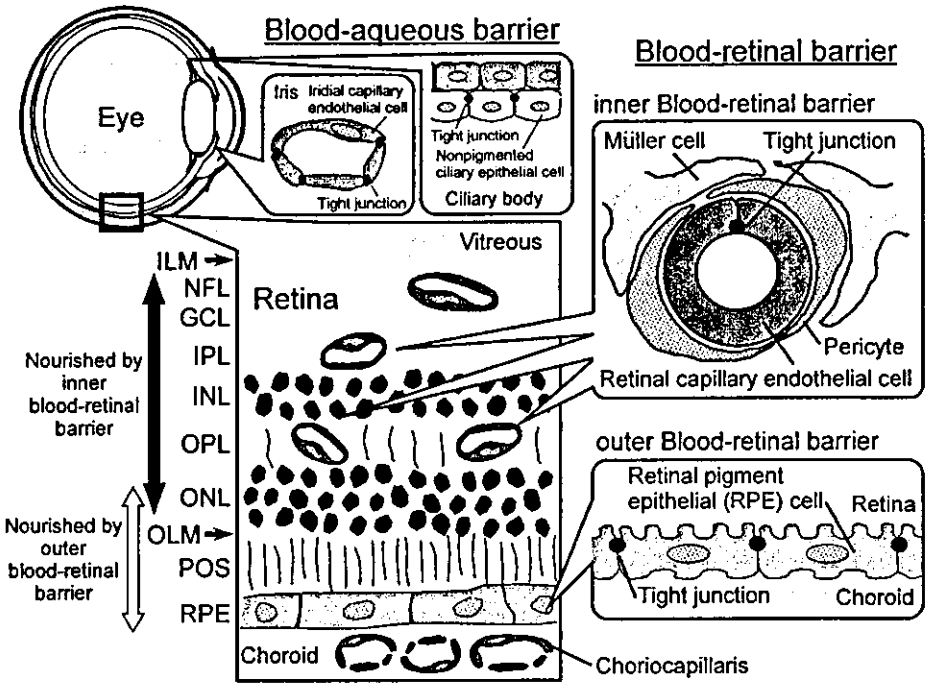

Fig. 1. Schematic Diagram of the Blood-Ocular Barrier

The retinal cell layers seen histologically consist of: RPE, retinal pigment epithelium; POS, photoreceptor outer segments; OLM, outer limiting "membrane"; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; NFL, nerve fiber layer; ILM, inner limiting "membrane".

Müller cell lines. Finally, the role of the inner BRB transporters and the paracrine interaction of pericytes will be discussed.

ESTABLISHMENT OF INNER BLOOD-RETINAL BARRIER CELL LINES

The rat immortalized retinal capillary endothelial cell line has been established by transfecting the retrovirus vector-encoded temperature-sensitive (ts) simian virus (SV) 40 large T-antigen gene to primary cultured retinal endothelial cells.[24] There is no report of the use of this cell line for transport functions after establishment. In general, introduction of a gene *in vitro* may lead to some unpredictable problems since the gene is introduced to a limited number of cells and is then inserted at different chromosomal positions. This may lead to the destruction of genes and the loss of original differentiated cell functions. The use of transgenic rats harboring the ts SV40 large T-antigen gene (tsA58 Tg rat) eliminates this concern and allows the establishing of cell lines from source tissues, which have very small dimensions (see other reviews).[25–27] As illustrated in Fig. 2, the ts SV40 large T-antigen gene is stably expressed in all tissues of the tsA58 Tg rat and the large T-antigen remains inactive at 37 °C and does not interact with retinoblastoma gene products (pRb) and p53, which normally regulate cell proliferation. Therefore, ts large T-antigen is not switched on at body temperature. On the other hand, the cultured cells can be easily immortalized by activation of the ts SV40 large T-antigen at 33 °C. The activated large T-antigen is thought to induce cell proliferation by interacting with pRb and p53.[25,28]

We have established conditionally immortalized rat retinal capillary endothelial cells (TR-iBRB),[29] pericytes (TR-rPCT),[30] and Müller cell lines (TR-MUL)[31] from tsA58 Tg rats. The procedures for establishing these three cell lines are shown in Fig. 3. Briefly, the eyes are enucleated from tsA58 Tg rats and the retinas are gently separated from the retinal pigment epithelial cell layer. In the case of TR-iBRB and TR-rPCT cells, the retinal capillary rich fraction is isolated and digested with collagenase/dispase. The digested capillary rich fraction (for TR-iBRB and TR-rPCT cells) and dissected retina (for TR-MUL cells) are seeded onto rat tail collagen type I-coated tissue culture dishes and tissue culture dishes, respectively. The cells are cultured in Dulbecco's modified Eagle's medium (DMEM) at 37 °C in a humidified atmosphere of 5% CO_2/air. After cell attachment, the temperature is reduced from 37 to 33 °C to activate the ts SV40 large T-antigen. After a few weeks, several types of cell colony appear in the dish. The colonies, which have the characteristic morphology of each cell, are surrounded by a cloning cylinder and selectively trypsinized. Following two or three passages, the cells are cloned from a single cell by colony formation and isolated twice from other cells using a cloning cylinder.

CHARACTERISTICS OF IMMORTALIZED INNER BLOOD-RETINAL BARRIER CELL LINES

TR-iBRB, TR-rPCT, and TR-MUL cells have a spindle-fiber shape, are multicellular nodules, or exhibit bipolar morphology, respectively, like their respective primary cultured cells (Fig. 3). TR-iBRB, TR-rPCT, and TR-MUL cells express a large T-antigen and grow well at 33 °C with a doubling-time of 18.8 h, 37.0 h, and 30.0 h, respectively. In contrast, growth of these cells is arrested at 37 °C and 39 °C due to a reduction in the expression of large T-antigen.[29–31]

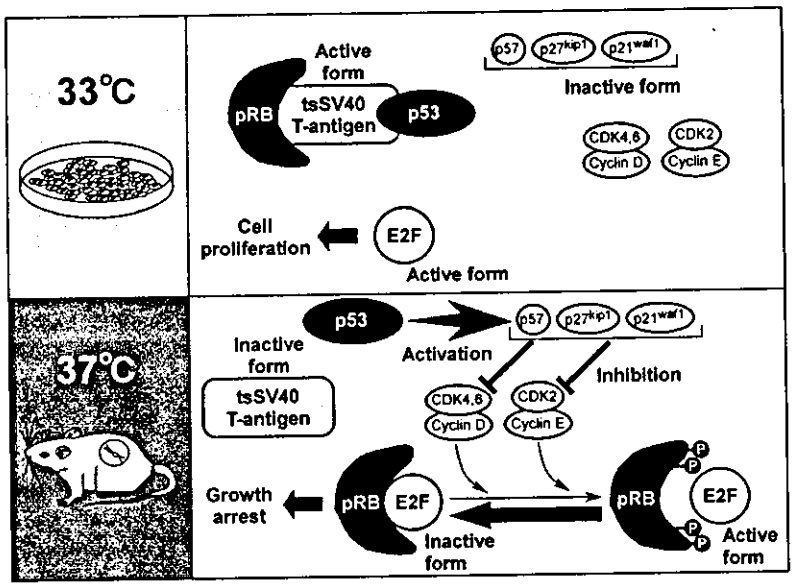

Fig. 2. The Role of Temperature-Sensitive SV40 Large T-Antigen (ts SV40 T-Antigen) in the Activation of Cell Proliferation
The tsSV40 T-antigen gene is ubiquitously expressed in the tsA58 Tg rat. The tsSV40 T-antigen is activated at 33 °C and inactivated at 37 °C.

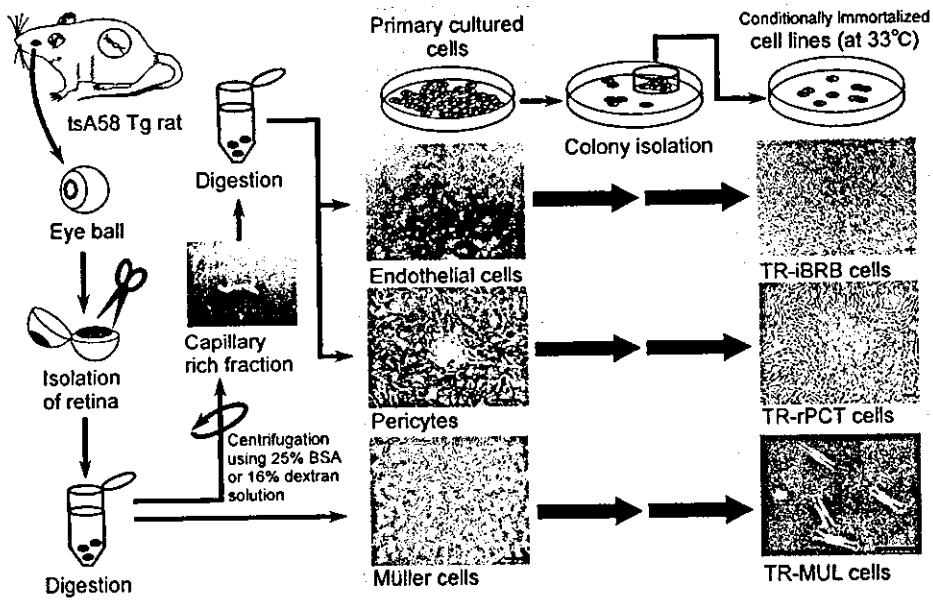

Fig. 3. Establishment of Conditionally Immortalized Inner BRB Cell Lines from the tsA58 Tg Rat
Scale bar = 100 μm.

Therefore, temperature-dependent growth of these cell lines corresponds to the temperature-sensitive phenotype of the large T-antigen of conditionally immortalized cell lines.[25]

TR-iBRB Cells TR-iBRB cells possess endothelial markers, such as von Willebrand factor (vWF) and a scavenger receptor for the uptake of acetylated low density lipoprotein (Ac-LDL). They also express vascular endothelial growth factor (VEGF) receptor-2 (KDR/Flk-1), which may play a critical role in binding to VEGF and in the development of neovascularization in diabetic retinopathy.[29] TR-iBRB cells have endothelial properties.

TR-rPCT Cells TR-rPCT cells are stained by von Kossa reagent (calcification), exhibit negation of contact inhibition and the mRNA expression of pericyte markers, such as rat intercellular adhesion molecule-1, platelet-derived growth factor-receptor β, angiopoietin-1, and osteopontin. However, they do not express vWF mRNA and exhibit uptake of Ac-LDL, suggesting that TR-rPCT cells are free from contamination by endothelial cells. TR-rPCT cells also exhibit α-smooth muscle actin expression and can be induced by transforming growth factor-β1.[30] TR-rPCT cells were initially established as a retinal pericyte cell line and have the properties of retinal pericytes.

