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Oxygen Tension Regulates the Maturation of the Blood–Brain Barrier

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The oxygen tension during the development of vascular systems influences vascular vessel formation through regulating angiogenesis. We studied the effect of hypoxia/reoxygenation (H/R) to explain its role in concert with astrocytes involvement in the development of the blood–brain barrier (BBB). On the basis of the fact that the disappearance of hypoxic regions and the decreased expression of vascular endothelial growth factor (VEGF) were observed by immunohistochemistry in a development-dependent manner in rat cerebral cortex, we examined the effects of astrocytes on the BBB-like properties of ECV304 cells by exposing astrocytes to H/R. Conditioned medium of reoxygenated astrocytes inhibited [^{3}H]thymidine incorporation and tube formation of ECV 304 cells. When astrocytes were exposed to reoxygenation, the expression of VEGF was reduced, whereas the expression of angiopoietin-1 and thrombospondin-1 was enhanced. Moreover, [^{3}H]sucrose permeability assay revealed that astrocytes enhance the barrier function of ECV 304 cells in coculture model within 5 h of reoxygenation. Correspondingly, the occludin expression of ECV 304 cells was slightly increased by the conditioned medium of reoxygenated astrocytes. In conclusion, our study suggests that reoxygenation of astrocytes may act as a significant driving force for the maturation of the BBB during brain development through oxygen-regulated gene(s).

The BBB protects the brain from the blood milieu and maintains homeostasis of the brain microenvironment, which is crucial for the proper function of the brain. During late embryonic and early postnatal period, the BBB is formed by brain capillary endothelial cells responding to the neural environment. The development of the BBB is a complex process which is generally classified into two major phases (1, 2). During the first phase, known as brain angiogenesis, brain endothelial cells (ECs) derived from the permeable vessels of perineural vascular plexus invade the avascular neuroectoderm and form the intraneural vessels by an angiogenic process. This process begins in the rat around embryonic day 11.5 and proceeds until around postnatal day 20. During the second phase, the differentiation of brain endothelium lacking a mature barrier in the early stages of brain angiogenesis to the "BBB-phenotype" involves the establishment of complex tight junctions between cells and transport systems for hydrophilic compounds required by the brain. It is reported that the cells closest to brain capillary ECs are the astrocytes in the central nerve system (CNS), whose end feet cover much of the capillary's basal surface (3, 4). Astrocytes are widely believed to be responsible for inducing and maintaining the expression of BBB features in brain endothelial cells (5), since the astroglial perivascular sheet is a unique feature of CNS capillaries, being formed at the same time as the permeability barrier develops (6). However, it has not been known quite clearly how BBB is induced and maintained by astrocytes at the cellular and molecular levels.

Recently, we reported that hypoxia, a state of low oxygen, exists widely in the developing embryonic tissues and may act as a signal for embryonic blood vessel formation *in vivo* (7). Those correspond to the fact that glial and neuronal precursors proliferate and migrate away from the existing blood vessels thereby creating oxygen gradients in the brain tissue (8, 9). In addition, it has been reported that astrocytes play a role as a hypoxic sensor, and that hypoxia stimulates astrocytes to produce VEGF which induces, in turn, active angiogenesis in a paracrine manner in retina development (10).

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However, once tube formation occurs, blood flow commences and local oxygen levels increase, resulting in the decrease of VEGF level (11). It is possible that there is reoxygenation, strictly speaking, oxygenation, in parenchymal cells (astrocytes in the brain), which triggers many different cellular responses. Until now, the reoxygenation conducted by most investigators has been implicating the pathological conditions such as edema and hemorrhage following ischemia/reperfusion (12, 13). However, reoxygenation seems to exist during normal physiological development, involving the maturation of blood vessels.

