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The immature endothelial cell in human glioma. Ultrastructural features of blood capillary vessels

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Abstract

New vessel formation is a prerequisite for the growth of a tumour mass. Growing evidence suggests that endothelial progenitor cells circulate in the blood and participate in that process. The purpose of the present study was ultrastructural and electron microscopic immunocytochemical examination of capillary blood vessels in human glioma. The results showed striking morphological changes in these vessels. Our observations indicate that tumours build vessels by cooption of pre-existing vasculature and de novo recruitment of endothelial progenitor cells. Immature endothelial cells characterized by fibrils in the cytoplasm and Flk-1 positive immunoreactivity were observed as small clusters or luminally localized individual endothelial progenitor cells that participate in intussusceptive vessel growth. This observation indicates that the tumour microenvironment determines biological and functional attributes of endothelial progenitor cells.

Key words: *endothelial progenitor cell, glioma angiogenesis, long-spacing collagen.*

Introduction

Tumour angiogenesis is a fast growing domain in tumour biology. The sprouting of new vessels out of pre-existing ones was considered as an exclusive way of tumour vascularisation. There are currently three hypotheses about the development and origin of tumour endothelial cells [8]. The first is that they develop from normal endothelial cells recruited into the tumour site from adjacent normal tissue. The second hypothesis is that tumour endothelial cells develop from an endothelial progenitor cell migrating to the tumour site and differentiated into vessels. This hypothesis is based on evidence that some tumour endothelial cells express markers of endothelial progenitor cells. A third theory is that tumour-associated endothelial cells develop from dedifferentiated tumour cells, and hence retain several of their properties (vasculogenic mimicry). At this time, it is likely that the tumour vasculature has multiple origins.

Electron microscopic analysis of the capillary endothelial proliferation in gliomas indicated that

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the blood vessels within the tumour contained essentially immature capillaries [7]. Previously, we studied by electron microscopic ultrastructural and immunocytochemical methods neovascularization with participation of characteristic immature endothelial cells (endothelial progenitor cell) [1,3]. These cells were characterized by endothelial-like features with fibrils in the cytoplasm. We showed that this class of endothelial-like cells contributes to new vessel formation. Our morphological and immunocytochemical studies showed different stages of new vessel formation.

The present study concerns the multistep nature of microvascular recruitment of ex vivo-expanded embryonic endothelial progenitor cells during tumour angiogenesis described by Vajkoczy et al. [11]. Using intravital fluorescence videomicroscopy the authors define the specific process of endothelial progenitor cell homing and incorporation into a functional vascular network.

Material and Methods

The patient Z.A., 68 years old, was admitted to the Neurosurgical Department of the Polish Academy of Science due to a brain tumour in the left hemisphere in the temporo-parietal region. The patient was transferred from the Internal Diseases Department where he was hospitalized for atrial fibrillation.

In brain tomography there was diagnosed, in the left cerebral hemisphere, a cystic shape tumour of 38 × 60 × 48 mm with an oedema around it, causing a mass effect exerting pressure on the left cerebral ventricles and shift of the midline structure of the brain. The tumour, in CT after contrast administration, is becomes more intense circumferentially.

During admission time, symptoms of psycho-organic syndrome, amnestic aphasia and small motor deficit of the right extremities were observed.

It was decided that, due to his neurological disease, he would undergo operative treatment. The anaesthesia risk was assessed at grade III/IV. After informing the patient about the high risk of the surgery we obtained his consent. Treatment with the drug acenocumarol was discontinued and was substituted by prolonged administration of fraxiparin during the perioperative period. When coagulation parameters were normalized the surgery was performed. We performed left craniotomy and partial resection of the tumour – morphologically glioma. The tissue was taken for histopathology examination and the small removed fragment was sent for ultrastructural morphological studies. The patient, after the surgery, was in good condition, conscious; his clinical condition, from the neurological point of view, did not change.

Material was processed for ultrastructural morphological studies and for immunocytochemical studies in transmission electron microscopy and analyzed in JEM-1200EX as described earlier [1].