TR-MUL Cells TR-MUL cells express typical Müller cell markers such as S-100, glutamine synthetase, and excitatory amino acid transporter (EAAT)1/GLAST, whereas EAAT2/GLT-1 and EAAT5 are not expressed. These transporters are detected in neuronal cells of the brain, but not in

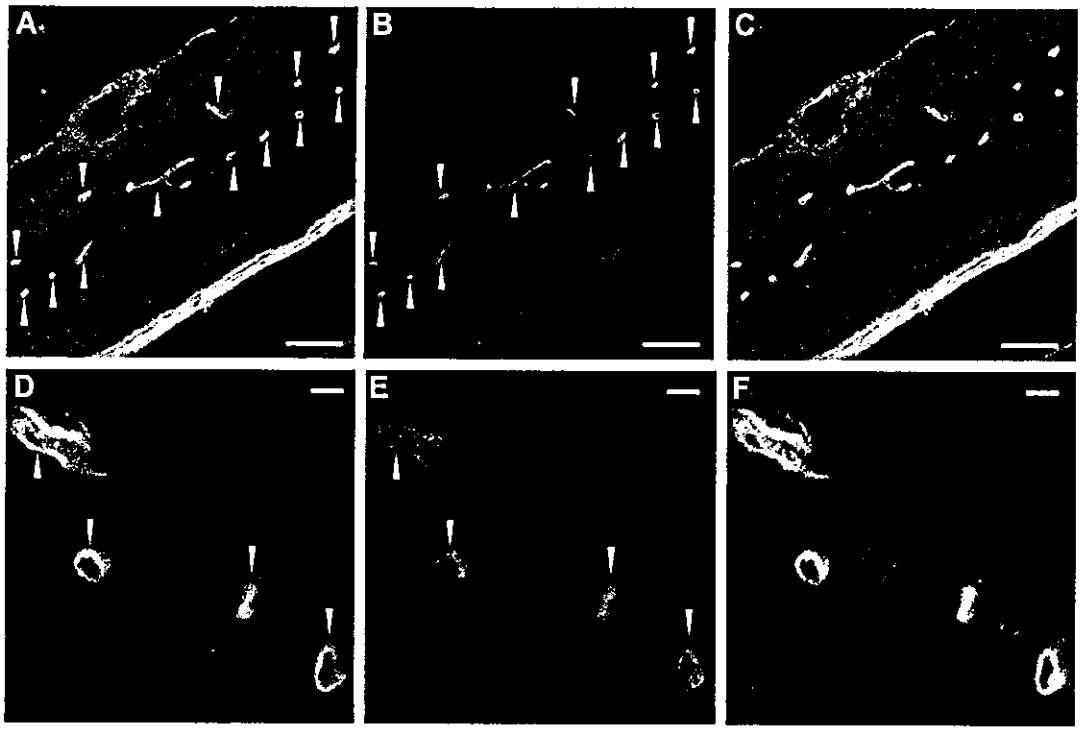

Fig. 4. Confocal Immunofluorescence Microscope Images of Single- and Dual-Labeled Rat Retinal Sections

The retinal capillaries (arrow heads) were predominantly stained with anti-GLUT1 antibody (green) (A and D) and anti-P-glycoprotein antibody (red) (B and E). Colocalization of GLUT1 and P-glycoprotein immunoreactivity is observed under low magnification (C) and P-glycoprotein immunoreactivity, rather than that of GLUT1, is observed inside under high magnification (F). P-Glycoprotein in the retinal endothelial cells is mainly localized at the luminal side. Scale bar = 50 μm for A, B, and C and 5 μm for D, E, and F.

glial cells of the retina.[32,33] TR-MUL cells also exhibit little or no expression of glial fibrillary acidic protein (GFAP).[31] The rat Müller cell line (rMC-1) was established by transfecting SV40 DNA to primary cultured Müller cells prepared from retinas of rats exposed to constant light for a period of 2 weeks.[34] The rMC-1 cells express GFAP, since light exposure may cause abnormalities including oxidative stress and the accumulation of GFAP in Müller cells.[35] Therefore, TR-MUL cells have the properties of normal Müller cells.

TRANSPORT FUNCTIONS OF THE INNER BLOOD-RETINAL BARRIER

D-Glucose Transport D-glucose transport from the circulating blood to the retina has been investigated since 1971 and D-glucose is known to be transported much more easily than L-glucose.[36] Betz and Goldstein were the first to report a carrier-mediated transport process for D-glucose at the inner BRB and showed that [^{14}C]3-*O*-methyl-D-glucose (3-OMG, a non-metabolizable analog of D-glucose) uptake by isolated retinal capillaries was saturable, and exhibited cytochalasin B- and phloretin-sensitivity, and could be inhibited by other hexoses such as D-glucose, 2-deoxy-D-glucose, and D-galactose.[20] Alm *et al.* confirmed the carrier-mediated transport of D-glucose across the rat BRB using the RUI method, and investigated the uptake of substrates by the retina across both the inner and outer BRB.[18] Their findings support the hypothesis that facilitative D-glucose transporters are involved in the blood-to-retina transport of D-glucose. Morphological evidence was provided by Takata *et al.* who demonstrated the immunolocalization of GLUT1 in the retina and showed that GLUT1 is localized at both the luminal (blood) and abluminal (retinal) sides of the inner BRB (retinal capillary) and at both the apical and basolateral sides of the outer BRB (RPE).[14] An illustration of GLUT1 expression at the rat inner BRB is shown in Figs. 4A and D. TR-iBRB cells express GLUT1 at 55 kDa and exhibit Na^+-independent 3-OMG uptake with a Michaelis constant (K_m) of 5.56 mM.[29] This is very similar to the K_m value of 7.81 mM obtained by Ennis *et al.* using a modification of the RUI method.[37] In the case of primary cultured bovine brain capillary endothelial cells, the mRNA expression of GLUT1 is down-regulated 100-fold less than that *in vivo*.[38] TR-iBRB cells express GLUT1, which is capable of transporting D-glucose.

Vitamin C Transport Vitamin C plays an important role in detoxifying free radicals in the retina and its concentration in the retina is more than 10-fold greater than that in the plasma.[39,40] It is well known that Na^+-dependent L-ascorbic acid transporter (SVCT) and GLUT have the reduced form of ascorbic acid (AA) and the oxidized form of dehydroxyascorbic acid (DHA), respectively, as substrates,[41,42] although nothing is currently known about either the vitamin C transport mechanism at the BRB or the relationship between the vitamin C concentration in the retina and retinal diseases. We believe that using a combination of both the *in vivo* integration plot analysis and TR-iBRB cells could identify the transport mechanism of vitamin C across the inner BRB.[43] The *in vivo* blood-to-retina influx transport of DHA and AA across the BRB was evaluated by means of the integration plot analysis after intravenous administration of [^{14}C]DHA and [^{14}C]AA to rats. The apparent influx permeability clear-

ance per gram retina of [^{14}C]DHA was found to be 2.44×10^3 μl/(min·g retina) and about 38-fold greater than that of [^{14}C]AA (65.4 μl/(min·g retina)). HPLC analysis revealed that most of the vitamin C accumulates in AA form in the retina, suggesting that vitamin C is mainly transported as the DHA form across the BRB and accumulates as the AA form in the rat retina. In order to clarify the transport mechanisms of vitamin C at the inner BRB, TR-iBRB cells have been used as an *in vitro* model of the inner BRB. The initial uptake rate of [^{14}C]DHA was 37-fold greater than that of [^{14}C]AA, which is in agreement with the results of *in vivo* studies. [^{14}C]DHA uptake by TR-iBRB cells took place in an Na^+-independent and concentration-dependent manner with a K_m of 93.4 μM and inhibition by substrates and inhibitors of GLUT. [^{14}C]DHA uptake was inhibited by D-glucose in a concentration-dependent manner with an IC_{50} of 5.56 mM. Quantitative real-time PCR and immunostaining analyses revealed that GLUT1 expression is greater than that of GLUT3 and SVCT2 in TR-iBRB cells.[43] The role of GLUT1 at the inner BRB is important in supplying D-glucose as well as vitamin C to the neural retina. The high plasma D-glucose concentration in diabetic mellitus may restrict the supply of vitamin C to the retina due to inhibition of GLUT1 and exacerbate diabetic retinopathy as the result of oxidative stress in the retina.

Efflux Transport for P-Glycoprotein P-gp, which is an ATP-dependent 170 kDa membrane glycoprotein, exhibits a protective role by restricting the entry of a wide variety of chemotherapeutic agents and hydrophobic compounds in tumor cells as well as normal tissues.[44] When cyclosporine A (CsA) is orally administered to the rabbit at dose of 20 mg/kg per day, although the blood level of CsA achieved is within the therapeutic window of 400—600 ng/ml, no CsA is detected in the intraocular tissues.[45] This suggests that P-gp at the blood-ocular barrier acts as an efflux pump for hydrophobic drugs (*e.g.*, CsA). In support of this functional evidence, immunofluorescence studies have shown that P-gp is present in rat retinal capillary endothelial cells[16,46] as well as brain capillary endothelial cells.[47] An illustration of P-gp expression and merged images of GLUT1 and P-gp at the rat inner BRB are shown in Fig. 4. P-gp is localized at the luminal side of the inner BRB, similar to that in the BBB[47], because the P-gp-immunoreactivity (red) is observed inside that of GLUT1 (Green, Fig. 4F). GLUT1 distribution at the abluminal side of the inner BRB is 3-fold greater than that at the luminal side.[48] TR-iBRB cells express P-gp at 170 kDa as well as mdr 1a, 1b, and 2 mRNA.[29] Although P-gp mRNA has not been identified yet in retinal capillary endothelial cells *in vivo*, murine brain endothelial cells contain predominantly mdr 1a.[49] Shen *et al.* reported that rhodamine 123 accumulation in the TR-iBRB cells was enhanced in the presence of inhibitors of P-gp.[50] This suggests that TR-iBRB cells exhibit efflux transport activity and can be used for screening the transport characteristics of drug candidates that pass through the inner BRB and for identifying those that are not substrates of P-gp.

