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DIVERSITY OF IMMUNOPHENOTYPES OF ENDOTHELIAL CELLS PARTICIPATING IN NEW VESSEL FORMATION FOLLOWING SURGICAL RAT BRAIN INJURY

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Surgical brain injury causes neovascularization in the disrupted brain parenchyma, which occurs with the participation of endothelial-like cells. Differentiation of angioblasts from embryonic mesothelial cells has been proposed on the ground of biochemical and antigenic similarities between mesothelial and endothelial cells. Therefore, a transient localization of cytokeratin, the main mesothelial intermediate filament protein, to some embryonic vessels and endothelial progenitors, prompted us to use it to identify the source of cells participating in vessel formation after surgical brain injury. To determine the immunophenotypes of immature endothelial cells involved in new vessel formation following surgical rat brain injury, we used immunohistochemical and electron microscopic immunocytochemical techniques. Subcellular localization of protein markers: Flk-1, cytokeratin, and vimentin was examined in the cells investigated. Our results confirmed the existence of a diversity of immunophenotypes of immature endothelial cells in case of surgical-related brain injury.

Key words: *AC133, angiogenesis, cytokeratin, endothelial cell, Flk-1, vimentin*

INTRODUCTION

In our previous study we have investigated neovascularization in the cerebral cortex of adult rats after surgical brain injury (1, 2). Using electron microscopic ultrastructural and immunocytochemical methods we have shown that the growth and development of new blood vessels are not restricted to angiogenesis, but also encompass vasculogenesis (2). The notion that vasculogenesis is observed only

during embryogenesis has been changed, since ample evidence shows that bone marrow-derived endothelial cells are present on the site of new blood vessel formation, which points to vasculogenesis in the postnatal neovascularization (3-6).

Most studies on endothelial progenitor differentiation are concentrated on the expression of endothelial markers. Both precursor and mature endothelial cells may express similar endothelial-specific markers, including vascular endothelial growth factors receptor-2 (VEGFR-2/Flk-1) (6, 7). Our previous data also indicate the presence of this protein in the endothelial-like cells (8). Other authors have shown that Flk-1 is critical for the normal development of both hematopoietic and endothelial lineage (9). Therefore, the first objective of present study was to investigate the expression of Flk-1 in the newly-formed capillaries localized in the proximity to the lesion.

In an earlier investigation (8) we analyzed ultrastructural features and changes in the Flk-1 and VEGF expression in the process of differentiation of immature and mature endothelial cells participating in new vessel formation after surgical brain injury. We found that endothelial-like cells are committed between immature and terminally differentiated endothelial cell stage (8). We suppose that there are endothelial stem cells in the blood that are present at different stages of morphogenetic differentiation and arrested at specific 'check points' of development. Brain trauma may contribute to extravasation of endothelial-like cells and the initiation of next steps in their development.

Although there is evidence for the presence of angioblasts during embryonic development, the finding of angioblast-like endothelial precursor cells in adult circulation has been hampered by the absence of specific phenotypic markers and functional assays to define these cells population (10, 11). Differentiation of angioblasts from the embryonic mesothelial cells was proposed on the account of biochemical and antigenic similarities between mesothelial and endothelial cells (12). A transient localization of cytokeratin, the main mesothelial intermediate filament protein, to some embryonic vessels and endothelial progenitors prompted us to use this marker to identify the source of cells participating in new vessel formation after surgical brain injury. Our reasoning was strengthened by the finding of cytokeratin in endothelial cells demonstrated by other research (13-16).

A second aim of our study was to investigate the expression of cytokeratin in the vessel walls. The cytoskeleton of blood vessel wall cells contains not only cytokeratin but also vimentin (17, 18) and both proteins may be used to characterize endothelial cells (19). Thus, vimentin, aside Flk-1, was yet another marker of the endothelial cells building capillary walls, currently studied.