For immunocytochemical studies, tissue was fixed in a mixture of 3.5% paraformaldehyde, 0.5% glutaraldehyde in 0.1 M sodium phosphate buffer (PBS) at pH 7.4 and 20°C (30 min). Then, the material was post-fixed in the same mixture for 2 hours at +4°C. Then, it was rinsed for 2 h in PBS, treated with 1% osmium tetroxide for 1 hour, dehydrated in increasing gradients of ethanol and finally embedded in Epon. Ultrathin sections were processed according to the post-embedding immunogold procedure. Briefly, the sections were mounted on formvar-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. For single labelling monoclonal mouse antibodies against Flk-1 (sc-6251, Santa Cruz Biotechnology, USA) were diluted in 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 with colloidal gold particles of 18 nm in diameter (Jackson ImmunoResearch, USA) diluted 1:50 PBS. After incubation for 1 hour in darkness, the grids were washed with PBS for 15 min, followed by distilled water for 15 min. Ultrathin sections were air-dried, and 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-Flk-1 antibody. The sections were examined and photographed using a JEOL 1200EX electron microscope.

Results

Electron microscope ultrastructural examination of capillaries in evaluated material revealed atypical alterations in the capillaries and the perivascular space. Among capillaries present in the analyzed material most of them possessed ultrastructural features of young blood vessels or newly formed ones.