L-Lactate Transport Under aerobic conditions, the retina produces more L-lactic acid than any other organ in the body and L-lactic acid is produced even under anaerobic conditions. Moreover, L-lactic acid appears to be required as an energy source, in addition to D-glucose, in photoreceptors.[51] Alm and Törnquist were the first to using the RUI method to show that L-lactic acid transport across the rat BRB exhibits saturability, pH-dependence, and is inhibited by pyruvate and 3-hydroxybutyrate.[19] In addition to this functional evidence, Gerhart *et al.* used immunoelectron microscopy to provide morphological evidence that MCT1 is localized at both the luminal and abluminal sides of the inner BRB and at the apical side of outer BRB (RPE).[15] The basolateral side of the outer BRB expresses MCT3.[52] TR-iBRB cells express MCT1 mRNA 33-fold more intensely than MCT2 mRNA.[53] In a study of the transport characteristics of L-Lactic acid at the inner BRB, [^{14}C]L-lactic acid uptake by TR-iBRB cells was shown to be a temperature-, H^+-, and concentration-dependent process with a K_m of 1.7 mM L-lactic acid. L-Lactic acid uptake was inhibited by a protonophore, MCT inhibitors, a number of other monocarboxylates and monocarboxylic drugs. Salicylic and valproic acids competitively inhibited this process with an inhibition constant of 4.7 mM and 5.4 mM, respectively. L-Lactic acid uptake is also inhibited by 5-(*N*,*N*-hexamethylene)-amiloride and Na^+/H^+ exchanger 1 (NHE1) mRNA is expressed in TR-iBRB cells, suggesting that NHE1 provides an H^+ gradient at the inner BRB.[53] The role MCT1 at the inner BRB may be important in regulating the L-lactate concentration in the retina and could be important for accurately assessing the efficacy of exogenous monocarboxylic drugs in the retina in general.

Amino Acid Transport Betz and Goldstein have carried out an amino acid transport study using [^{14}C]α-(methylamino)isobutyric acid and isolated retinal capillaries.[20] It is worth noting that Na^+-dependent α-(methylamino)isobutyric acid transport was found at the inner BRB, although so far there have been no reports of the presence of such neutral amino acids in the retina and serum. In the support of *in vitro* experiments, Törnquist and Alm used the RUI method to show that the transport of L-phenylalanine, L-arginine, and taurine from the blood to the retina across the rat BRB is a carrier-mediated transport process.[54] At the BBB, system L (LAT1),[55] system y^+ (CAT1),[56] and system β/TAUT[57] are present and transport neutral, cationic, and β amino acids, respectively. RT-PCR analysis has been used to show that TR-iBRB cells express LAT1, CAT1 and TAUT mRNA. At present, the amino acid transporters at the inner BRB remain largely unknown and the characterization of each form of amino acid transport is expected in the near future.

L-Cystine Transport and Glutathione Biosynthesis Glutathione (GSH), which is a tripeptide composed of L-glutamic acid (L-Glu), L-cysteine, and glycine, plays an important role in protecting cells against free radical peroxides, and other toxic agents.[58] To protect the retina against light-induced oxidative stress and maintain the intracellular GSH at an appropriate level, transport into the retina of one of the constituent amino acids, cyst(e)ine, is critical for the health of the retina. *In vivo* integration plot analysis shows that L-cystine uptake by the eye and brain is activated by pretreatment with diethyl maleate (DEM), a reagent used to deplete intracellular GSH in order to induce oxidative stress.[59] This enhanced uptake is inhibited in the presence of L-Glu and L-α-aminoadipic acid, substrates for system x_c^-, which is composed of 4F2hc and xCT.[60] This suggests that L-cystine influx transport *via* system x_c^- is activated by DEM at the BBB and BRB *in vivo*. TR-iBRB and TR-MUL cells express xCT

and 4F2hc mRNA and L-cystine uptake by TR-iBRB cells takes place in an Na^+-independent and concentration-dependent manner with a K_m of 9.2 μM and is inhibited by system x_c^- substrates and inhibitors.[31,61] DEM treatment causes significant induction of xCT mRNA, L-cystine uptake and increases the GSH concentration in TR-iBRB and TR-MUL cells. Understanding the supply pathway of sulfur amino acid precursors and the cellular mechanisms of GSH homeostasis in the retina would be of great value in devising GSH-based treatment for retinal diseases and protecting the eyes from light-induced oxidative stress.

Creatine Transport Creatine plays a vital role in the storage and transmission of phosphate-bound energy due to the conversion of creatine to phosphocreatine.[62] In order to maintain a high concentration of creatine and ATP homeostasis in the retina, the transport of creatine from the circulating blood to the retina across the BRB is important in the treatment of gyrate atrophy of the choroids and retina with hyperornithinemia (GA). This results in high ornithine and low creatine concentrations in the body fluids and leads to chorioretinal degeneration.[63] An *in vivo* intravenous administration study demonstrated that [^{14}C]creatine is transported from the blood to the retina against the creatine concentration gradient that exists between the retina and blood.[64] [^{14}C]Creatine uptake by TR-iBRB cells took place an Na^+- and Cl^--dependent manner and was inhibited by creatine transporter (CRT) inhibitors. CRT mRNA and protein was expressed in the retina and TR-iBRB cells and immunoelectron microscopy investigations revealed localization of CRT immunoreactivity at both the luminal and abluminal sides of the inner BRB.[64] Thus, the processes of creatine transport and CRT expression at the inner BRB are important for understanding the mechanism governing the supply of creatine to the neural retina and could help in the design of improved treatments for GA.

Organic Anions Transport Vitreous fluorophotometry has been used to determine the transport of fluorescein, an organic anion, in the blood-to-vitreous direction as well as the vitreous-to-blood direction in humans.[65,66] Engler *et al.* found that the fluorescein transport in the vitreous-to-blood direction is more than 100-fold greater than that in the opposite direction. It was inhibited in the presence of probenecid, suggesting that fluorescein transport across the BRB is involved in the carrier-mediated organic anion transport process.[66] Betz and Goldstein demonstrated that *p*-aminohippuric acid (PAH) uptake by isolated retinal capillaries was slightly greater than that of the extracellular marker, sucrose, and inhibited by fluorescein and penicillin.[20] These findings suggest that one or more organic anion transport processes are present at the inner BRB. Recently, organic anion transporter polypeptide (oatp)2 has been shown to be present at the rat inner and outer BRB[67] and immunofluorescence has been used to show that oatp12 (oatp-E) is expressed at the rat outer BRB.[68] Oatp1, oatp3, and oatp9 mRNAs are also expressed in the retina, although the localization of these transporters is presently unknown.[69—71] Oatps do not transport PAH and organic anion transporter (OAT) has PAH as a substrate.[72] The expression and functions of OATs and other transporters, such as multidrug resistance associated protein (MRP), at the inner BRB remain largely unknown at present and there is a need to investigate the efflux transport mechanisms of neurotransmitters and their metabolites.

CELL-TO-CELL INTERACTIONS AND DIFFERENCES

The Inner BRB Specific Gene As described earlier, GLUT1,[14] MCT1,[15] P-gp,[16] and CRT[64] transporters are expressed and function at the inner BRB as well as the BBB.[10,47,73,74] Nevertheless, the retina is especially differentiated for vision. This prompts the hypothesis that the inner BRB expresses different molecules than the BBB. In addition to TR-iBRB, TR-rPCT, and TR-MUL cells, we have established BBB cell lines, a conditionally immortalized rat brain capillary endothelial cell line (TR-BBB),[75] pericytes (TR-PCT),[76] and an astrocyte cell line (TR-AST)[77] from tsA58Tg rats. The background of gene expression in these cell lines is similar, since these lines were established from the same rat strain using the same procedure. Therefore, the difference in expressed genes between these cell lines may indeed reflect differences between the inner BRB and BBB *in vivo*. A comparison of expressed genes between TR-iBRB cells and TR-BBB cells was performed using mRNA differential display analysis and quantitative real-time PCR analysis.[78] Although no difference in transporters between these two cell lines was observed under these conditions, 8 clones were identified as highly expressed genes in TR-iBRB cells including GATA-binding protein-3, cytosolic branched chain amino transferase, and M-cadherin (cadherin-15). The expression of rat M-cadherin in TR-iBRB cells was much greater than that in TR-BBB cells and greater expression of M-cadherin may indeed be involved in the unique functions exhibited by the inner BRB.[78]

Cell-to-Cell Interactions Retinal endothelial cells are surrounded by retinal pericytes and Müller cells as shown in Fig. 1. Although the overall retinal microvascular biology may be a function of the paracrine interaction between endothelial cells and the two other types of cells, the signal transduction mechanisms for tight junction regulation of endothelial cells and the exact cause of diabetic retinopathy remain largely unknown at present. TR-rPCT cells reduce the number of TR-iBRB cells in a contact co-culture in comparison with that of a single culture of TR-iBRB cells.[30] This suggests that retinal pericytes may regulate the growth of retinal endothelial cells. Clearly more mechanistic studies and identification of regulatory factor(s) are required for the development of new treatments for diabetic retinopathy in the near future.

CONCLUSIONS

The development of inner BRB cell lines helps in investigating the transport mechanisms operating at the inner BRB. Up until 5 years ago, knowledge of inner BRB transport was very limited compared with that at the BBB. This began to change when inner BRB cell lines were developed to allow the identification of transporters and their regulation at the inner BRB under physiological and pathophysiological conditions. The transporters identified in recent years are summarized in Fig. 5. Although these transporters are the same as in the BBB, several genes at the inner BRB are different from the BBB and may be involved in specific functions in the inner BRB and retina. The study of cell-to-cell interac-

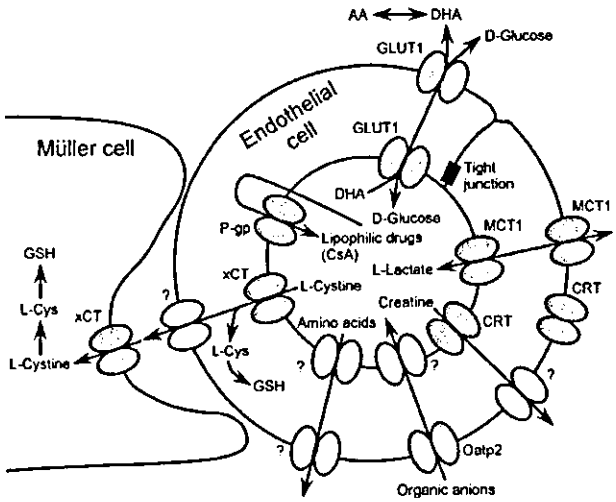

Fig. 5. Putative localization of inner BRB transporters

tions between retinal endothelial cells and other types of cells is in its infancy. We hope to identify regulation factor(s) of endothelial growth secreted from pericytes and the interactions between retinal endothelial cells and Müller cells. As more information becomes available regarding inner BRB transport and cell-to-cell interactions, compared with the thoroughly investigated BBB, we may be able to design simpler and more effective routes for drug delivery to the retina and develop improved treatments for diabetic retinopathy.