Therefore, we hypothesize that astrocytes under reoxygenation, subsequently derived from the occurrence of perfusion (blood flow) through the permeable vessels formed after hypoxia-driven angiogenesis, cause the cease of the brain angiogenesis and in turn make endothelial cells have the BBB characteristics. To prove our hypothesis, we adopted the method conducted by Hori *et al.* (14), in which conditioned medium is rapidly replaced by fresh medium after specific period of hypoxia and then, the cultures are returned to the ambient atmosphere (reoxygenation) in order to minimize hypoxic effect during the reoxygenation and to observe the effect of astrocytes alone.

MATERIALS AND METHODS

Immunohistochemistry. Pregnant Sprague–Dawley rats at the time of embryonic day 13 and 18, postnatal day 3 and day 21, and adult rats were injected with hypoxia marker, pimonidazole hydrochloride (60 mg/kg rat) intravenously. Pimonidazole hydrochloride (Hypoxyprobe-1) and mouse monoclonal antibody were supplied by NPI, Inc. (Belmont, MA). At 2 h after injection, rats were anesthetized and perfusion-fixed by transcardiac perfusion with 4% formaldehyde in phosphate buffer, pH 7.2. Immunohistochemistry was performed as described (7).

Cell culture. A human endothelial-like cell line, ECV 304, which is known to form tight junction, and monkey kidney fibroblast-like cell line, COS-7, were obtained from Dr. Chi-Bom Chae (Pohang University of Science and Technology, Korea). Primary rat astrocytes were obtained from neonatal Sprague–Dawley rats with some modifications, according to MaCarthy *et al.* (15). More than 95% astrocytes were yielded by this procedure routinely.

Exposure to hypoxia/reoxygenation and preparation of astrocytes conditioned medium (ACM). Cultured astrocytes were seeded at a density of 5×10^4 cells/cm^2. When cultures achieved confluence, they were exposed to hypoxia in a hypoxia chamber (Forma Scientific Inc) which maintained a humidified atmosphere with low oxygen tensions (5% CO_2, 1% O_2, and 94% N_2). After exposure to normoxia or hypoxia for 48 h, each culture was returned to the ambient atmosphere just by replacing the conditioned medium with fresh medium and continuously incubated for the indicated times under the same condition. The conditioned media at 48 h of normoxia or hypoxia (for further study, each CM was mixed with the same volume of fresh DMEM supplemented with 10% FBS and referred to 48N-ACM and 48H-ACM, respectively) and at additional 5 h under the ambient atmosphere (referred to 5N-ACM and 5R-ACM, respectively) were collected.

[^{3}H]Methylthymidine incorporation assay. To measure cell proliferation, ECV 304 cells were seeded at a density of 3×10^4 cells/cm^2. The cells were incubated for 24 h after adding the indicated ACM. One μCi/ml of [^{3}H]methylthymidine (Amersham Pharmacia Biotech) was added at the final 4 h prior to the assay. The cpm values from cultures were counted with liquid scintillation counter (Wallac) and expressed as percentage of the control, depending on the mean cpm of triplicate.

Tube formation assay. 250 μl of Matrigel (10 mg/ml) (Becton–Dickinson) was pipetted into a 24-well culture plates and polymerized for 30 min at 37°C. ECV 304 (1×10^5 cells) was seeded on the surface of Matrigel. Then, after adding the indicated ACM or 3 ng/ml of bFGF (Upstate Biotech) or 10 ng/ml of VEGF (R&D Systems), the cells were incubated. Morphological changes of cells were photographed at $\times$40 magnification.

Immunoprecipitation and immunoblotting. Cultured astrocytes were exposed to the H/R described above. For immunoprecipitation analysis of VEGF, equal amount of total protein or ACM was incubated with anti-VEGF antibody (1 μg; Santa Cruz) at 4°C overnight and further incubated with protein A-agarose beads (Upstate Biotech). The immunoprecipitated complex was analyzed by Western blotting. For Western analysis of VEGF, Ang-1 and TSP-1 in the astrocytes, the blocked membranes were incubated with anti-VEGF, anti-Ang-1 (Santa Cruz) and anti-TSP-1 (Neomarker) antibodies, respectively. The immunoreactive bands were visualized using chemiluminescent reagent according to the manufacturer's instruction (Amersham Pharmacia Biotech). The loading control was done by anti-α-tubulin monoclonal antibody (Upstate Biotech). For the detection of occludin, ECV 304 cells were used by seeding at a density of 5×10^4 cells/cm^2 and allowed to attach for 2 h. Then, the cells were incubated for 24 h after adding the indicated ACM and solubilized in 6 M urea buffer (16). Western blotting was performed with anti-occludin polyclonal antibodies (Santa Cruz).