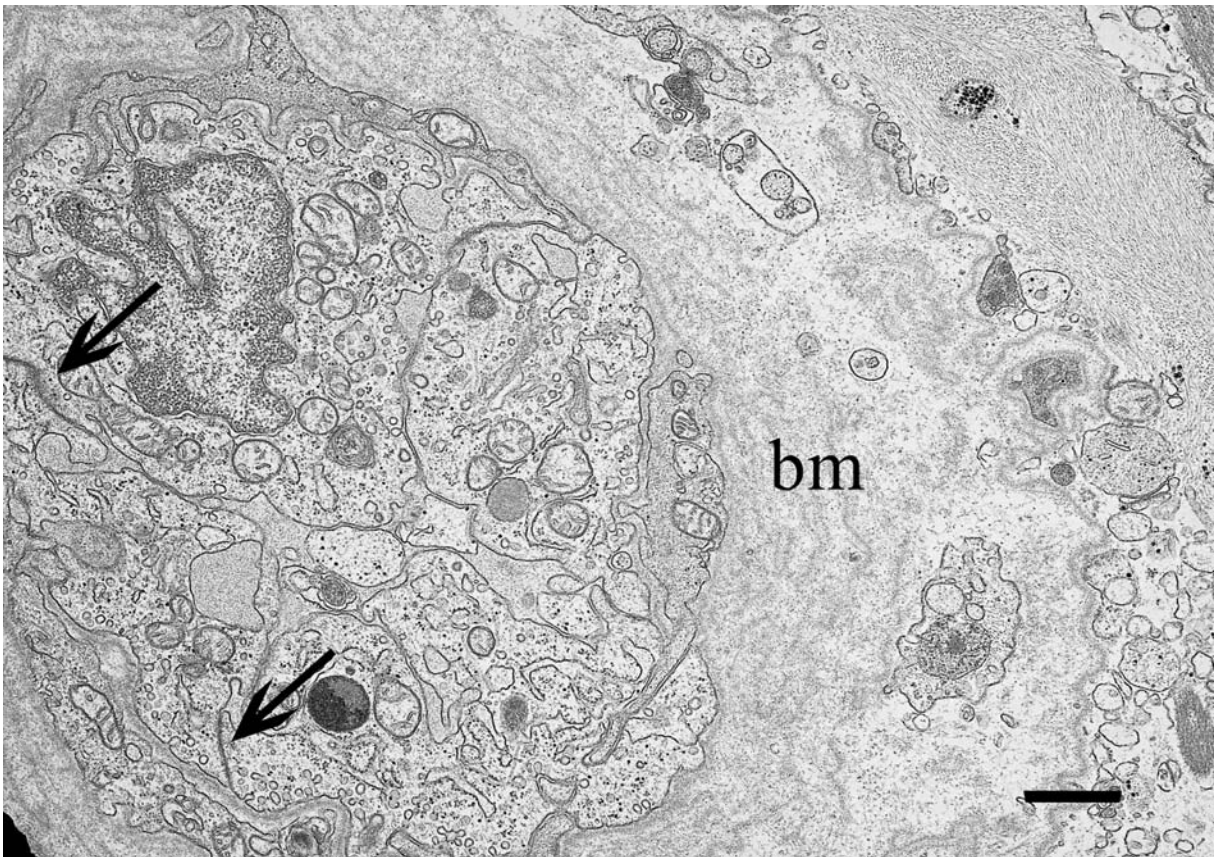

Fig. 1. Young capillary vessel characterized by narrow lumen and hypertrophic endothelial cells is observed. Zonulae occludens (arrows) are seen between endothelial cells. Basement membrane-like material (bm) formed from amorphic substance with collagen fibrils of varied transverse diameter is present around capillary. Bar = 1 µm

The capillaries, mostly composed of high, hypertrophic endothelium, were often characterized by a narrow lumen. They formed clusters surrounded by irregular basal laminae, and large extravascular spaces. Between adjacent endothelial cells zonulae occludentes frequently were formed (Fig. 1). An unusual characteristic of capillary endothelial cells in the analyzed specimens was numerous fine fibrils, with their size corresponding to the intermediate filaments (Figs. 2, 3). These single cells characterized by endothelial-like features were often observed in the lumen of capillaries (Fig. 2) or outside capillaries (Fig. 3). The immature endothelial cells also showed unusually numerous pinocytic vesicles (Figs. 1, 2) and Weibel-Palade bodies in the cytoplasm (Fig. 4). Large nuclei with numerous indentations around their circumference were seen in these cells (Fig. 1). While screening the material we often found transendothelial extravasation of endothelial progenitors (Fig. 4).

The abluminal surface of the capillaries was surrounded by solid, markedly thickened, often branched basal membrane-like material formed from an amorphic substance. Its three-dimensional structure became indistinct, and collagen fibrils of varied transverse diameter were seen between the lamina externa and the lamina interna. Of note, marked proliferation of the basal membrane was seen, often with formation of numerous layers. Between the layers of basement membrane endothelial-like cells often were present (Figs. 1, 2).

A very interesting feature of endothelial-like cells forming new vessels in glioma is the presence of several banded structures, so-called fibrous long-spacing collagen (Fig. 5). They were spindle shaped, about 5 µm in length, and showed cross bands of 300-nm-wide intervals with fine intraperiodic bands. These collagen fibres appear in Golgi-associated vacuoles.

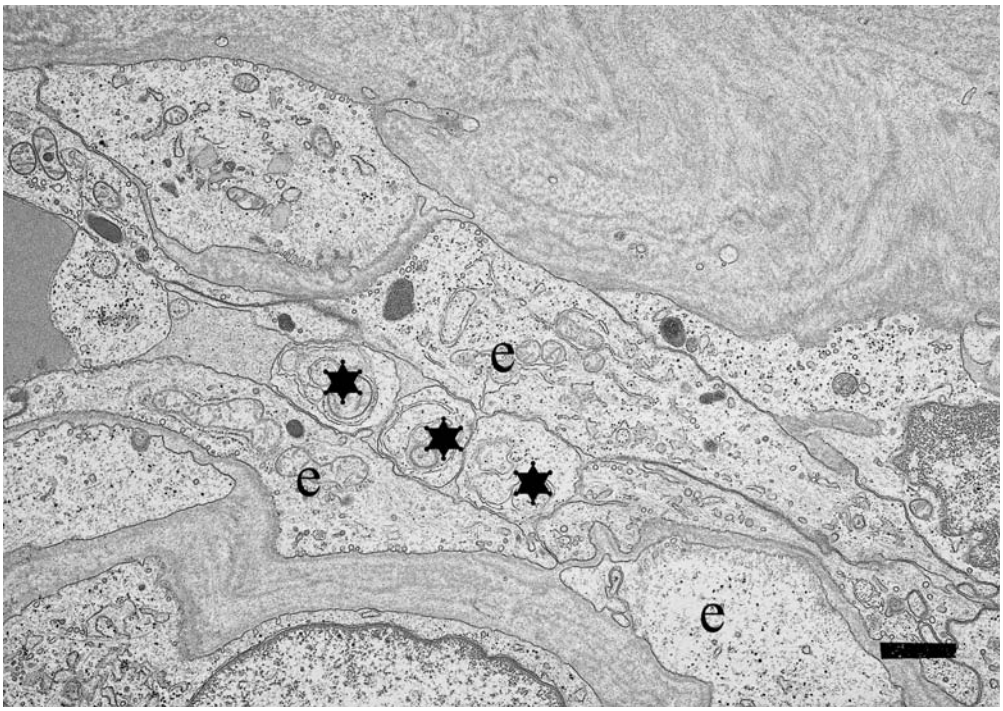

Fig. 2. Part of capillary vessel formed from endothelial progenitors (e). Immature endothelial cells are also present in the lumen of that vessel (asterisk). Bar = 1 µm