Immature endothelial cells incorporate into the sites of active neovascularization in animal models of ischemia and brain injury (5). AC133, an orphan receptor, is connected to endothelial progenitors. However, its expression is lost once these cells differentiate into more mature endothelial cells (7). Based on the ultrastructural identification of immature endothelial cell type, we also attempted to characterize the AC133 expression in these cells by immunogold electron microscopy.

MATERIAL AND METHODS

Surgical procedure

Study protocol was approved by a local Ethics Committee. All experimental procedures were in accord with the guidelines of the Polish Council for Animal Care and Use of Laboratory Animals.

Male adult Wistar rats (200-250g) were anesthetized with Nembutal (80 mg/kg, i.p.). Traumatic brain injury was induced in the fronto-temporal region of the cerebral cortex after scull openning as described earlier (2). In sham operated controls, the same procedure was applied except that no hemisection was done. After the operation and recovery from anesthesia, the rats remained under standard laboratory conditions for 2 or 4 days. The experimental groups consisted of 10 operated animals, 6 sham-operated and 6 non-operated rats; the latter 2 groups were used as controls.

Immunohistochemistry

Sham operated and brain-lesioned rats (2 days and 4 days after surgery) were anesthetized with Nembutal (80 mg/kg, i.p.) and perfused for 2-3 min through the ascending aorta with 0.01 M phosphate buffered saline (PBS) containing heparin, followed by ice-cold fixative (4% paraformaldehyde in 0.1 M PBS for 20 min). The brains were removed, postfixed in the same fixative for 2 h, immersed in 10% sucrose in phosphate buffer, followed by 20% and 30% sucrose solution infiltrations in the following days. Subsequently, brains were cut on a cryostat (Leica, Wetzlar, Germany) and 25 μm thick free-floating sections which were mounted on glass slides and were processed for immunohistochemistry. To reduce the background staining, sections were preincubated with 3% serum in PBS + 0.2% Triton X-100 (PBS+T) for 20 min. The following primary antibodies (Abs) were used: anti-Flk-1 (1:400), anti-vimentin (1:200), and anti-pan-cytokeratin (1:200; all obtained from Santa Cruz Biotechnology, Santa Cruz, CA).

Secondary antibodies conjugated to horse radish peroxidase (HRP), 1:1000, were used in the next step of the reaction. HRP reaction product was visualized with SG vector reagent (Vector Labs, Burlingame, CA). Between all incubations, sections were washed (3 times for 5 min each). All washings and dilutions of Abs were carried out with the use of PBS+T containing 1% serum. For negative controls the primary Abs were routinely omitted. This procedure completely eliminated the staining.

Immunocytochemistry

For immunocytochemical electron microscopic studies, material was fixed in 4% paraformaldehyde in 0.1 M PBS pH 7.4 for 2 h at 4°C. Then, it was rinsed in PBS for 30 min, treated with 1% OsO_4 for 30 min, dehydrated in sequential ethanol gradients, and embedded in Epon 812. For immunoelectron study, ultrathin sections were processed according to the postembedding immunogold procedure. Briefly, the sections were mounted on formvar-coated nickel grids, incubated in 10% hydrogen peroxide for 10 min, rinsed in water for injection and PBS for 15 min and blocked for 10 min in 1% bovine serum albumin (BSA) in PBS.

For single labeling, monoclonal mouse antibodies against Flk-1 and vimentin (sc-6251 and sc-6260, respectively; Santa Cruz Biotechnology, Santa Cruz, CA) or rabbit antibody AC133 (ab 16518; Abcam, UK) were 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 or anti- rabbit conjugated to colloidal gold particles of 12 nm (both from Jackson ImmunoResearch, West Grove, PA), in case of AC133, diluted 1:50 in PBS. After incubation for 1 h in darkness, the grids were washed with PBS for 15 min. Ultrathin sections were air-dried, stained for 10 min with 4.7% uranyl acetate and for 2 min with lead citrate.