[^{3}H]Sucrose permeability assay. ECV 304 cells were plated at 5×10^4 cells/cm^2 on 12-mm collagen-coated transwell filters (0.4 μm pore size; Costar) with M199 containing 10% FBS. Astrocytes (5×10^4 cells/cm^2) were seeded in DMEM containing 10% FBS in the 12-well plate. When they were grown to confluence, the astrocytes were exposed to either hypoxia or normoxia for 48 h. For the permeability assay, coculture experiments were performed within 3 days after ECV 304 cells reaching a confluent monolayer. The transwell containing confluent ECV 304 cells was transposed to 12-well which had astrocytes preincubated under either normoxia or hypoxia described above. Before transposing, the conditioned media of both upper and lower chamber were aspirated and replaced by fresh media immediately. This fresh medium for upper chamber having ECV 304 cells contained [^{3}H]sucrose (1 μCi/ml of medium; Amersham Pharmacia Biotech). To minimize the effect of serum and glucose, a 1:4 mixture of DMEM supplemented with 10% FBS and DMEM-base was used for the coculture. The samples for the assay were withdrawn from the lower chamber at the indicated times. The volume in the lower chamber was not changed significantly ($<$5%) by sampling during the experiment. The amount of radioactivity at the indicated times diffused into the lower chamber was measured by liquid scintillation counter (Wallac). For the control, the same experiments were performed on COS-7 cells preincubated under normoxia or hypoxia for 24 h. The activity was expressed as the mean cpm of triplicate.

Data analysis and statistics. Data are presented as mean $\pm$ SD or as percentage of control. Statistical comparisons between groups were performed using the Student *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

Hypoxic Regions and the Expression of VEGF/VPF Disappear in a Development-Dependent Manner in Rat Cerebral Cortex

To observe the change of hypoxic states of the cerebral cortex during brain development, we performed

immunohistochemistry using hypoxia-specific anti-pimonidazole adduct antibody, and VEGF antibody (7). Between embryonic day 13 (E13) and 18 (E18), hypoxic immunoreactive regions were detected in the marginal layer and the ependymal layer (ventricular neuroepithelium) of the cortex, where cells proliferate and contribute to make local formation of low oxygen (Figs. 1A and 1B). At postnatal day 3 (P3), the hypoxic regions were spread in the whole cerebral cortex (Fig. 1C). However, hypoxic region was hardly detected in the cerebral cortex of postnatal day 21 (P21) at which the brain angiogenesis becomes decreased profoundly (Fig. 1D) and in the cerebral cortex of the adult stage where the BBB had formed (Fig. 1E). The immunoreactivity to VEGF was colocalized with that of pimonidazole in the cerebral cortex of E13, E18, and P3 (Figs. 1F, 1G, and 1H). The actively proliferous regions of pia mater and choroid plexus were also stained with hypoxic marker and VEGF antibodies (data not shown). While expression of VEGF was observed at a low level in P21 brain (Fig. 1I), immunoreactivity to VEGF was not detected in adult brain (Fig. 1J) (17). This result indicates that there is spatial and temporal existence of hypoxic and VEGF expression regions, and that both immunoreactivities disappeared after forming blood vessels.

The Conditioned Medium from Astrocytes under Reoxygenation Inhibits the Proliferation and the Tube Formation of ECV 304 Cells

To distinguish angiogenic from anti-angiogenic (or angiostatic) effects of conditioned medium (CM) from astrocytes under normoxia, hypoxia and reoxygenation on ECV 304 cells, we tested the effect of ACM on the proliferation of ECV 304 cells by [^{3}H]thymidine incorporation assay (Fig. 2A). The cells with 48H-ACM showed a relatively higher increase of DNA synthesis, compared with that of the cells with 48N-ACM, whereas the cells with 5R-ACM showed decreased DNA synthesis, compared with that of the cells with 5N-ACM. This result suggests that the astrocytes under hypoxia enhance the proliferation of ECV 304 cells, whereas the astrocytes under reoxygenation inhibit the proliferation.

