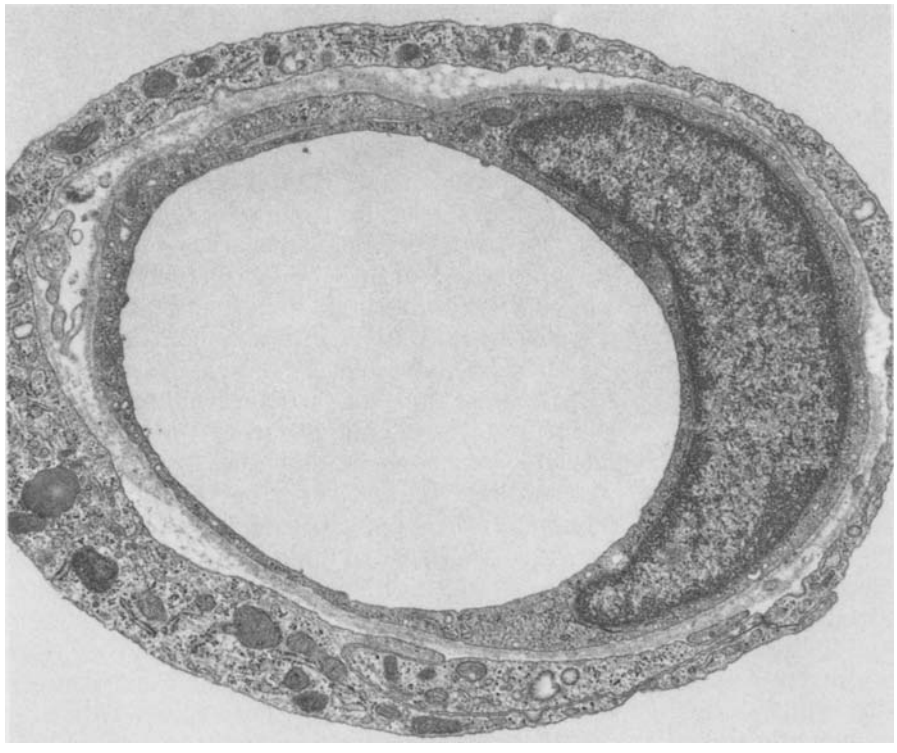

Fig. 3. A regenerating capillary observed in a 21-day-old infarct. A seamless endothelial cell is shown, with a moderately hypertrophic nucleus and irregular BM. TEM ×14700

toplasm of venules located in the superficial portion of 14-day-old lesions. The endothelial BMs are still thick, but the multilayered appearance has become obscure. Pericytes enveloped by a BM are hypertrophic and have electron-lucent cytoplasm and prominent nuclei.

Among these vessels, SEs are recognized more frequently than among younger vessels (Fig. 3 and Table 1).

Discussion

Endothelial swelling as an initial degenerative change in the affected area of cerebral infarcts has been noticed 5 h after anoxic ischaemia in rats (Hills 1964) and 12 h after the MCA occlusion in monkeys (Garcia et al. 1971). These endothelial cells subsequently undergo advanced necrosis with extensive alteration of cytoplasmic organelles and nuclei, and disappear up to 7 days after injury (Garcia et al. 1971). However, descriptions of the endothelial BM in these studies were unfortunately fragmentary. In association with endothelial cell damage, the BM loses its trilayered pattern and changes into a dense amorphous band that varies in thickness (Hills 1964; Calhoun and Mottaz 1966). In addition to these changes, the present study revealed that the BM was not phagocytosed simultaneously with the endothelial debris and that it remained in the extracellular space, although it might finally have disappeared. In fact, no remnant of the BM was observed in lesions 10 days old or more.

However, regenerating capillaries were recognized in the reactive zone. In previous studies, Hills (1964) observed endothelial hypertrophy occurring within 48 h, and Garcia et al. (1971) pointed out the beginning of capillary reconstruction at 7 days. Our results showed that microscopic hypertrophy of endothelial cells began at 3 days after injury. This time difference in the onset of regeneration may vary with the pathogenesis of the lesions induced in experimental animals, or the species of experimental animals employed.

Associated with this capillary regeneration, it has been shown that the BM takes on a reduplicated profile or a wavy form (Calhoun and Mottaz 1966; Brierley and Brown 1981). The significance of the altered BM, however, remains unresolved. The present study indicated the possibility that the remnant BM might play an important role in the growth of endothelial buds, for the following reason. Although newly formed endothelial cells in cross-section are usually associated with multiple BMs, they can be divided into outer and inner layers in tangential section. At the sprouting portion a peculiar wavy profile of the outer BM is noticeable, and a single, but thickened BM is predominant at the perikaryon, where the outer and inner basal laminas are often fused. In addition, the outer basal lamina usually shows a homogeneous electron-dense band, which is extremely similar to that of the remnant BM. Only the innermost BM has a trilayered profile like that seen in the normal structure. Therefore, most of the multiple BM around the endothelial cells appearing in cross-section may indicate wavy infoldings of the outer BM, which is ultrastructurally derived from the remnant BM. Thus, in order to elucidate the precise mechanism of capillary regeneration in lesions, it seems necessary to conduct further studies including endothelial cell kinetics or analyses of constitutional macromolecules of the BM, such as type IV and VI collagens, laminin, fibronectin and proteoglycan (Laurie et al. 1983; Abrahamson 1986; McComb et al. 1987; Salonen et al. 1987), under pathological conditions.