For double labeling with mouse anti-Flk-1 (dilution 1:20) and rabbit anti pan-cytokeratin (sc-15367, Santa Cruz Biotechnology, Santa Cruz, CA) (dilution 1:20) the sections were incubated with Flk-1 overnight at 4°C and next with pan-cytokeratin for 2 h at 37°C. Both secondary antibodies (donkey anti-rabbit 12 nm) and goat anti-mouse 18 nm (both from Jackson ImmunoResearch, West Grove, PA) were applied at 1:50 dilution for 1 h at 37°C.

Control immunoelectron microscopy was performed with irrelevant primary antibodies followed by the corresponding secondary anti-mouse and anti-rabbit-labelled antibodies. In negative control the primary antibodies were omitted. No labeling was demonstrated in either type of control.

RESULTS

Light microscopy studies

In the light microscopic investigations we focused on the immunoreactivity profiles of the vessels localized close to the brain injury, since we found a massive loss of brain parenchyma around the affected tissue area. Tissue atrophy was observed in all cortical layers progressing in time down to the corpus callosum. In the cortex of sham-operated animals (not shown), intact control rats and on the contralateral side of the operated rats, we found only a weak expression of Flk-1 and vimentin in the vessel walls (*Fig. 1, Panels A and C*) and no expression of cytokeratin immunoreactivity (*Fig. 1, Panel E*). In brain parenchyma of the intact rats a small number of vimentin IR astroglial cells with regular appearance was detected (*Fig. 1, Panel C*).

At both post-injury time points examined, many Flk-1 and vimentin positive (Flk-1+ and vimentin+) vessels were observed in the area adjacent to the lesion (*Fig. 1, Panels B and D*). Deposits of immunoreactive material were localized in the vessel walls, but we also found many vimentin IR astrocytes that had a hypertrophic appearance (*Fig. 1, Panel D*). The staining observed in these astrocytes was higher than that in the control ones. Many vimentin+ astrocytes wound the immunopositive vessels. In the tissue adjacent to the lesion, the Flk-1 immunopositive neurons also were detected (data not shown). In some slices, a weak immunoreactive cytokeratin signal was detected in individual vessels. The immunostaining was observed in the outer side of vessel walls (*Fig. 1, Panel F*). light microscopic findings prompted us to examine the ultrastructural features and immunophenotypes of blood vessels in the area of lesion.

Immunoelectron microscopy studies

Electron microscopic investigations showed that the border zone of the injured region of the adult rat cerebral cortex consisted of viable parenchyma characterized with edema and leukocyte invasion. An extensive formation of new blood vessels was observed. New vessels were formed from cells that revealed endothelial-like features. Morphologically immature endothelial-like cells resembled endothelial cell as identified by their characteristic ultrastructural