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A pericyte-derived angiopoietin-1 multimeric complex induces occludin gene expression in brain capillary endothelial cells through Tie-2 activation *in vitro*

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Abstract

Although tight-junctions (TJs) at the blood–brain barrier (BBB) are important to prevent non-specific entry of compounds into the CNS, molecular mechanisms regulating TJ maintenance remain still unclear. The purpose of this study was therefore to identify molecules, which regulate occludin expression, derived from astrocytes and pericytes that ensheathe brain microvessels by using conditionally immortalized adult rat brain capillary endothelial (TR-BBB13), type II astrocyte (TR-AST4) and brain pericyte (TR-PCT1) cell lines. Transfilter co-culture with TR-AST4 cells, and exposure to conditioned medium of TR-AST4 cells (AST-CM) or TR-PCT1 cells (PCT-CM) increased occludin mRNA in TR-BBB13 cells. PCT-CM-induced occludin up-regulation was significantly inhibited by an angiopoietin-1-neutralizing antibody, whereas the up-regulation by AST-CM was not. Immunoprecipitation and western blot analyses confirmed that multimeric angiopoietin-1 is secreted from TR-PCT1 cells, and induces occludin mRNA, acting through tyrosine phosphorylation of Tie-2 in TR-BBB13 cells. A fractionated AST-CM study revealed that factors in the molecular weight range of 30–100 kDa led to occludin induction. Conversely, occludin mRNA was reduced by transforming growth factor β1, the mRNA of which was up-regulated in TR-AST4 cells following hypoxic treatment. In conclusion, *in vitro* BBB model studies revealed that the pericyte-derived multimeric angiopoietin-1/Tie-2 pathway induces occludin expression.

Keywords: astrocyte, blood–brain barrier, multimeric angiopoietin-1, occludin, pericyte, Tie-2.

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Tight-junctions (TJs) form barriers between adjacent brain capillary endothelial cells (BCECs) at the blood–brain barrier (BBB) and play an important role in preventing non-specific paracellular transport in order to protect the CNS. Brain disorders, such as brain tumors, infarcts and encephalitis, cause TJ disruption to allow BBB leakage (Davies 2002). Therefore, clarifying the mechanism of TJ maintenance is important for understanding and treating CNS diseases associated with BBB leakage.

BCECs are surrounded by pericytes and astrocyte foot processes. The overall brain microvascular biology is a function of the paracrine interactions between BCECs and the two other types of cells (Pardridge 1999; Gaillard

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Abbreviations used: AST (PCT)-CM, conditioned medium of TR-AST (TR-PCT) cells; BBB, blood–brain barrier; BCEC, brain capillary endothelial cell; CM, conditioned medium; ECGF, endothelial cell growth factor; JAM, junctional adhesion molecule; PI3-kinase, phosphatidylinositol 3-kinase; PTyr, phosphotyrosine; TEER, transendothelial electrical resistance; TGF-β1, transforming growth factor β1; TJ, tight-junction; TR-AST, conditionally immortalized rat astrocyte cell line; TR-BBB, conditionally immortalized rat brain capillary endothelial cell line; TR-PCT, conditionally immortalized rat pericyte cell line; VEGF, vascular endothelial growth factor.

et al. 2000; Abbott 2002). Astrocytes are known to induce TJs of non-CNS endothelial cells *in vivo* (Janzer and Raff 1987). As for brain pericytes, their recruitment and association with microvessels are also key processes in normal vascular development and maintenance. It has been shown that platelet-derived growth factor-B knock-out mice lack brain pericytes, and die due to hemorrhage (Lindahl *et al.* 1997). Therefore, it is conceivable that paracrine interactions between BCECs, and astrocytes and pericytes, play important roles in maintaining TJs at the BBB.

Occludin (Furuse *et al.* 1993; Hirase *et al.* 1997), which is a transmembrane protein, is exclusively localized at the TJ strands of the BBB, together with junctional adhesion molecule (JAM) (Martin-Padura *et al.* 1998), claudin-5 and claudin-12 (Nitta *et al.* 2003). These transmembrane proteins are important in regulating the paracellular permeability by interacting with each other in both a homophilic and heterophilic manner between adjacent cells, and by becoming linked to the cytoskelton through a complex of accessory proteins such as ZO-1/ZO-2/ZO-3. Since occludin expression is down-regulated in various brain disorders accompanied by TJ disruption (Huber *et al.* 2001; Davies 2002), the expression level of occludin is important for TJ maintenance at the mature BBB. Therefore, identifying physiological occludin-regulators will provide deeper insight into the maintenance and recovery of the TJ properties at the BBB.

Angiopoietin-1, a ligand of tyrosine kinase Tie-2, is known to be an anti-permeability factor in the peripheral vascular system (Davis *et al.* 1996). It has recently been reported that administration of angiopoietin-1 reduces BBB leakage in the ischemic brain (Zhang *et al.* 2002), suggesting that angiopoietin-1 has an anti-permeability effect on the BBB as well as the peripheral vascular system. In contrast, transforming growth factor β1 (TGF-β1) and vascular endothelial growth factor (VEGF) are vascular permeability factors, and have been reported to be increased in the brain in various neurodegenerative diseases (Kalaria *et al.* 1998; Lesne *et al.* 2002). We hypothesize that these soluble factors are secreted from astrocytes and/or brain pericytes in the mature CNS, and involved in TJ regulation at the BBB under physiological and pathophysiological conditions.

Induction and maintenance of the TJ properties at the BBB depend critically on the local conditions and maturational state. In the present study, in order to clarify the astrocyte- and pericyte-derived factors involved in TJ maintenance, we selected conditionally immortalized brain endothelial (TR-BBB) (Hosoya *et al.* 2000b), type II astrocyte (TR-AST) (Tetsuka *et al.* 2001), and brain pericyte (TR-PCT) (Asashima *et al.* 2002; Asashima *et al.* 2003) cell lines, which had been established from adult transgenic rats harboring temperature-sensitive simian virus 40 large T-antigen (Obinata 1997; Takahashi *et al.* 1999) and which retain their *in vivo* function well (Terasaki and Hosoya 2001; Terasaki *et al.* 2003).

The purpose of the present study was to clarify the mechanism of occludin induction and identify occludin-inducing molecules by using conditionally immortalized BBB cell lines, which are of the same maturational stage, strain, and genetic background. The change in occludin expression level was quantified in TR-BBB13 cells during transfilter co-culture with TR-AST4 cells and on treatment with the conditioned medium of TR-AST4 cells (AST-CM) or TR-PCT1 cells (PCT-CM). We also investigated the effect of angiopoietin-1 in conditioned medium, TGF-β1 and VEGF on occludin expression, and Tie-2 activation by PCT-CM to clarify the signal pathway for occludin regulation.

Materials and methods

Animals

Male Wistar rats, weighing 250–300 g, were purchased from Charles River (Yokohama, Japan). The investigations using rats described in this report conformed to the guidelines established by the Animal Care Committee, Graduate School of Pharmaceutical Sciences, Tohoku University.

Reagents

Endothelial cell growth factor (ECGF) was purchased from Boehringer Mannheim (Mannheim, Germany); benzylpenicillin potassium and streptomycin sulfate were purchased from Wako Pure Chemical Industries (Osaka, Japan); recombinant human angiopoietin-1 was purchased from Genzyme Techne (Minneapolis, MN, USA); recombinant human VEGF and TGF-β1 were purchased from Peprotech EC (London, UK). All other chemicals were commercial products of analytical grade.

Cell cultures

TR-BBB13, TR-AST4 and TR-PCT1 cells were conditionally immortalized BCEC, astrocyte and pericyte cell lines, respectively (Hosoya *et al.* 2000b; Tetsuka *et al.* 2001; Asashima *et al.* 2002) and used as *in vitro* BBB model (Terasaki *et al.* 2003). TR-BBB13 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Nissui Pharmaceutical, Tokyo, Japan) supplemented with 20 mM sodium bicarbonate, 15 ng/mL ECGF, 100 U/mL benzylpenicillin potassium, 100 μg/mL streptomycin sulfate and 10% fetal bovine serum (Moregate, Bulimba, Australia) (culture medium-A). The culture medium-B for TR-AST4 cells and TR-PCT1 cells consisted of culture medium-A without ECGF. TR-BBB13 cells and TR-PCT1 cells were seeded onto rat tail collagen type I-coated tissue culture dishes (BD Biosciences, Franklin Lakes, NJ, USA). These cells were maintained at 33°C, which is a permissive temperature at which temperature-sensitive SV40 large T-antigen is activated, in a humidified atmosphere of 95% air and 5% CO_2. The experimental culture temperature was also 33°C because a long-term culture needs the cell growth conditions. The cells retain the expression of specific markers at 33°C (Hosoya *et al.* 2000b; Tetsuka *et al.* 2001; Asashima *et al.* 2002).

Transfilter co-culture of TR-BBB13 cells and TR-AST4 cells

TR-BBB13 cells were cultured with TR-AST4 cells in a transfilter co-culture system. In this system, TR-AST4 cells were seeded [5×10^4 cells per insert (4.3 cm^2)] on the backside membrane of a collagen type I-coated transfilter, a cell culture insert (pore size: 3.0 μm, BD Biosciences) in culture medium-B. After 24-h culture, the insert was transferred to a 6-well plate and TR-BBB13 cells were seeded (5×10^4 cells per insert) on the upper side of the insert. In single culture, TR-BBB13 cells were seeded (5×10^4 cells per insert) on the upper side of the insert without TR-AST4 cells on the backside. The cells were cultured at 33°C and the culture medium-B was renewed every other day. After a predetermined time period, transendothelial electrical resistance (TEER) in TR-BBB13 cells was measured using Millicell-ERS equipment (Millipore, Bedford, MA, USA), and after that the cells were collected with a cell scraper.

Preparation of AST-CM and PCT-CM

TR-AST4 cells or TR-PCT1 cells were cultured in culture medium-B without serum. After 24 h, conditioned medium (CM) was collected, concentrated up to 20-fold in a Centriprep-10 (10-kDa cut-off) (Millipore, Bedford, MA, USA) and stored at −20°C until studied. Control CM was prepared by the same procedure using culture medium-B without serum. The CM was adjusted to an appropriate concentration by diluting the 20-fold concentrated CM with culture medium-B without serum.