With regard to capillary growth into the lesion, Mitchell et al. (1978) observed the process of repair of cold lesions induced in mice and pointed out that newly forming capillaries grew predominantly into the lacunae composed of astrocyte processes and lined by their BM. Regenerating vessels are known to be usually enveloped by the processes of macrophages and/or reactive astrocytes (Mitchell et al. 1978; Brierley and Brown 1981), and we were able to confirm these findings. However, the lacunae were not recognizable 5 to 7 days after MCA occlusion, when the most active capillary regeneration and ingrowth in the lesion were observed.

The present study demonstrated for the first time that SEs were formed in regenerating capillaries. The incidence of SEs appearing in the reactive zone ranged from 23% to 42%. These values are similar to those observed in normal adult rat brain (Oldendorf et al. 1977; Bär et al. 1984).

The SEs present in the vascular system seem to be at least involved in the mechanism of endothelial growth and formation of the vascular lumen. Generally, most capillaries in cross-section observed by electron microscopy have one to four interendothelial contacts (tight junctions), forming the vascular lumen. In the process of capillary formation in the normal brain, the capillary buds (sprouting sites) are composed of a number of endothelial cells and their lumina are derived from local dilatation of the intercellular clefts between these cells (Schoefl 1963; Ashton et al. 1972).

However, when capillary buds arise in a single

endothelial cell, tube formation for the vascular lumen is restricted to only part of the cytoplasm. In such cases, the resulting capillaries are seamless (Clark and Clark 1939; Wolff 1964). Thus, the capillary lumen is constituted by dual structural designs (Bär et al. 1984). According to a chronological study of capillary development, the frequency of SEs was increased in association with the formation of late-arising-type capillaries, indicating that SE formation is also related to the intensive formation of true capillaries (Wolff et al. 1972). By using the silver nitrate perfusion method which stains the endothelial margin, the SEs were found to be mainly located at the junctional portions of capillary networks, where many capillary branchings and capillary-venule junctions were established during the late stage of development.

The fact that SEs are present in regenerating capillaries may suggest that the regeneration mechanism involved in lesion repair is similar to developmental angiogenesis, indicating that a dual capillary structure is necessary for the formation of the reactive vascular network.

With regard to reactive neovascularization, Burger et al. (1983) have obtained interesting findings. They observed corneal neovascularization toward a lesion induced in the center of the cornea by silver nitrate cauterization, and showed that the first new vascular buds emerged from the pericorneal venules and capillaries using a scanning electron microscopy of vascular casts. They emphasized that not only capillaries but also venules were an important source of new vessels. Similar results were observed by Beggs and Waggener (1978), who studied traumatic lesions in cats by electron microscopy.

Although we do not yet have any direct evidence to indicate that the sprouting originated from venules, the histology including ultrastructural features showed that many regenerating capillaries were connected to dilated venules in both the reactive zone and the leptomeninges covering the lesion. Up to now, it has been thought that capillary sproutings may connect with other capillaries or venules (Wolff et al. 1972; Bär et al. 1984). However, in the regenerating vessels, at least, venules may play an important role as the site or origin of vascular buds, in addition to capillaries.

In conclusion, the present study showed two different findings. The BM and SEs appeared in degenerating and subsequent regenerating capillaries. The SEs, which exhibit a dual structure in the process of endothelial growth and tube formation in normal angiogenesis, are also recognizable in reactively regenerating capillaries in the reactive zone of experimental cerebral infarcts indicating that the regenerating process of reactive blood vessels is similar to that of normal development. The remnant BMs resulted from endothelial degeneration are apparently different from the BM-like materials which are seen in normal developing endothelium (Bär and Wolff 1972), but may play a role as guide tubes for the ingrowth of regenerating endothelial cells. Reactively proliferating microvasculature may prove to be an excellent model as the developing brain for elucidating the biological mechanism of vascular regeneration.