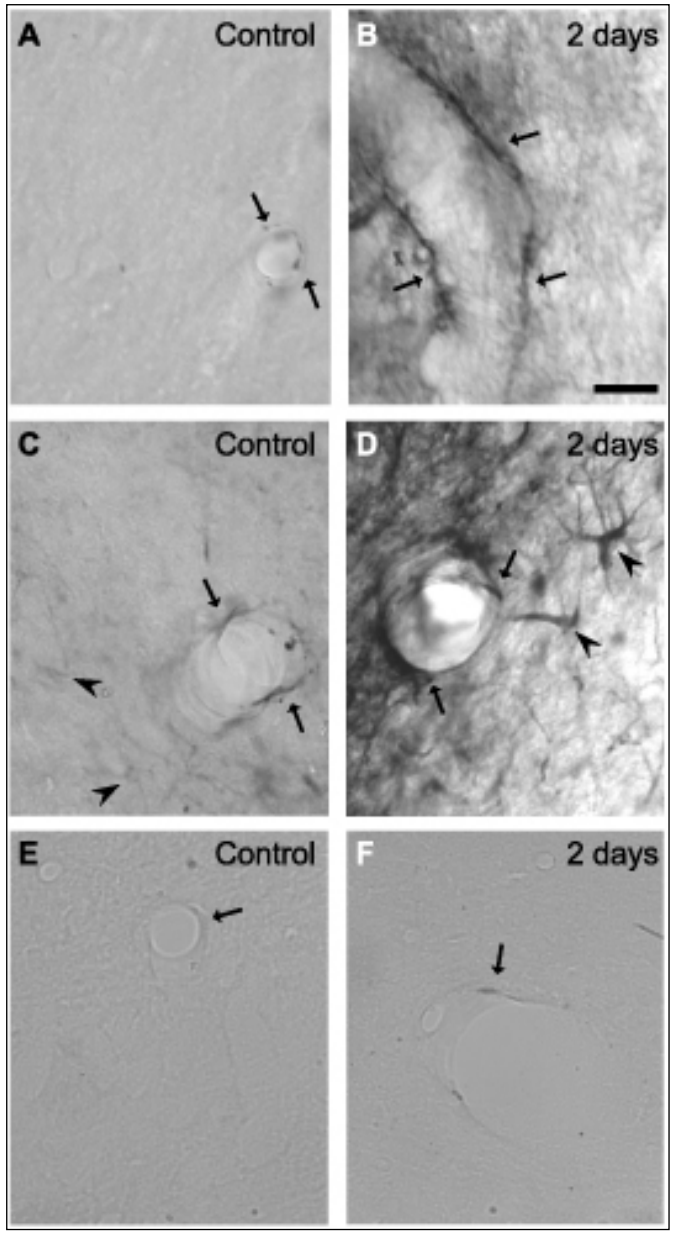

Fig. 1. Flk-1 (Panels A and B), vimentin (Panels C and D) and pan-cytokeratin (Panels E and F) immunoreactivity in the intact and injured rat cortex. Blood vessel walls (arrows) in the control cortex showed only weak immunostaining for Flk-1 (Panel A) and vimentin (Panel C). Vimentin positive astrocytes (Panel C; arrowheads) show normal appearance. All blood vessels detected in the control cortex were cytokeratin negative (Panel E). In vessel walls (arrows) located in the perilesion cortical region, strong immunoreactive signals for Flk-1 (Panel B) and vimentin (Panel D) and a weak signal of cytokeratin (Panel F) were detected. Arrowheads point to vimentin positive astrocytes with hypertrophic appearance (Panel D). Vimentin positive vessels were often surrounded by the immunoreactive astrocytes. Bar, 20 μm.

features, but additionally they possessed fibrils in the cytoplasm. Double labeling immunoelectron microscopy revealed subcellular colocalization of Flk-1 and cytokeratin (*Fig. 2*), where Flk-1 and cytokeratin were represented by 18 nm and 12 nm gold particles, respectively. The immunogold reactivity showing

cytokeratin was found in the cytoplasm rich in intermediate filaments and in the basement membrane of capillary vessel. High density of cytokeratin IR particles is shown in *Fig. 3*. The immunoreactivity linked to vimentin was expressed rather at a low level in the cytoplasm of endothelial-like cells, as shown in *Fig. 4*.

Thus, endothelial-like cells showed weak vimentin, moderate Flk-1, and heavy cytokeratin immunolabeling. In contrast, the immunostaining for AC133 in individual immature endothelial cells was highly variable. The cells showed weak, medium or heavy immunogold signal, as exemplified in *Fig. 5*.

DISCUSSION

Disruption of brain parenchyma allows endothelial progenitors to penetrate through vessel walls. Light microscopy data of this study showed the presence of immunopositive deposits for Flk-1, vimentin, and occasionally cytokeratin in blood vessel walls following surgically-induced injury. Flk-1 is traditionally