Fractionation of CM using size exclusion membranes

CM was concentrated up to 20-fold using a graded series of molecular weight cut-off filters as follows (Millipore). The CM was concentrated using a Centriplus-100 (100-kDa cut-off). The retentate was kept as the >100-kDa fraction and the filtrate was sequentially concentrated using a Centriprep-50 (50-kDa cut-off) (to obtain a 50- to 100-kDa fraction), Centriprep-30 (30-kDa cut-off) (30- to 50-kDa fraction) and Centriprep-10 (10- to 30-kDa fraction).

Treatment with AST-CM, PCT-CM, angiopoietin-1, VEGF or TGF-β1

TR-BBB13 cells were treated with AST-CM, PCT-CM or fractionated AST-CMs for 24 h at 33°C. TR-BBB13 cells were treated with angiopoietin-1 (0, 0.1, 1, 10, 100 and 500 ng/mL), VEGF (0, 0.1, 1 and 10 ng/mL) or TGF-β1 (0, 0.1, 1 and 10 ng/mL) for 24 h at 33°C.

Hypoxic conditions

Hypoxic conditions were achieved with an anaerobic chamber and BBL GasPak Plus (BD Biosciences), which catalytically reduces the oxygen level to less than 10 p.p.m. within 90 min (Shimizu *et al.* 1996). TR-AST4 cells were cultured for 24 h under these hypoxic conditions.

Angiopoietin-1 inhibitory study

AST-CM and PCT-CM were pre-treated with 0.625 μg/mL antibody against angiopoietin-1 (Chemicon, Temecula, CA, USA) or normal rabbit IgG (control) for 16 h at 4°C. TR-BBB13 cells were cultured with the angiopoietin-1-neutralized-AST-CM or PCT-CM for 24 h at 33°C.

RT-PCR analysis

Total RNA was extracted from rat tissues, TR-AST4 cells and TR-PCT1 cells using an RNeasy kit (Qiagen, Tokyo, Japan) according to the manufacturer's protocol. Single-stranded cDNA was made from 1 μg total RNA by RT (ReverTraAce, Toyobo, Osaka, Japan) using oligo dT primer. The sequences of primers were as follows: sense primer 5′-AGGAGACGGAATACAGGGCT-3′ and antisense primer 5′-CCGGGTTGTGTTGGTTGTAG-3′ for TGF-β1 (GenBank Accession Number; NM021578); sense primer 5′-CTCAGTGGCTGCAAAAACTTG-3′ and antisense primer 5′-CAGAATTTCATTTGTCTGTTGGA-3′ for angiopoietin-1 (GenBank Accession Number; AB080023); sense primer 5′-TTTGAGACCTTCAACACCCC-3′ and antisense primer 5′-ATAGCTCTTCTCCAGGGAGG-3′ for β-actin (GenBank Accession Number; NM031144). The PCR was performed using GeneAmp (PCR system 9700, Perkin-Elmer, Norwalk, CT, USA) with TGF-β1, angiopoietin-1 and β-actin specific primers through 30 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 60°C, and synthesis for 1 min at 72°C. The sizes of the expected RT-PCR products of TGF-β1, angiopoietin-1 and β-actin were 416, 275 and 352 bp, respectively. The RT-PCR of each sample RNA without the RT was used as a negative control. The RT-PCR products were separated by electrophoresis on an agarose gel in the presence of ethidium bromide (0.6 μg/mL) and visualized using an imager (EPIPRO 7000; Aisin, Aichi, Japan). The PCR products were subcloned into a plasmid vector using pGEM-T Easy Vector System I (Promega, Madison, WI, USA) and then sequenced from both directions using a DNA sequencer (CEQ2000XL DNA Analysis System; Beckman Coulter, Fullerton, CA, USA). Sequence comparisons were made using the GENETYX software package, version 6.1.0 (Genetyx, Tokyo, Japan).

Quantitative real-time PCR analysis

Total RNA was extracted from TR-BBB13 cells using an RNeasy kit according to the manufacturer's protocol. RNA integrity was checked by electrophoresis on an agarose gel. Quantitative real-time PCR analysis was performed using an ABI PRISM 7700 sequence detector system (PE Applied Biosystems, Foster City, CA, USA) with 2× SYBR Green PCR Master Mix (PE Applied Biosystems) as per the manufacturer's protocol. To quantify the amount of specific mRNA in the samples, a standard curve was generated for each run using pGEM-T Easy Vector containing occludin, JAM or β-actin (dilution ranging from 0.1 fg/μL to 1 ng/μL). This enabled standardization of the initial mRNA content of TR-BBB13 cells relative to the quantity of β-actin. The control lacking the RT enzyme was assayed in parallel to monitor any possible genomic contamination. PCR was performed through 40 cycles of 95°C for 30 s, 60°C for 1 min, and 72°C for 1 min after pre-incubation at 95°C for 10 min using specific primers. The sequences of primers were as follows: sense primer 5′-GCCTTTTGCTTCATCGCTTCC-3′ and antisense primer 5′-AACAATGATTAAAGCAAAAGCCAC-3′ for occludin (GenBank Accession Number, AB016425); sense primer 5′-ACAGCCATGAGGTCAGAGGCT-3′ and antisense primer 5′-ACCTAGAAGACATTGAAGGCATC-3′ for JAM (GenBank Accession Number, AF276998). The β-actin primers are given above.

Western blot analysis

The membrane and whole cell lysate fractions of rat brain, isolated rat brain capillary, and TR-BBB13 cells were prepared using the procedure described in a previous report (Hosoya *et al.* 2000a). The AST-CM or PCT-CM was suspended in 10% trichloroacetic acid/acetone, with or without 20 mM dithiothreitol for 1 h at −20°C, followed by centrifugation at 15 000 *g* for 15 min. The pellet was washed with acetone, with or without 20 mM dithiothreitol, then lyzed with lysis buffer (10 mM Tris-HCl pH 7.4, 1% sodium dodecyl sulfate, 1 mM EDTA and 10% glycerol), with or without 5% 2-mercaptoethanol. Protein concentrations were determined by a DC protein assay kit (Bio-Rad, Hercules, CA, USA). Membrane lysate (20 μg per lane) was used for detecting occludin protein in rat tissues and the cell lines (Fig. 1b). Whole cell lysate (50 μg per lane) was used for investigating the effect of the transfilter co-culture on occludin expression (Fig. 1c). Protein samples were electrophoresed on gradient sodium dodecyl sulfate–polyacrylamide gel (Bio-Rad) and subsequently electrotransferred to nitrocellulose membranes. Membranes were treated with blocking buffer (4% skimmed milk in 25 mM Tris-HCl pH 8.0, 125 mM NaCl, 0.1% Tween 20) for 2 h at room temperature and incubated with anti-occludin antibody (0.1 μg/mL; Zymed, San Francisco, CA, USA), anti-β-actin antibody (1 : 2000; Sigma, St.Louis, MO, USA), anti-angiopoietin-1 antibody (1 μg/mL; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-Tie-2 antibody (1 μg/mL; Santa Cruz Biotechnology) for 16 h at 4°C as the primary antibody. The membranes were washed three times with blocking buffer and incubated with horseradish peroxidase-conjugated second antibody. The bands were visualized with an enhanced chemiluminescence kit (SuperSignal; Pierce, Rockford, IL, USA). The relative densities of the bands were measured using NIH image software (National Institutes of Health, Bethesda, MD, USA).

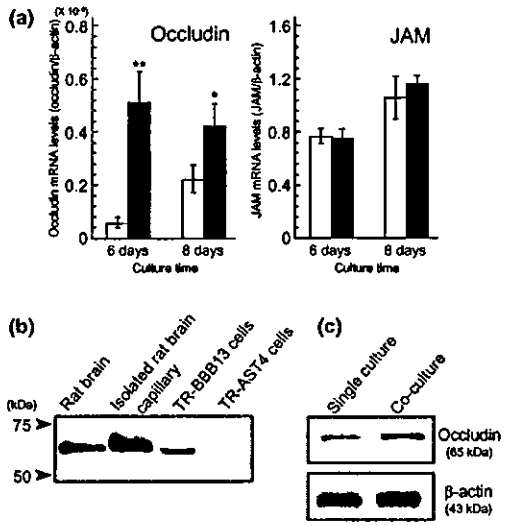

Fig. 1 Induction of occludin expression in TR-BBB13 cells transfilter co-cultured with TR-AST4 cells. (a) TR-BBB13 cells were transfilter co-cultured with TR-AST4 cells (black column) or single-cultured (open column) for 6 or 8 days. The occludin and JAM mRNA levels were determined by quantitative real-time PCR analysis. Each mRNA expression level was normalized with respect to the β-actin mRNA expression. Each column represents the mean ± SEM ($n = 3$). $^{**}p < 0.01$, $^{*}p < 0.05$, significantly different from the single culture. (b) Western blot analysis of occludin in rat brain and isolated rat brain capillary (positive controls), TR-BBB13 cells and TR-AST4 cells (a negative control). (c) Western blot analysis of occludin (upper) and β-actin (lower) in single-cultured TR-BBB13 cells and TR-BBB13 cells co-cultured with TR-AST4 cells for 8 days. The ratio of occludin to β-actin density in co-culture was 1.77-fold greater than that in single culture ($n = 3$).

Analysis of tyrosine phosphorylation of Tie-2 protein

TR-BBB13 cells were cultured with two-fold concentrated AST-CM, PCT-CM or 300 ng/mL angiopoietin-1 for 24 h, then collected and suspended in lysis buffer (10 mM Tris-HCl pH 7.5, 1% Triton X-100, 0.5% Nonidet P-40, 1 mM EDTA, 150 mM NaCl, 5 mM sodium pyrophosphate, 10 mM *p*-nitrophenyl phosphate, 10 mM β-glycerophosphate, 50 mM sodium fluoride and 1 mM sodium orthovanadate) for 30 min on ice, followed by centrifugation at 15 000 *g* for 15 min. The Tie-2 protein was immunoprecipitated from the cell lysate using protein Glutathione Sepharose 4B gel beads (Amersham Biosciences, Picataway, NJ, USA) coated with anti-Tie-2 antibody. After electrophoresis under reducing conditions using a gradient gel polyacrylamide (Bio-Rad), the immunoprecipitated Tie-2 proteins were transblotted onto a nitrocellulose membrane. The membrane was incubated with either 1 μg/mL anti-Tie-2 antibody or anti-phosphotyrosine (PTyr) antibody in a blocking buffer for 1 h. The membrane was washed and incubated with horseradish peroxidase-conjugated IgG, then developed as described above. The relative densities of the bands were measured using NIH image software (National Institutes of Health).