The effect of ACM on morphological changes of ECV 304 cells on the surface of three-dimensional Matrigel was investigated. The formation of tubular structure was observed by phase-contrast microscope (Fig. 2B). At 9 h after seeding, 48H-ACM-treated ECV 304 cells showed stronger and many tube-like structures compared to those of both control and 48N-ACM-treated ECV 304 cells, indicating that the astrocytes under hypoxia enhances the formation of tubular structures in ECV 304 cells. In contrast, both 5N-ACM and 5R-ACM significantly suppressed ECV 304 cells to produce tubular structure. However, a significant difference of capacity to inhibit tube formation was not found between them.

Reoxygenation Inhibits the Expression of VEGF and Enhances the Expression of Angiopoietin-1 as Well as of Thrombospondin-1 in Astrocytes

As seen in Fig. 3A, astrocytes under hypoxia overexpressed VEGF, however, the expression of VEGF sustained during the early period of reoxygenation was reduced to the normoxia level after 3 h- exposure to reoxygenation. Beads alone and rabbit IgG antibody were used as negative controls and no bands were detected (data not shown). Then, the expression of Ang-1 was analyzed by Western blot analysis in both cell lysate and CM. Figure 3B showed that the expression of Ang-1 was significantly decreased under hypoxia. However, its expression was not changed under normoxia as well as under reoxygenation. In addition, the expression of TSP-1 was increased after 0.5 h-exposure to reoxygenation and remained to increase significantly during reoxygenation (Fig. 3C).

Taken together, our data demonstrate that the decrease of VEGF, and the increases of Ang-1 and TSP-1 resulting from exposure to reoxygenation may lead to an imbalance that angiogenic inhibitors are predominant and thus allow astrocytes to contribute to the cessation of brain angiogenesis as well as the stabilization and maturation of vessels.

Reoxygenated Astrocytes Inhibit the [^{3}H]Sucrose Permeability of ECV 304 Cells

To examine whether any change of the balance between angiogenic activators and inhibitors observed in astrocytes under reoxygenation influences the barrier function of ECV 304 cells, we performed [^{3}H]sucrose permeability assay with coculture system. As shown in Fig. 4A, astrocytes at 5 h of reoxygenation showed a relatively higher inhibitory effect on the permeability of [^{3}H]sucrose than the astrocytes under normoxia did. This inhibitory effect by reoxygenated astrocytes was initiated after 3 h of reoxygenation exposure (data not shown). The permeability of coculture of ECV 304 cells with astrocytes, regardless of oxygen state, was decreased compared with that of ECV 304 cells alone. However, non-CNS COS-7 cells under both conditions of normoxia and reoxygenation did not show any inhibitory effect (Fig. 4B).

The Conditioned Medium from Astrocytes under Reoxygenation Increases the Occludin Expression of ECV 304 Cells

We investigated whether the decrease of the [^{3}H]sucrose permeability was accompanied by an increase of occludin in ECV 304 cells. As shown in Fig. 5, the cells