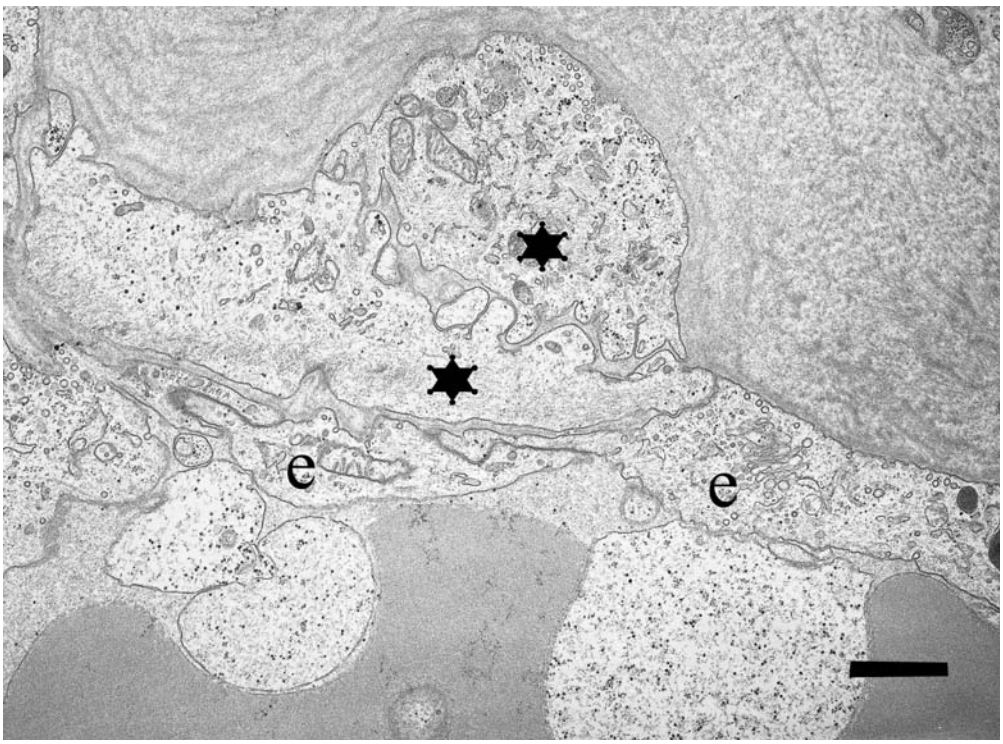

Fig. 3. Part of capillary vessel composed of immature endothelial cells (e) with fibrils in the cytoplasm. Single endothelial cells with features of endothelial progenitor are present beyond the endothelial lining (asterisks). Bar = 1 µm