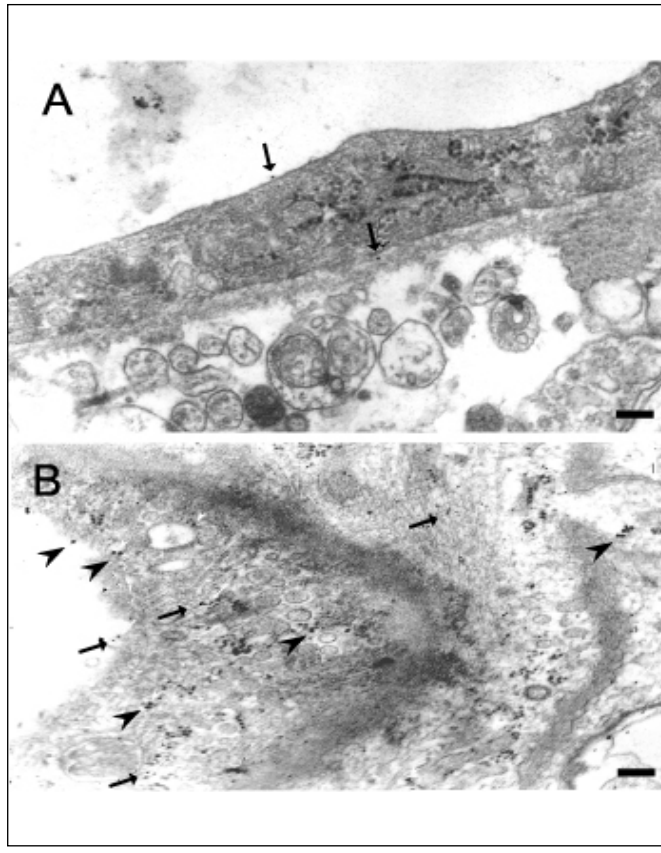

Fig. 2. Panel A - Subcellular localization of Flk-1 and pan-cytokeratin in the control cortex. Flk-1 immunoreactivity (arrows) is seen as single gold particles (18nm). Cytokeratin immunoreactivity is not present. Panel B - Subcellular localization of Flk-1 and pan-cytokeratin in blood vessel composed of cell that revealed endothelial-like features, 2 days after operation. Flk-1 immunoreactivity (arrowheads) is present as single or double immunogold particles (18nm) in the cytoplasm of the endothelial-like cell. Immunoreactivity for cytokeratin (arrows) represented by 12nm gold particles are seen in the cytoplasm and in the basement membrane of the cell. Bar, 200 nm.

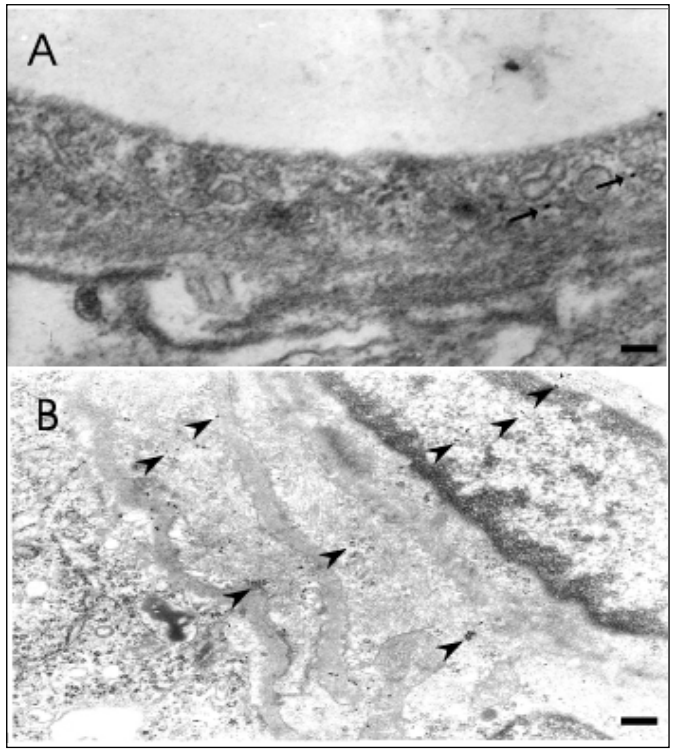

Fig. 3. Panel A - Subcellular localization of pan-cytokeratin (arrows) in blood vessel in the control cortex. Cytokeratin IR is not present. Panel B - Subcellular localization of pan-cytokeratin in blood vessel composed of endothelial-like cells. Two days after operation, high immunoreactivity for cytokeratin (arrowheads) was observed in the cytoplasm of the endothelial-like cell rich in fibrils and in the basement membrane. Some immunogold particles are present in the nucleus of the endothelial-like cell. Bar, 200 nm.

linked to the endothelium, but our and other recent data show the possibility of its expression also in neurons, which should be explored in more detail in future studies. Cytokeratin and vimentin are not specific for the endothelial cells. Therefore, these proteins cannot be used as exclusive endothelial cell markers. From the light microscopic examination we inferred that the cells immunoreactive for the markers we investigated were probably endothelial cells. The inference was confirmed under electron microscopy.