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References

Abrahamson DR (1986) Recent studies on the structure and pathology of basement membranes. J Pathol 149:257–278

Ashton N, Tripathi B, Knight G (1972) Effect of oxygen on the developing retinal vessels of rabbits. I. Anatomy and development of the retinal vessels of the rabbit. Exp Eye Res 14:214–221

Bär T, Wolff JR (1972) The formation of capillary basement membranes during internal vascularization of the rat's cerebral cortex. Z Zellforsch 133:231–248

Bär T, Güldner FH, Wolff JR (1984) "Seamless" endothelial cells of blood capillaries. Cell Tissue Res 235:99–106

Beggs JL, Waggener JD (1978) Posttraumatic vascular regeneration in the spinal cord: The growth sequence and permeability properties of new vessels. J Neuropathol Exp Neurol 37:589

Blakemore WF (1971) The ultrastructural appearance of astrocytes following thermal lesions of the rat cortex. J Neurol Sci 12:319–332

Brierley JB, Brown AW (1981) The origin of lipid phagocytes in the central nervous system: I. The intrinsic microglia. J Comp Neurol 211:397–406

Burger PC, Chandler DB, Klintworth GK (1983) Corneal neovascularization as studied by scanning electron microscopy of vascular casts. Lab Invest 48:169–180

Calhoun CL, Mottaz JH (1966) Capillary bed of the cerebral cortex. The fine structure in experimental cerebral infarction. Arch Neurol 15:320–328

Chen ST, Tsu CY, Hogan EL, Maricq H, Balentine JD (1986) A model of focal ischemic stroke in the rat: Reproducible extensive cortical infarction. Stroke 17:738–743

Clark ER, Clark EL (1939) Microscopic observations on the growth of blood capillaries in the living mammal. Am J Anat 64:251–299

Garcia JH, Cox JV, Hudgins WR (1971) Ultrastructure of the microvasculature in experimental cerebral infarction. Acta Neuropathol 18:273–285

Garcia JH, Kamijo Y (1974) Cerebral infarction. Evolution of histopathological changes after occlusion of a middle cerebral artery in primates. J Neuropathol Exp Neurol 15:320–328

Güldner FH, Wolff JR (1973) Seamless endothelia as indicators of capillaries developed from sprouts. Bibl Anat 12:120–123

Hills CP (1964) Ultrastructural changes in the capillary bed of the rat cerebral cortex in anoxic ischemic brain lesions. Am J Pathol 44:531–551

Ikuta F, Yoshida Y, Ohama E, Oyanagi K, Takeda S, Yamazaki K, Watabe K (1983) Revised pathophysiology on BBB damage: The edema as an ingeniously provided condition for cell motility and lesion repair. Acta Neuropathol (Suppl) VIII:103–110

Klatzo I (1967) Neuropathological aspects of brain edema. J Neuropathol Exp Neurol 26:1–14

Laurie GW, Leblond CP, Martin GR (1983) Light microscopic immunolocarization of type IV collagen, laminin, heparan sulfate proteoglycan and fibronectin in the basement membranes of a variety of rat organs. Am J Anat 167:71–82

McComb RD, Moul JM, Bigner DD (1987) Distribution of type VI collagen in human gliomas: Comparison with fibronectin and glioma-mesenchymal matrix glycoprotein. J Neuropathol Exp Neurol 46:623–633

Mitchell J, Weller RO, Evans H (1978) Capillary regeneration following thermal lesions in the mouse cerebral cortex. Acta Neuropathol 44:167–171

Oldendorf WH, Cornford ME, Brown WJ (1977) The large apparent work capability of the blood-brain barrier: A study of the mitochondrial content of capillary endothelial cells in brain and other tissues of the rat. Ann Neurol 1:409–417

Persson L, Hansson HA (1976) Reversible blood-brain barrier dysfunction to peroxidase after a small stab wound in the rat cerebral cortex. Acta Neuropathol 35:333–342

Reese TC, Karnovsky MJ (1967) Fine structural localization of a blood-brain barrier to exogenous peroxidase. J Cell Biol 34:207–217

Salonen V, Peltonen J, Röyttä M (1987) Laminin in traumatized peripheral nerve: Basement membrane changes during degeneration and regeneration. J Neurocytol 16:713–720

Schoefl GI (1963) Studies on inflammation. III. Growing capillaries: Their structure and permeability. Virchows Arch [A] 337:97–141

Tamura A, Graham DI, McCulloch J, Teasdale GM (1981) Focal cerebral ischemia in the rat: I. Description of technique and early neuropathological consequences following middle cerebral artery occlusion. J Cereb Blood Flow Metab 1:53–60

Wolff JR (1964) Ein Beitrag zur Ultrastruktur der Blutkapillaren: Das nahtlose Endothel. Z Zellforsch 64:290–300

Wolff JR, Bär T (1972) 'Seamless' endothelia in brain capillaries during development of the rat's cerebral cortex. Brain Res 41:17–24

Wolff JR, Moritz A, Güldner FH (1972) 'Seamless' endothelia within fenestrated capillaries of duodenal villi (rat). Angiologica 9:11–14

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