Data analysis

Unless otherwise indicated, all data represent the mean ± SEM. An unpaired, two-tailed Student's *t*-test was used to determine the significance of differences between two groups means. One-way ANOVA followed by the modified Fisher's least-squares difference method was used to assess statistical significance of differences among means of more than two groups.

Results

Induction of occludin expression in TR-BBB13 cells by transfilter co-culture with TR-AST4 cells

The effect of transfilter co-culture with TR-AST4 cells on the expression levels of occludin and JAM was examined in TR-BBB13 cells (Fig. 1). The cells were co-cultured for 6 and 8 days, since it has been reported that cell-to-cell contact was observed between endothelial cells and astrocytes using the same transfilter pore size membrane for over 4 days (Hayashi *et al.* 1997). As shown in Fig. 1(a), the occludin mRNA levels in 6- and 8-day co-cultured TR-BBB13 cells were significantly increased compared with single culture (9.14- and 1.91-fold, respectively). In contrast, the JAM mRNA level was not changed significantly at either time point. To clarify whether occludin protein was increased

concomitantly with the induction of occludin mRNA, the expression of occludin protein was examined in TR-BBB13 cells for 8 days (Figs 1b and c). A single band at 65 kDa was detected in TR-BBB13 cells, corresponding in size to the bands from rat brain and isolated rat brain capillary used as positive controls, whereas no band was detected in TR-AST4 cells (Fig. 1b). Moreover, TR-AST4 cells did not express occludin mRNA (data not shown). The ratio of occludin to β-actin density in co-culture was 1.77-fold greater than that in single culture (Fig. 1c). The TEER in TR-BBB13 cells was unchanged by co-culture (106 ± 2 and 106 ± 1 ohm cm^2) compared with single culture (103 ± 1 and 109 ± 2 ohm cm^2) for 6 and 8 days, respectively.

Induction of occludin mRNA in TR-BBB13 cells by treatment with AST-CM or PCT-CM

To determine the regulatory effects of soluble factors secreted from TR-AST4 and TR-PCT1 cells, the expressional change of occludin and JAM mRNAs was examined in TR-BBB13 cells treated with AST-CM or PCT-CM (Fig. 2). The occludin mRNA level in TR-BBB13 cells was significantly increased by treatment with both CMs (Fig. 2a). Following treatment with AST-CM, the occludin mRNA level was increased in a concentration-dependent manner, and the occludin mRNA level was increased and reached a plateau at two-fold concentrated CM in the case of PCT-CM treatment. In contrast, the JAM mRNA level was not significantly changed by the presence of one- to five-fold concentrated AST-CM or PCT-CM compared with each control medium (Fig. 2b).

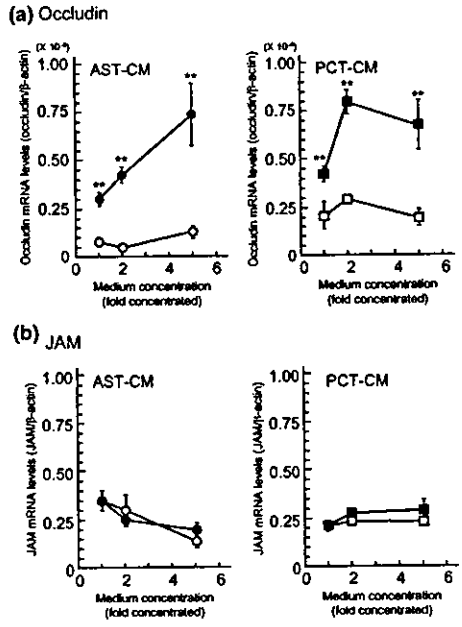

Fig. 2 Induction of occludin mRNA in TR-BBB13 cells by treatment with AST-CM or PCT-CM. Effects of AST-CM (●) or PCT-CM (■) on the occludin (a) and JAM (b) mRNA levels in TR-BBB13 cells for 24 h. ○, control medium for AST-CM; □, control medium for PCT-CM. The occludin and JAM mRNA levels were determined by quantitative real-time PCR analysis. Each point represents the mean ± SEM ($n = 3$). Each mRNA expression level was normalized with respect to the β-actin mRNA expression. $^{**}p < 0.01$, significantly different from the control.

Regulation of occludin mRNA by angiopoietin-1, VEGF and TGF-β1

The effects of 24-h treatment of angiopoietin-1, VEGF and TGF-β1 on the occludin mRNA level were examined in TR-BBB13 cells (Fig. 3). The occludin mRNA level significantly increased following treatment with >1.0 ng/mL angiopoietin-1 compared with non-treated cells (1.73-fold by 1.0 ng/mL; 1.76-fold by 10 ng/mL; 2.09-fold by 100 ng/mL; 2.20-fold by 500 ng/mL) (Fig. 3a). Conversely, the occludin mRNA level was significantly reduced following the addition of VEGF (1.0 ng/mL, by 25.9%; 10 ng/mL, by 35.9%) or TGF-β1 (0.1 ng/mL, by 17.1%; 1.0 ng/mL, by

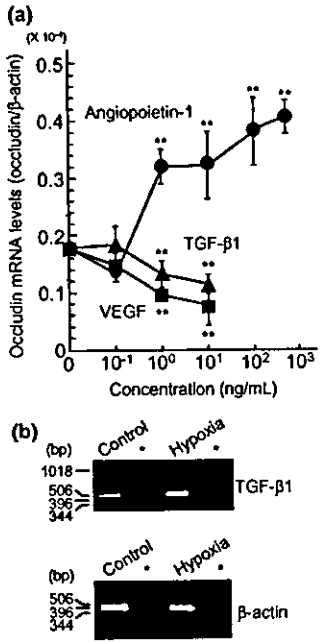

Fig. 3 Effects of angiopoietin-1, VEGF and TGF-β1 on the occludin mRNA level in TR-BBB13 cells (a) and effect of hypoxia on the expression level of TGF-β1 mRNA in TR-AST4 cells (b). (a) Effects of angiopoietin-1 (●), TGF-β1 (▲) and VEGF (■) on the occludin mRNA level in TR-BBB13 cells for 24 h. The occludin mRNA levels were determined by quantitative real-time PCR analysis. Each mRNA expression level was normalized with respect to the β-actin mRNA expression. $^{**}p < 0.01$, significantly different from the control. (b) RT-PCR analysis of TGF-β1 and β-actin in TR-AST4 cells under normal conditions (control) and 24-h hypoxic conditions. *Respective RT(−) for left-hand lane.

46.3%; 10 ng/mL, by 57.4%) for 24 h (Fig. 3a). The JAM mRNA level remained unchanged under any of these conditions (data not shown). To clarify the involvement of TGF-β1 under pathophysiological conditions, the effect of hypoxic conditions on the mRNA expression level of TGF-β1 was determined in TR-AST4 cells by RT-PCR analysis. The mRNA expression of TGF-β1 was increased in TR-AST4 cells following a 24-h period of hypoxia as shown in Fig. 3(b).

Effect of anti-angiopoietin-1 antibody on induction of occludin mRNA in TR-BBB13 cells by AST-CM and PCT-CM

To clarify the contribution of angiopoietin-1 to the induction of occludin mRNA in TR-BBB13 cells by AST-CM and PCT-CM, angiopoietin-1 activity was neutralized with anti-angiopoietin-1 antibody. The induction of occludin mRNA after 24-h treatment with 10 ng/mL angiopoietin-1 was reduced by 79.0% following pre-treatment with the antibody, indicating that the antibody possesses angiopoietin-1-neutralizing activity (Fig. 4a). The occludin mRNA level was not inhibited in TR-BBB13 cells by AST-CM pre-treated with the antibody (Fig. 4b). In contrast, following stimulation with one-, two- and five-fold concentrated PCT-CM pre-treated with anti-angiopoietin-1 antibody, the occludin mRNA level was inhibited by 62.6, 61.4 and 45.1%, respectively (Fig. 4c).

Induction of occludin mRNA in TR-BBB13 cells by fractionated AST-CMs

To determine the approximate molecular weight of TR-AST4 cell-derived soluble factors regulating the occludin mRNA level, a study using fractionated AST-CMs was performed (Fig. 5). The occludin mRNA level was increased by 2.50-, 1.99- and 2.44-fold following treatment with AST-CM (> 10-kDa CM), 30- to 50-kDa and 50- to 100-kDa AST-CM, respectively (Fig. 5a). In contrast, the JAM mRNA level in TR-BBB13 cells was not changed by any of the fractionated AST-CMs (Fig. 5b).

Identification of angiopoietin-1 secretion from TR-PCT1 cells

The mRNA expression of angiopoietin-1 was detected in TR-PCT1 cells at the same level as in rat lung, used as a positive control, but was not detected in TR-AST4 cells (Fig. 6a). Western blot analysis was performed for PCT-CM with or without reducing agents in order to clarify whether angiopoietin-1 secreted from TR-PCT1 cells forms a complex via disulfide bonds, as reported previously (Procopio *et al.* 1999) (Fig. 6b). Several bands at >250 kDa were detected in PCT-CM under non-reducing conditions. Recombinant human angiopoietin-1, used as a positive control, was detected at >250 kDa under non-reducing conditions. The reducing conditions led to a reduction in the apparent molecular weight of bands. The shifted band was single, and detected at 65 and 75 kDa in PCT-CM and recombinant human angiopoietin-1, respectively.