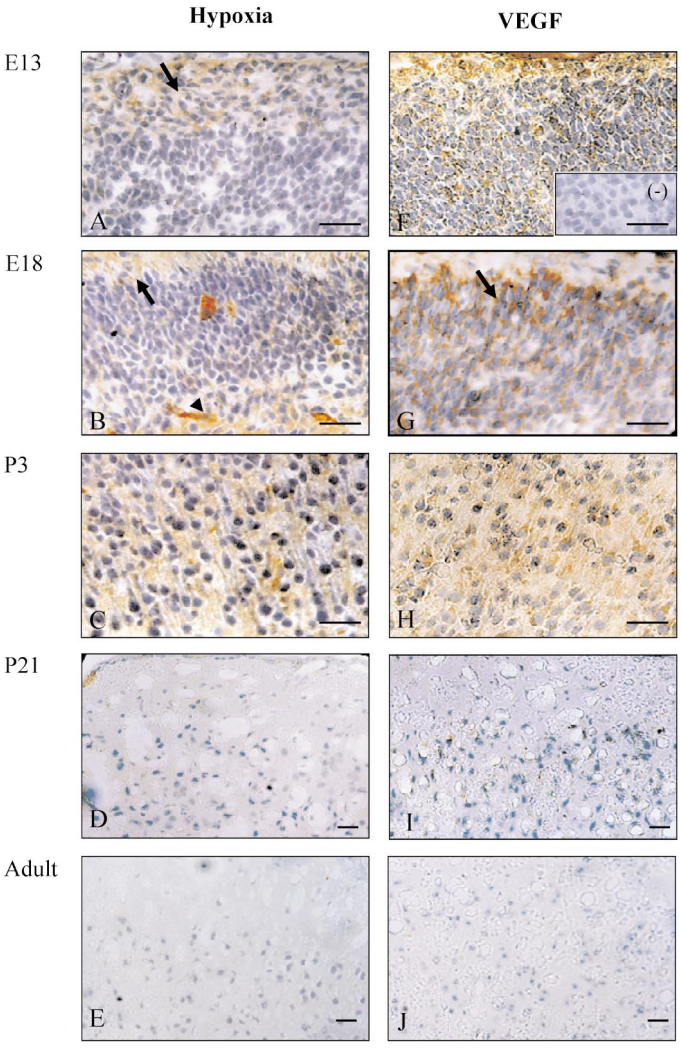

FIG. 1. Immunohistochemical staining of hypoxic regions by hypoxic marker, pimonidazole, and VEGF in developing rat brain. Positive immunoreactivity was represented by brown color of the DAB. When negative control was performed by mouse IgG isotype instead of hypoxic marker and VEGF antibody with the tissue of embryonic day 13, no positive staining was found (inset in F (−)). (A–E) Sections from the cerebral cortex at embryonic day 13 (E13) and at embryonic day 18 (E18) showed that hypoxic regions in the marginal layer (arrow) and the ependymal layer (arrowhead) (A and B, respectively). Broad staining was observed in the cerebral cortex at postnatal day 3 (P3) (C). In cerebral cortex at postnatal day 21 (P21) (D), and adult rats brain (E), stainings of hypoxia were not found. Scale bar, 100 μm. (F–J) The VEGF staining in the sections at E13 (F), E18 (G), and P3 (H) were strong in cerebral cortex consistent with hypoxic region, whereas weak or no staining of VEGF was observed in cerebral cortex at P21 (I) and adult rat brain (J). Scale bar, 100 μm.

treated with 48H-ACM for 24 h showed the decrease of expression of occludin compared with those of 48N-ACM-treated cells and the control, whereas the expression of occludin in ECV 304 cells treated with 5R-ACM for 24 h was increased compared with those of 5N-ACM and the control, respectively.

DISCUSSION

Most studies on the development of the BBB have mainly examined the effect of astrocytes without considering the effect of different oxygen tension on astrocytes and the BBB. Here, we investigated the effect of oxygen tension to explain its role in concert with astrocytes involvement in the development of the BBB and found that reoxygenation of astrocytes might act