In our previous report, we observed endothelial cells leaving blood vessels (8). That finding indicates that neovascularization is a continuous phenomenon, which leads to the establishment of a mature endothelial phenotype. In the present study, we investigated changes in the endothelial-like cells with time passing after brain tissue injury, starting 2 days post-injury. The earliest stadium was a primary capillary vessel formed from the endothelial-like cell with high immunoreactivity for VEGF and Flk-1 (8). In the embryonic development, vasculogenesis begins when VEGF binds to its receptor - Flk-1 (20). This signal triggers the differentiation of mesodermal cells into endothelial cells and subsequent proliferation of the later cell type. Our earlier morphological and immunocytochemical studies indicated that different stages of new vessel

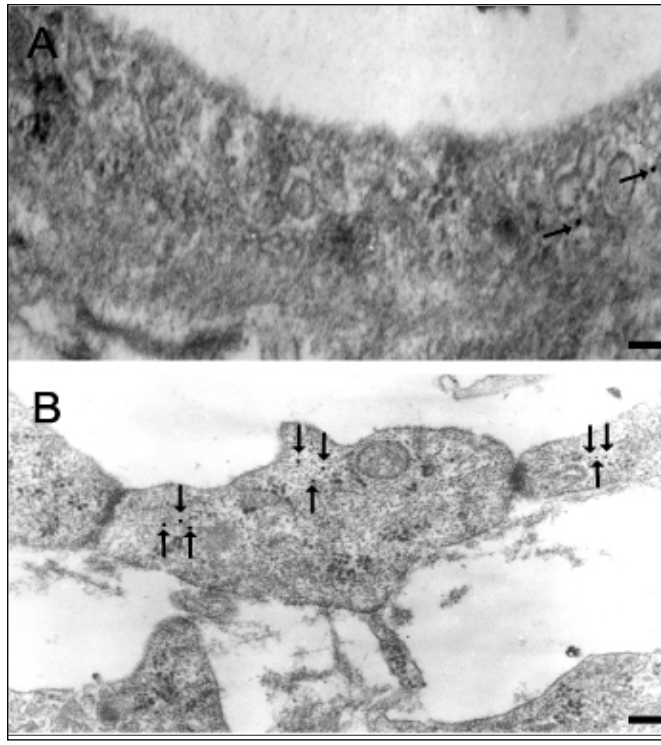

Fig. 4. Panel A - Subcellular localization of vimentin in a blood vessel in the control cortex (arrows). Panel B - Subcellular localization of vimentin in the endothelial-like cell, 4 days after surgical injury. Single immunogold particles (arrows) are present in the cytoplasm. Basement membrane material in the vicinity of the blood vessel is present. Bar, 200 nm.

formation are connected with different expression levels of Flk-1 and VEGF (8). A high immunochemical reactivity seen on Western blots confirmed morphological and immunocytochemical observations (8). The majority of cells with hematopoietic potential have been shown to be Flk-1 positive during the early stages of differentiation and Flk-1 negative at later stages (8, 21).

A multistep process of microvascular formation and alterations in the endothelial-like cell immunophenotype found in our earlier studies, make us suggest that the endothelial-like cell is a kind of transition endothelial cell between the progenitor endothelial cell and the terminally differentiated endothelium. Brain trauma may contribute to the extravasation of endothelial-like cells and the initiation of the following steps in their development.