Expression and tyrosine phosphorylation of Tie-2 in TR-BBB13 cells treated with PCT-CM or angiopoietin-1

Western blot analysis revealed that Tie-2 protein, a tyrosine kinase receptor of angiopoietin-1, was expressed in TR-BBB13 cells and had the same molecular weight as that expressed in rat brain and isolated rat brain capillary, used as

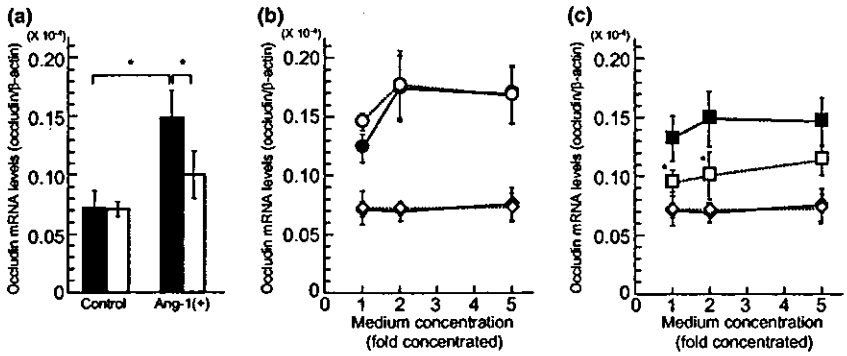

Fig. 4 Effect of anti-angiopoietin-1 antibody on occludin induction by treatment with AST-CM or PCT-CM. (a) TR-BBB13 cells were cultured in the presence [Ang-1(+)] or absence (control) of recombinant human angiopoietin-1 pre-treated with anti-angiopoietin-1 antibody (open columns) or normal rabbit IgG (black columns). (b) TR-BBB13 cells were cultured with AST-CM (circle symbols) or control medium (diamond symbols) pre-treated with anti-angiopoietin-1 antibody (open symbols) or normal rabbit IgG (closed symbols). (c) TR-BBB13 cells were cultured with PCT-CM (square symbols) or control medium (diamond symbols) pre-treated with anti-angiopoietin-1 antibody (open symbols) or normal rabbit IgG (closed symbols). The occludin mRNA levels were determined by quantitative real-time PCR analysis. Each column or point represents the mean ± SEM ($n = 3$). Each mRNA expression level was normalized with respect to the β-actin mRNA expression. $^*p < 0.05$, significantly different from the control.

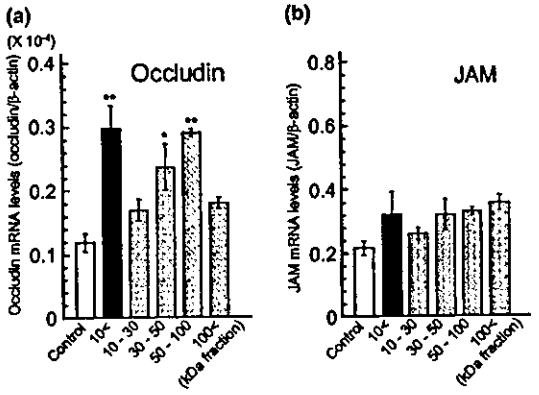

Fig. 5 Effects of fractionated AST-CMs on the occludin (a) and JAM (b) mRNA levels in TR-BBB13 cells. TR-BBB13 cells were cultured with fractionated AST-CM [> 10 (black column), 10–30, 30–50, 50–100 and > 100 kDa fractionated CM (gray columns)] for 24 h. Open column indicates treatment with control medium (serum-free). The occludin and JAM mRNA levels were determined by quantitative real-time PCR analysis. Each column represents the mean ± SEM ($n = 3$). Each mRNA expression level was normalized with respect to the β-actin mRNA expression. $^{**}p < 0.01$, $^{*}p < 0.05$, significantly different from the control.

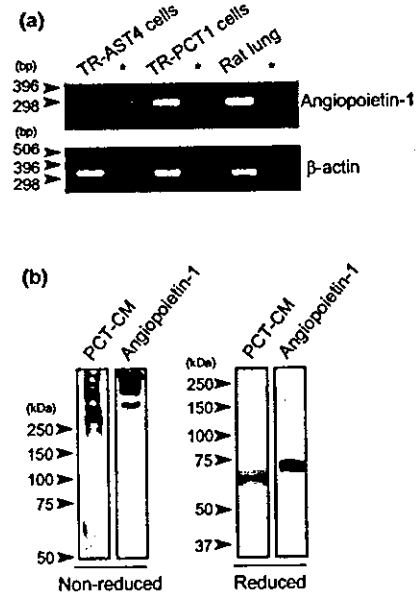

Fig. 6 Secretion of angiopoietin-1 from TR-PCT1 cells. (a) RT-PCR analysis of angiopoietin-1 and β-actin mRNA in TR-AST4 cells, TR-PCT1 cells and rat lung as a positive control. *Respective RT(−) for left-hand lane. (b) Western blot analysis of angiopoietin-1 in PCT-CM in the presence (Reduced) or absence (Non-reduced) of reducing agents (dithiothreitol and 2-mercaptoethanol). Recombinant human angiopoietin-1 was used as a positive control.

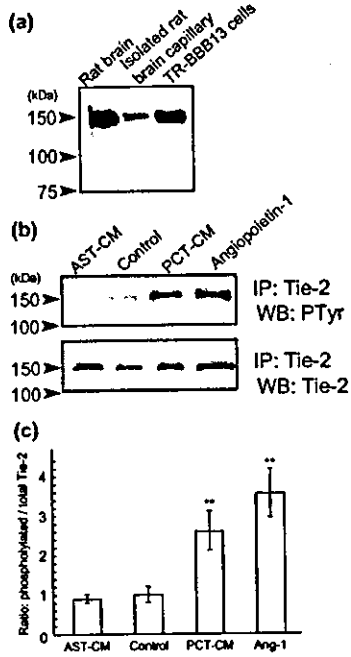

Fig. 7 Expression of Tie-2 (a) and effects of PCT-CM and recombinant human angiopoetin-1 on Tie-2 tyrosine phosphorylation (b and c) in TR-BBB13 cells. (a) Western blot analysis of Tie-2 in rat brain and isolated rat brain capillary as positive controls, and TR-BBB13 cells. (b) TR-BBB13 cells were cultured with two-fold concentrated AST-CM, PCT-CM or 300 ng/mL recombinant human angiopoietin-1. After 24 h, cellular extracts from the treated cells were immunoprecipitated (IP) with anti-Tie-2 antibody followed by western blot analysis (WB) with anti-PTyr antibody (upper) or anti-Tie-2 antibody (lower). A typical result from five experiments is shown. (c) The ratio of tyrosine phosphorylated Tie-2 density to total Tie-2 density. Ang-1, recombinant human angiopoietin-1. Each column represents the mean ± SEM ($n = 5$). $^{**}p < 0.01$, significantly different from non-treated cells (control).

positive controls (Fig. 7a). The tyrosine phosphorylation of Tie-2 protein after 24-h treatment with two-fold concentrated PCT-CM was examined by immunoprecipitation and western blot analyses (Figs 7b and c). The immunoprecipitated Tie-2 protein was electrophoresed and immunoblotted with anti-PTyr antibody. As shown in Fig. 7(b) (upper panel), the tyrosine phosphorylation of Tie-2 protein increased in TR-BBB13 cells treated with PCT-CM or 300 ng/mL recombinant human angiopoietin-1 (a positive control) compared with that in non-treated TR-BBB13 cells. In contrast, the phosphorylated Tie-2 protein was unchanged in TR-BBB13 cells treated with AST-CM. The amount of Tie-2 protein was not affected by any of the treatment conditions (Fig. 7b, lower panel). The ratio of tyrosine phosphorylated Tie-2 to total Tie-2 density in TR-BBB13 cells treated with AST-CM, PCT-CM or recombinant human angiopoietin-1 was, respectively, 0.89-, 2.60- or 3.55-fold greater than that in non-treated TR-BBB13 cells (Fig. 7c).

Discussion

The present study demonstrated that soluble factors secreted from TR-AST4 cells and TR-PCT1 cells induced occludin expression in TR-BBB13 cells. Angiopoietin-1 in PCT-CM predominantly induced occludin expression via Tie-2 tyrosine phosphorylation in TR-BBB13 cells, and was secreted from TR-PCT1 cells as a multimeric disulfide-linked complex (an active form of angiopoietin-1), but not secreted from TR-AST4 cells. This is the first direct evidence concerning the signaling pathway for occludin induction from brain pericytes to BCECs.

The amounts of occludin mRNA and protein were increased in TR-BBB13 cells in transfilter co-culture with cells of the type II astrocyte cell line, TR-AST4 cells (Fig. 1). Type II astrocytes appear to exist in the mature CNS, corresponding to mature fibrous astrocytes mainly localized in white matter (Miller and Raff 1984). This result suggests that fibrous astrocytes ensheathing microvessels at least play some role in maintaining occludin expression at the BBB, mediated by releasing factors and/or a direct contact effect. The concentration-dependent induction of occludin mRNA by AST-CM revealed that soluble factors secreted from TR-AST4 cells induced the occludin gene expression in TR-BBB13 cells (Fig. 2). Direct contact with TR-AST4 cells was also possibly involved in the occludin induction in TR-BBB13 cells, since the membrane pore size (3.0 μm) of the cell culture insert used in the present study was the same as that used in the report in which direct cell-to-cell contact was demonstrated by electron microscopic analysis (Hayashi *et al.* 1997). Moreover, PCT-CM increased the occludin mRNA level, as well as AST-CM (Fig. 2), suggesting that soluble factors secreted from both type II astrocytes and brain pericytes contribute to the induction of occludin expression at the BBB.

Following treatment with angiopoietin-1, the occludin mRNA level was increased in TR-BBB13 cells (Fig. 3a). The expression of occludin mRNA tends to increase in the range of 1–500 ng/mL angiopoietin-1 treatment, and its mRNA level of 500 ng/mL was significantly different from that of 1 ng/mL. This trend is compatible with the reported K_D value of angiopoietin-1 for Tie-2 (about 3 nM, it is calculated as 173 ng/mL when the molecular weight of angiopoietin-1 is 57.7 kDa) (Maisonpierre *et al.* 1997). This result raised the possibility that the occludin gene induction by AST-CM and PCT-CM was partly mediated by angiopoietin-1. The inhibition study using anti-angiopoietin-1-neutralizing antibody revealed that angiopoietin-1 is mainly responsible for the occludin-inducing activity produced by PCT-CM, as shown in Fig. 4(c). Moreover, RT-PCR analysis confirmed that TR-PCT1 cells expressed angiopoietin-1 mRNA (Fig. 6a). Therefore, angiopoietin-1 is suggested to be the predominant occludin-inducing factor secreted from brain pericytes.

In contrast, occludin induction by AST-CM was not inhibited by treatment with angiopoietin-1-neutralizing antibody (Fig. 4b), and TR-AST4 cells did not express angiopoietin-1 mRNA (Fig. 6a). These results indicate that the soluble factors inducing occludin mRNA in AST-CM are different from angiopoietin-1. The absence of angiopoietin-1 in TR-AST4 cells reflects that in mature astrocytes; it has been reported that the mRNA expression level of angiopoietin-1 in astrocytes is low after 3 weeks after birth (Acker *et al.* 2001). During the present study, Lee *et al.* reported that angiopoietin-1 secreted from astrocytes under reoxygenated conditions induces occludin expression in the developing BBB (Lee *et al.* 2003). Taken together, these results suggest that pericyte-derived angiopoietin-1 and astrocyte-derived undefined factors act to induce occludin expression in the mature BBB. Moreover, angiopoietin-1 seems to be a crucial factor for occludin induction at both the mature and developing BBB, although the cell types secreting angiopoietin-1 seem to change with aging.