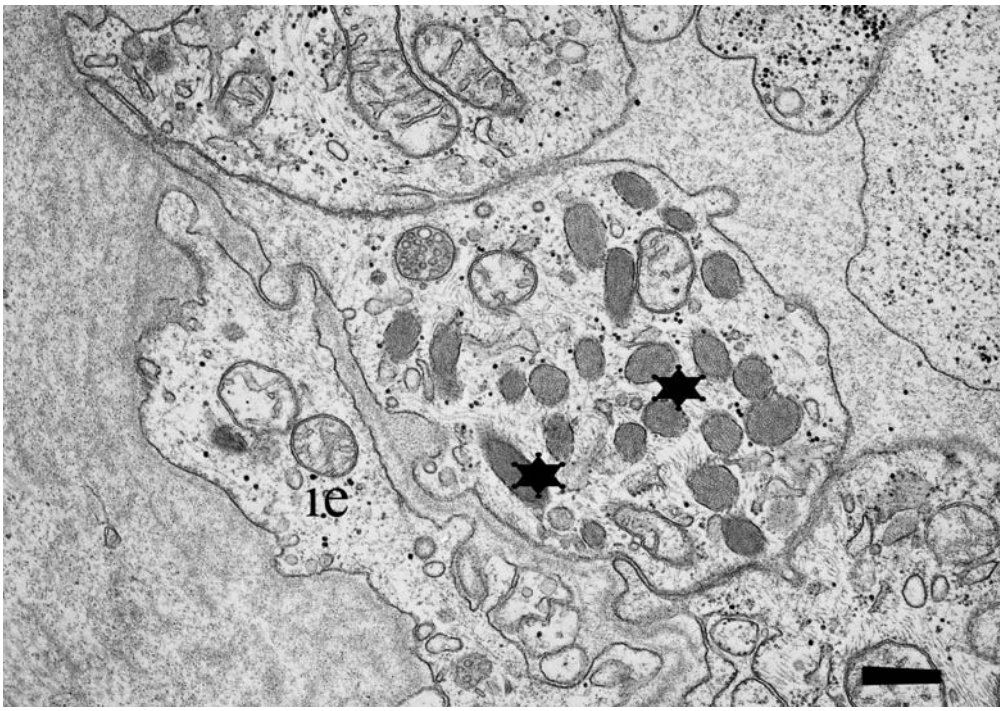

Fig. 4. Weibel Palade bodies are present inside the endothelial progenitor cell (asterisks). An immature endothelial cell is also seen in extracellular material (ie). Bar = 500 nm

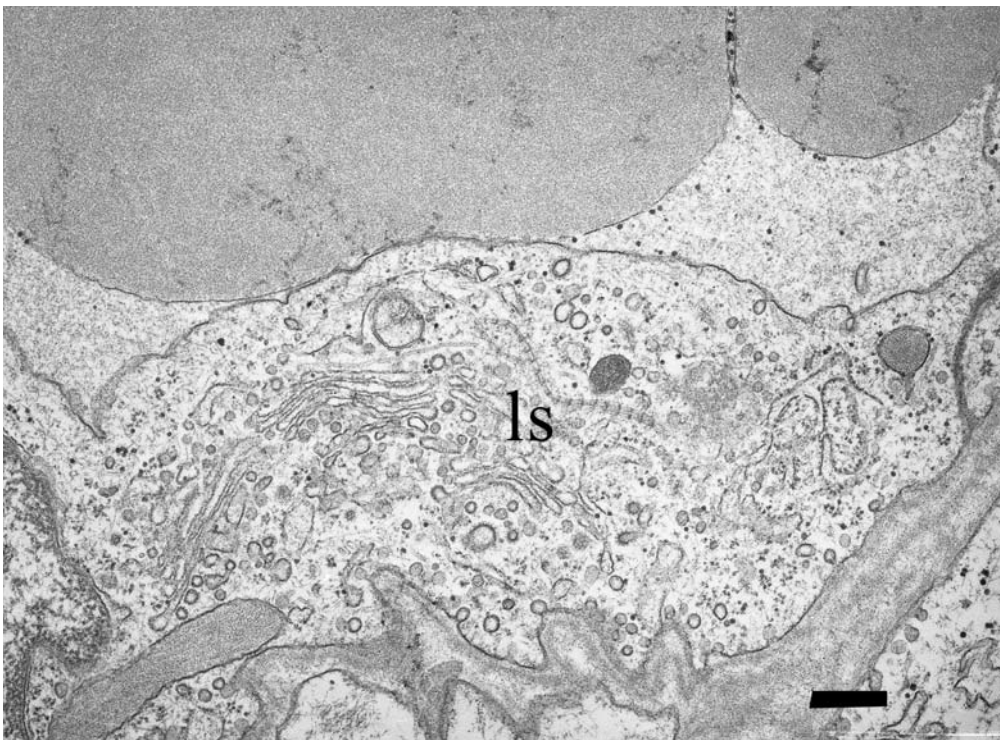

Fig. 5. Long spacing collagen (ls) in the area of Golgi zone of endothelial progenitor cell is seen. Bar = 500 nm