Flk-1 is characteristic for the hemangioblast, a bipotent stem cell. Like hemangioblasts, endothelial-like cells express Flk-1. Current concepts about vasculogenesis and regeneration hold that different types of cells that participate in new vessels formation co-exist in circulating blood (7, 22). In the present study, we showed a high immunoreactivity of cytokeratin and a weak expression of vimentin in endothelial-like cells. Several studies have demonstrated the expression of cytokeratin in the endothelial cells (13, 14, 15). Our results were in line with those data. Cytokeratin forms the intermediate filaments and is detected

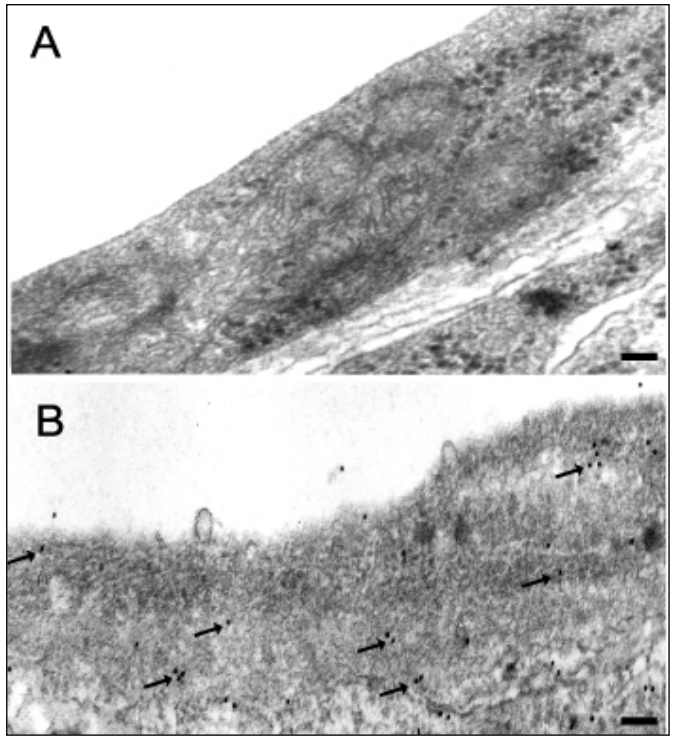

Fig. 5. Panel A - Subcellular localization of AC133 in the control cortex. AC133 IR in a blood vessel is not present. Panel B -Subcellular localization of AC133 in the endothelial-like cell, 2 days after surgical injury (example). Immunogold particles (arrows) are present in the cytoplasm of the endothelial-like cell. Bar, 200nm.

in the cytoplasm, cell membrane, and on the surface of endothelial cells (15). Several groups reported its presence in the nucleus (23, 24). Wand et al (24) have suggested its association with DNA.

Vimentin, another cytoskeleton protein, is present in the endothelium (14), but also in astrocytes. We observed vimentin positive astrocytes in the control brain parenchyma. Following the lesion, increased number of vimentin IR astrocytes was observed and the level of the immunoreactive signal was elevated. IR astrocytes had a hypertrophic appearance, which is considered a typical reaction of astrocytes in response to injury. Howell et al (14) have reported co-expression of cytokeratin and vimentin in the endothelial cells. Our results confirmed those findings.

Based on the ultrastructural features and immunogold staining for Flk-1 in immature endothelial cells we detected few a different types of endothelial progenitors. In the last step of our experiments, we tried to identify the source of endothelial progenitors following the injury. AC133 is a surface glycoprotein of yet unknown function which helps define angioblast-like endothelial progenitors and distinguish them from mature endothelial or monocytic cells (7). Our results showed a few types of immature endothelial-like cells that expressed AC133 to a variable extent. Asahara and Kawamoto (7) have suggested that the level of expression of this marker is characteristic for a specific endothelial progenitor.

Gill et al (25) have postulated a fast mobilization of Flk-1 and AC133 positive endothelial precursors following vascular trauma (25).

In conclusion, our previous and present data indicate that brain trauma or vascular injury may mobilize a group of endothelial progenitors being on a different level of maturation, which can be illustrated by a different level of expression of several markers, whose role would be to help form new vessels and participate in the repair process.

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