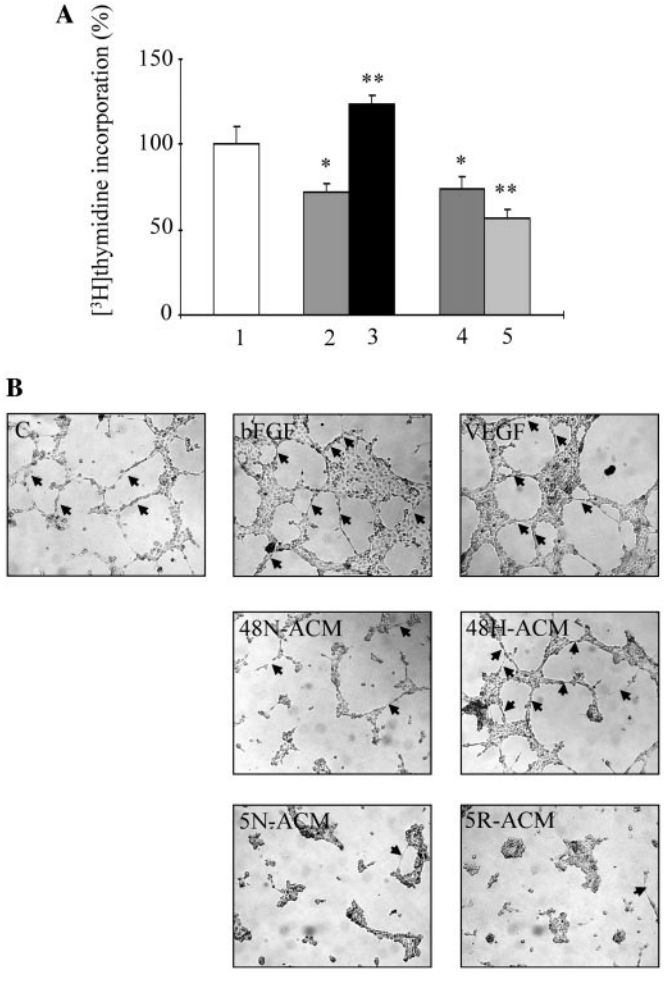

FIG. 2. Effect of ACM on the proliferation and the tube formation of ECV 304 cells. (A) ECV 304 cells were incubated with each ACM as described under Materials and Methods. After 24 h incubation, [^{3}H]thymidine incorporation assay was performed. 1, non-conditioned normal growth medium; 2, 48N-ACM; 3, 48H-ACM; 4, 5N-ACM; 5, 5R-ACM. Data represent the mean ± SD of triplicate, and similar results were obtained in two different experiments. *$P < 0.05$ compared to control. **$P < 0.05$ to each N-ACM. (B) ECV 304 cells were plated on Matrigel-coated wells at a density of 1×10^5 cells/24-well in the absence (nonconditioned normal growth medium (C)) and presence of each astrocytes conditioned medium (48N-ACM, 48H-ACM, 5N-ACM and 5R-ACM). 3 ng/ml of bFGF or 10 ng/ml of VEGF was used as positive controls. Arrows indicate the tube formed by ECV304 cells responding to each astrocyte conditioned medium and positive controls (VEGF and bFGF). Photographs were taken after 9 h in culture (magnifications, ×40). Each sample was assayed in duplicate, and the assays were repeated three times.

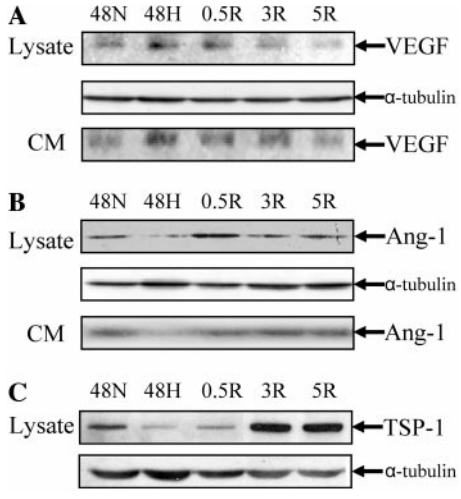

FIG. 3. Effect of reoxygenation on expressions of VEGF, Ang-1, and TSP1 in astrocytes. Astrocytes were exposed to normoxia alone for 48 h (48N), hypoxia alone for 48 h (48H), and hypoxia followed by reoxygenation for 0.5, 3, and 5 h (0.5R, 3R, 5R). At each time point, the cell lysate (lysate) and/or conditioned medium (CM) was used. VEGF (A) was immunoprecipitated and then detected by Western blot analysis. Ang-1 (B) and TSP-1 (C) were detected by Western blot analysis.

as a physiological trigger for developmental brain vessel maturation. This is the first report to study the effect of reoxygenation of astrocytes on the BBB development.