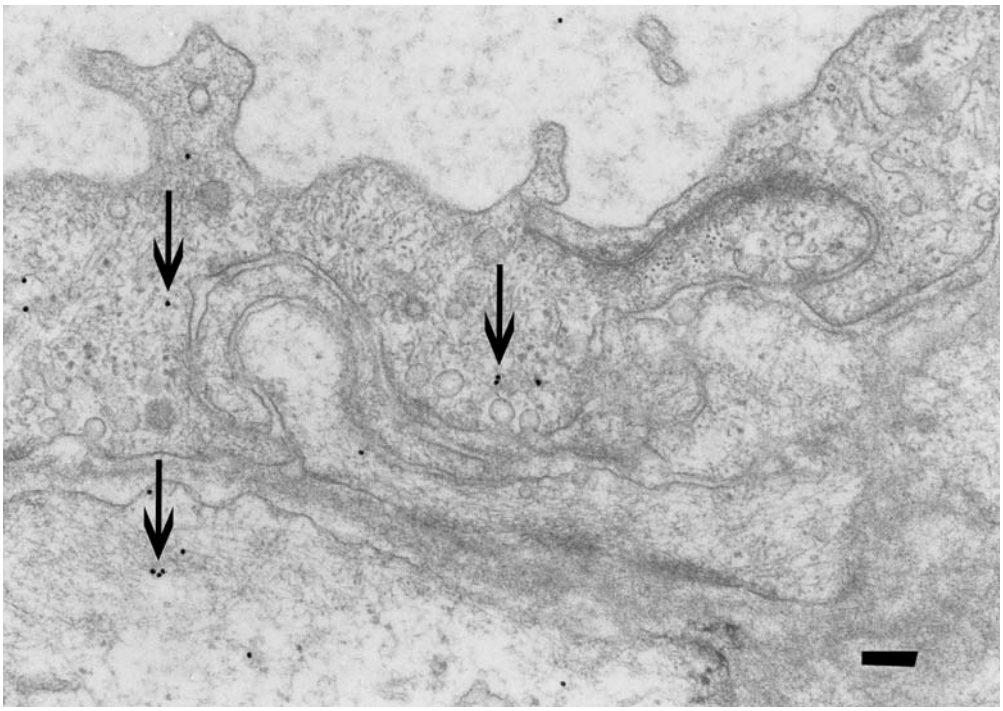

Fig. 6. Endothelial progenitor cell with Flk-1 immunoreactivity (arrows). Bar = 200 nm

Our observations of endothelial progenitors involved in new vessel formation in the space of tumour angiogenesis were confirmed by an ultrastructural immunocytochemical study. Single labelling immunoelectron microscopy revealed subcellular localization of Flk-1 in the cytoplasm of endothelial progenitor cells (Fig. 6).

In the lumen of the capillary there also occurred endothelial progenitor cells characterized by ultrastructural features different from those observed earlier. In the cytoplasm of these cells we observed some vacuoles, probably the place of the new vessel lumen (Fig. 7).

Ultrastructural features of these cells situated in the lumen of the vessel probably point to recruitment of immature endothelial cells to sites of angiogenesis.

Discussion

The purpose of the present study was to investigate the ultrastructural features of capillaries in the area of human brain glioma. The results showed atypical morphological changes in these vessels.

Gliomas are among the highest vascularized tumours. It was hypothesized that patients with gliomas have increased levels of circulating endothelial progenitor cells. The data are suggestive of increased mobilization of endothelial progenitors contributing to neoplastic vasculogenesis in glioma [12].

Electron microscopic analysis of capillary formation in this case indicated that the complex vascular structures within the tumour were composed essentially of progenitor endothelial cells. Our data show that cells with ultrastructural features of endothelial progenitors are recruited to the tumour periphery preceding vessel formation. As described by Vajkoczy et al. [11], participation of endothelial progenitors in tumour angiogenesis takes place in a multistep process, including: arrest and homing of endothelial progenitors within the angiogenic microvasculature; transendothelial extravasation into the perivascular space; extravascular formation of cellular clusters; creation of vascular sprouts; and incorporation into a functional microvasculature.

Previously, we reported that endothelial progenitors are migratory endothelial cells with characteristic ultrastructural features and the capacity to circulate, proliferate and differentiate into mature endothelial cells. They have neither acquired the characteristic markers of mature endothelium nor formed lumina before they were terminally diffe-

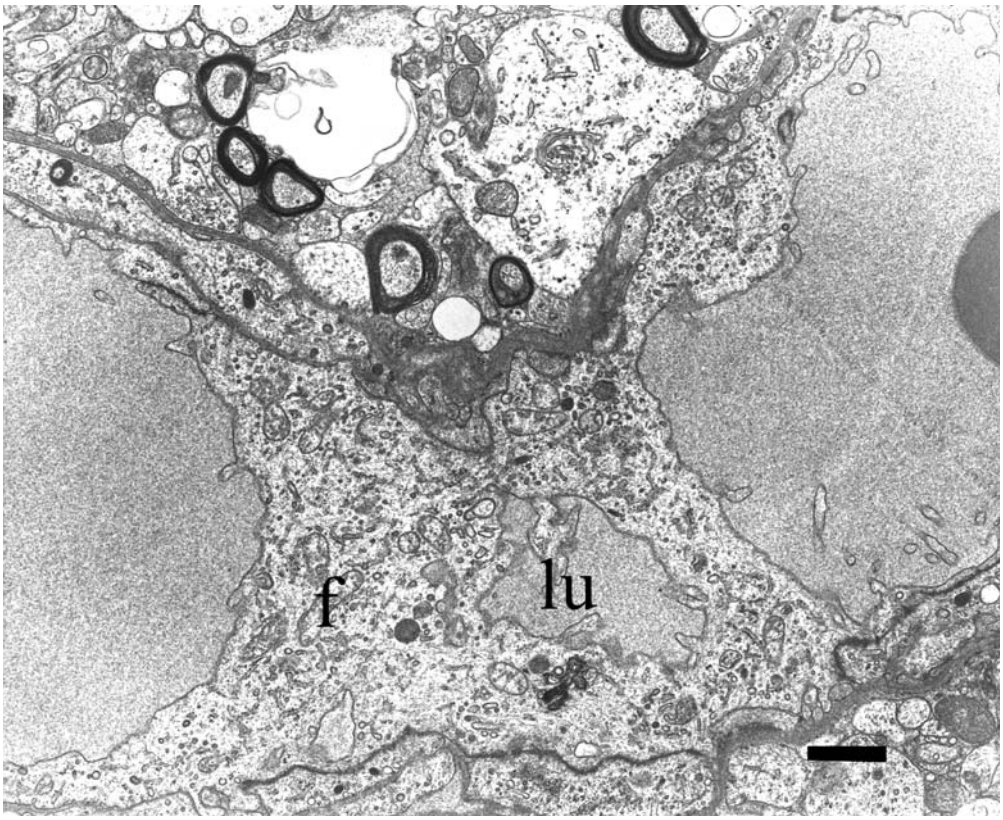

Fig. 7. Immature endothelial cell with fibrils (f) in the cytoplasm creating a bridge between two capillaries. Inside we can see a vacuole (lu); probably it is the new vessel lumen. Bar = 1 µm

rentiated [1,2]. The present study showed that endothelial progenitors could divide and form a bridge before ultrastructural features of immaturity were lost. We suppose that the glioma environment can advance maturation of endothelial progenitors before differentiation is terminated.

Ultrastructural examination of new vessel formation in this case of human glioma seems to confirm observations about two patterns of endothelial progenitor behaviour described earlier [11]: the first, cellular network formation by progenitor small clusters that were incorporated into the tumour microvasculature; and the second, individual endothelial progenitor cells luminally localized that take part in intussusceptive vessel growth.

In human glioma we observed endothelial progenitor cells characterized by long-spacing collagen in the cytoplasm. The term fibrous long-spacing collagen has been employed to designate collagen where the periodicity is markedly greater than the 64 nm periodicity of the native collagen fibril [6,9]. So far fibrous long-spacing collagen has been observed mainly in extracellular spaces [10]. The occurrence of fibrous long-spacing collagen in the cytoplasm of endothelial progenitors may express intracellular aggregation of procollagen microfibrils [4,5].

Our observations indicate that the tumour microenvironment determines the biological and functional attributes of endothelial progenitors.

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