Our immunoblot assay revealed that hypoxia-induced VEGF expression was rapidly decreased by reoxygenation in a time-dependent manner in astrocytes (Fig. 3A), although the VEGF expression was sustained at the early stage of reoxygenation. The VEGF expression at the early stage of reoxygenation might occur due to the stimulation of reactive oxygen intermediates during exposure to H/R and/or to the synthesis and the release of VEGF that has been transcriptionally induced under hypoxia. The pattern of VEGF expression in response to oxygen tension was consistent with the result from the histological study (Fig. 1) in which the immunoreactivities to VEGF and pimonidazole disappeared in a development-dependent manner in rat cerebral cortex. These data indicates that reoxygenation causes downregulation of VEGF under the level required to sustain angiogenesis and initiates the maturation of vessels.

Along with the VEGF expression pattern, we observed the constitutive expression of Ang-1 under normoxia and reoxygenation. However, the expression of Ang-1 was reduced significantly under hypoxia. This result is in accordance with the observation that Ang-1 mRNA was decreased under hypoxia in rat C6 glioblastoma cells (18). The induced expression of VEGF and reduced expression of Ang-1 under hypoxia might be involved in promoting brain angiogenesis. However, the reduced expression of VEGF and enhanced expression of Ang-1 under reoxygenation might be involved in inducing the maturation of brain endothelium, since it was reported that the enhanced expression of Ang-1 contributes to the formation of leakage-resistant blood vessels (19). In addition, it is possible that the highly expressed Ang-1 under reoxygenation involves the interactions among endothelial cells, the surrounding extracellular matrix and supporting cells (astrocytes in this case) as observed by Suri *et al.* (20) in mice lacking Ang-1.

It is also suggested that astrocytes are likely to be involved in endothelial differentiation and function in the central nervous system via extracellular matrix molecule secretion (21). Thrombospondin-1 (TSP-1) is a potent natural inhibitor of angiogenesis that modulates endothelial cell adhesiveness, motility and pro-

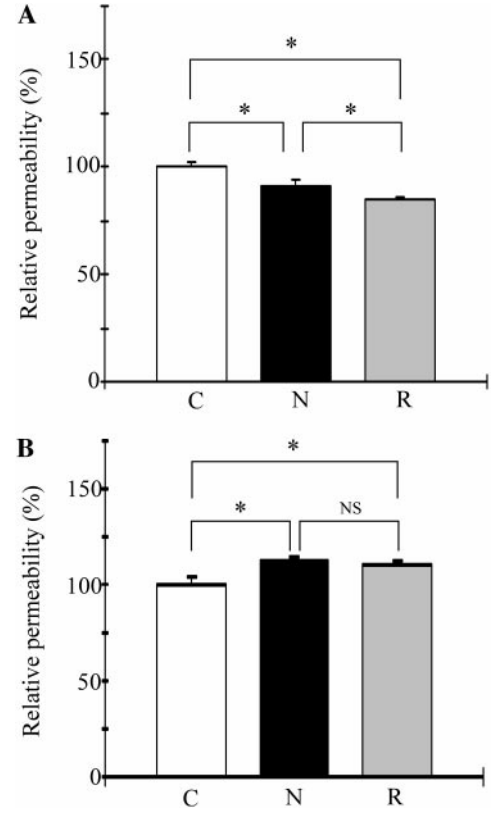

FIG. 4. Effect of reoxygenated astrocytes on the permeability of ECV 304 cells in coculture model. After transposing the transwell containing confluent ECV 304 cells into 12-well which had astrocytes preincubated under either normoxia or hypoxia for 48 h as described under Materials and Methods, permeability assays were carried out by adding fresh medium containing [^{3}H]sucrose to the compartment above the monolayer. In each case, the transfer of [^{3}H]sucrose to the compartment below the monolayer was assessed at 5 h. (A) C: ECV 304 cells alone in the absence of astrocytes. N: coculture of ECV 304 cells in the presence of astrocytes preincubated under normoxia. R: coculture of ECV 304 cells in the presence of astrocytes preincubated under hypoxia. (B) Non-CNS cells, COS-7, was used for coculture. Data represent the mean ± SD of triplicate. * and NS means $P < 0.05$ and not significant, respectively. Results are representative of three independent experiments.

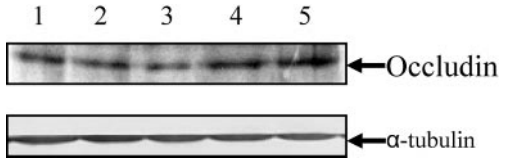

FIG. 5. Effect of ACM on the expression of occludin in ECV 304 cells. Confluent ECV 304 cells were incubated with the indicated ACM for 24 h. The cell lysate was subjected to Western blot analysis. Lane 1, nonconditioned normal growth medium; lane 2, 48N-ACM; lane 3, 48H-ACM; lane 4, 5N-ACM; lane 5, 5R-ACM.

teolytic activity, and is expressed highly in developing brain (22). We showed that exposure of astrocytes to reoxygenation simultaneously induced TSP-1 and suppressed VEGF. Our observations suggest that reoxygenation signal in the brain microenvironment induces TSP-1 and inhibits VEGF expression, by promoting the switch from angiogenic to angiostatic phenotype and allowing ECs to be a differentiated, quiescent state which is involved in the cease of brain angiogenesis. On the other hand, TSP-1 is a major activator of transforming growth factor-β (TGF-β) *in vivo* (23). TGF-β as well as Ang-1 plays a role in stabilizing vessels (11). The previous report demonstrated, as well, that TGF-β importantly contributes to the angiostatic role of astrocytes (24). Therefore, it seems that TSP-1 contributes, at least partly, to reoxygenation-induced vessel maturation through TSP-dependent TGF-β activation (25) in the BBB development. According to our findings that Ang-1 and TSP-1 are highly expressed in astrocytes under reoxygenation, we propose that reoxygenation of astrocytes plays a critical role in inhibiting vascular growth and inducing vascular stabilization by expression of angiogenic inhibitors, i.e., TSP-1.

The brain endothelial cells are coupled with each other to form the BBB by tight junctions with extremely low permeability (6). Several lines of evidence suggest that occludin play a crucial role in the function of tight junctions and control of vascular permeability (26). In this study, we investigated the effect of reoxygenated astrocytes on the barrier function of endothelial cells with the [^{3}H]sucrose permeability assay and Western blot analysis of occludin. We observed that reoxygenated astrocytes diminished the sucrose permeability of ECV 304 cells in coculture system. This coincided with the observations that the occludin level in ECV 304 cells was slightly increased by treatment of 5R-ACM for 24 h. Many studies demonstrated that VEGF disrupts tight junctions and increases the permeability of endothelial cells (27). The effects of 5R-ACM on the occludin expression and of reoxygenated astrocytes on the [^{3}H]sucrose permeability of ECV 304 cells to a certain extent come from the decrease of VEGF expression in a time-dependent manner in astrocytes under reoxygenation.

Our results suggest that reoxygenated astrocytes may, at least in part, contribute to the BBB development through two ways: one way is through the cessation of brain angiogenesis by producing angiogenic inhibitors (e.g., Ang-1 and TSP-1), which consequently suppress the activities of angiogenic activators (e.g., VEGF). The other way is through the maturation and stabilization of permeable vessels by initiating the differentiation of ECs to BBB-like phenotype (i.e., overexpression of occludin and decrease of vascular permeability). Therefore, we propose that reoxygenation may act as an important signal for inhibiting angiogenesis and for inducing tissue-specific endothelial differentiation. Further investigations are required to elucidate properly the roles of the astrocytes under reoxygenation in the brain angiogenesis and the development of the BBB, in detail.

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