The fractionated AST-CM study revealed that the 30- to 50-kDa and 50- to 100-kDa fractions contain factors involved in inducing occludin expression (Fig. 5), although these factors remained undefined in the present study. Interleukin-15 and prolactin have been reported to induce occludin expression in epithelial cells (Stelwagen *et al.* 1999; Nishiyama *et al.* 2001). However, the size of the secreted interleukin-15 and prolactin is less than 20 kDa. Accordingly, unknown factors other than interleukin-15 and prolactin induce occludin expression in TR-AST4 cells. Identification of the occludin-inducing factors using AST-CM should provide a better understanding of the physiological regulators of expression of occludin secreted from type II astrocytes.

VEGF and TGF-β1 are known to be increased in the brain in various neurodegenerative diseases (Kalaria *et al.* 1998; Lesne *et al.* 2002). Following treatment with VEGF, the occludin mRNA level was reduced in a concentration-dependent manner in TR-BBB13 cells (Fig. 3a). This result is consistent with a previous report on primary bovine BCECs (Wang *et al.* 2001). TGF-β1 also reduced the occludin mRNA level in a concentration-dependent manner in TR-BBB13 cells (Fig. 3a). This suggests that the BBB disruption induced by TGF-β1 occurs at least partly by a reduction in occludin. The TGF-β1 mRNA level was enhanced in TR-AST4 cells during a 24-h period of hypoxia (Fig. 3b). This was the case for the *in vivo* response of astrocytes under ischemic conditions (Knuckey *et al.* 1996), suggesting that type II astrocytes may reduce occludin expression in BCECs under ischemic conditions by producing TGF-β1. In the light of these findings, the occludin expression is regulated by astrocyte- and pericyte-derived factors under physiological and pathophysiological conditions (Fig. 8).

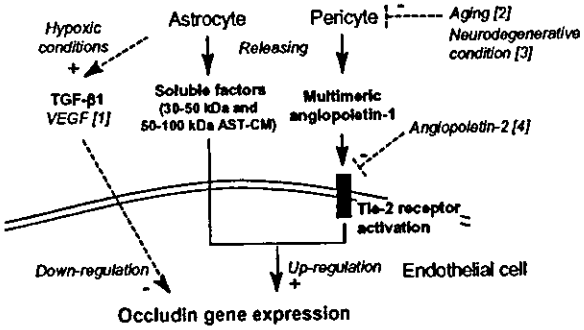

Fig. 8 Postulated mechanism of occludin gene regulation by astrocytes and pericytes for maintaining the adult BBB. [1], Kalaria *et al.* (1998); [2], Heinsen and Heinsen (1983); [3], Verbeek *et al.* (1997); [4], Maisonpierre *et al.* (1997).

JAM expression was unchanged by TR-AST4 cells and TR-PCT1 cells (Figs 1 and 2). This suggests that regulation of JAM expression at the BBB is not regulated by type II astrocytes and brain pericytes, unlike that of occludin. JAM plays a role in inflammatory transmigration of leukocytes as a ligand of integrins (Ostermann *et al.* 2002). The function of JAM in leukocyte migration may not be influenced by microenvironmental stimuli such as astrocytes and pericytes, VEGF and TGF-β1. Although the concentrated-CM tends to reduce JAM mRNA levels in the left panel of Fig. 2(b), it is not significantly different from non-concentrated-CM. The concentrated-CM would not affect the JAM mRNA in TR-BBB13 cells because other concentrated-CMs did not change JAM or occludin mRNA levels from one- to five-fold concentrated-CM (Fig. 2).

Induction of occludin expression in brain microvessels possibly enhances the tightness of TJs, since overexpression of occludin in MDCK cells has been reported to elevate their TEER (McCarthy *et al.* 1996). However, the TEER in TR-BBB13 cells was unchanged by the induction of occludin expression following the transfilter co-culture. Claudin-5 is involved in TJ formation at the BBB, and the BBB of the corresponding knock-out mouse showed a loss of tightness for solutes with a permeability under 800 Da (Nitta *et al.* 2003). Therefore, it is conceivable that TJ formation at the BBB is necessary for claudin-5 as well as occludin. Indeed, the expression level of claudin-5 mRNA in TR-BBB13 cells was very low compared with that of occludin mRNA (data not shown).

The multimeric character of the angiopoietin-1 secreted from pericytes was demonstrated using TR-PCT1 cells (Fig. 6). The 65-kDa protein in PCT-CM is possibly monomeric angiopoietin-1 since it is the same size as recombinant rat angiopoietin-1 reported previously (Iizasa *et al.* 2002). The size-difference between PCT-CM (65 kDa) and recombinant human angiopoietin-1 (75 kDa) is possibly due to the degree of glycosylation exhibited by difference in species and host cells. The low mobility of the bands detected under non-reducing conditions indicates that angiopoietin-1 forms a disulfide-linked complex, mostly larger than tetrameric. Tetrameric angiopoietin-1 has been recently reported to be the minimal size required for Tie-2 activation (Davis *et al.* 2003). Therefore, a multimeric complex of angiopoietin-1 endogenously secreted from TR-PCT1 cells is very likely to activate the Tie-2 receptor.

Tyrosine phosphorylation of Tie-2 in TR-BBB13 cells was induced by PCT-CM as shown in Fig. 7. Therefore, the signaling pathway from angiopoietin-1 multimers in PCT-CM to occludin mRNA induction through Tie-2 autophosphorylation is clearly demonstrated. The p85 subunit of phosphatidylinositol 3-kinase (PI3-kinase), the adaptor proteins Grb2, Grb7 and Grb14, and the protein tyrosine phosphatase Shp2 can interact with Tie-2 in a phosphotyrosine-dependent manner (Jones *et al.* 1999), and are potential downstream molecules of Tie-2. Among these molecules, PI3-kinase has been reported to influence TJ function although this is controversial. It has been reported that PI3-kinase activation increases the amount of occludin localized at the cell surface (Little *et al.* 2003) although this, conversely, leads to TJ disruption (Woo *et al.* 1999). Further studies are needed to elucidate the signal molecules involved in occludin induction following the angiopoietin-1/Tie-2 signal.

The present study suggests that the attenuation or inhibition of the angiopoitin-1/Tie-2 pathway between BCECs and brain pericytes leads to dysfunction of the TJs at the BBB in disease. There are two possible ways of modifying the pathway; one is changing the secretion of angiopoietin-1 from pericytes, and the other is inhibition of the interaction between angiopoietin-1 and Tie-2 (Fig. 8). As far as the latter is concerned, angiopoietin-2 is known to suppress angiopoietin-1 activity as an antagonist of Tie-2 (Maisonpierre *et al.* 1997). In PCT-CM, angiopoietin-1 is possibly a dominant factor compared with angiopoietin-2, since angiopoietin-1-dependent occludin induction is seen following treatment with PCT-CM (Fig. 4c). The expression level of angiopoietin-2 is induced under hypoxic conditions in brain, whereas angiopoietin-1 expression is unchanged (Mandriota *et al.* 2000). Therefore, it is conceivable that angiopoietin-2 affects occludin expression under pathophysiological conditions by modifying the angiopoietin-1/Tie-2 pathway. The occludin regulation by angiopoietin-1 and angiopoietin-2 is very important for a better understanding of the regulation of TJs at the BBB. Mouse angiopoietin-3 and human angiopoietin-4, other members of the angiopoietin family, are also ligands of Tie-2 (Valenzuela *et al.* 1999). There is little possibility that angiopoietin-3 and angiopoietin-4 modulate the TJ function at the BBB because their expression was not detected in the brain (Valenzuela *et al.* 1999), whereas the contribution of angiopoietin-3 and angiopoietin-4 to occludin regulation is still unknown.

Pericyte degeneration seen with aging (Heinsen and Heinsen 1983; Peters *et al.* 1991) and in neurodegenerative conditions (Verbeek *et al.* 1997) is associated with increased permeability of the BBB. Moreover, it has been suggested that the reduction of the occludin content at the rat BBB takes place in an age-dependent manner (Mooradian *et al.* 2003). The findings of the present study indicate that one of the mechanisms of occludin reduction would be a decrease in angiopoietin-1 from pericytes, and this may partly explain the increased permeability following pericyte loss (Fig. 8).

In conclusion, *in vitro* BBB model studies have demonstrated that soluble factors (the molecular weight range of candidates is 30–100 kDa) secreted from type II astrocytes and multimeric angiopoietin-1, activating the Tie-2 receptor via tyrosine phosphorylation, secreted from pericytes induce occludin expression. In contrast, VEGF and TGF-β1, the mRNA of which was up-regulated in type II astrocytes following hypoxic treatment, reduced occludin mRNA. These findings provide important information about the molecular mechanism of TJ regulation based on paracrine interactions between BECEs, and astrocytes and pericytes under both physiological and pathophysiological conditions, and should help in the design of molecules to block TJ disruption and, thereby, protect the CNS.

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Functional expression of rat ABCG2 on the luminal side of brain capillaries and its enhancement by astrocyte-derived soluble factor(s)

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Abstract

The purpose of the present study was to clarify the expression, transport properties and regulation of ATP-binding cassette G2 (ABCG2) transporter at the rat blood–brain barrier (BBB). The rat homologue of ABCG2 (rABCG2) was cloned from rat brain capillary fraction. In rABCG2-transfected HEK293 cells, rABCG2 was detected as a glycoprotein complex bridged by disulfide bonds, possibly a homodimer. The protein transported mitoxantrone and BODIPY-prazosin. In rat brain capillary fraction, rABCG2 protein was also detected as a glycosylated and disulfide-linked complex. Immunohistochemical analysis revealed that rABCG2 was localized mainly on the luminal side of rat brain capillaries, suggesting that rABCG2 is involved in brain-to-blood efflux transport. For the regulation study, conditionally immortalized rat brain capillary endothelial (TR-BBB13), astrocyte (TR-AST4) and pericyte (TR-PCT1) cell lines were used as an *in vitro* BBB model. Following treatment of TR-BBB13 cells with conditioned medium of TR-AST4 cells, the Ko143 (an ABCG2-specific inhibitor)-sensitive transport activity and rABCG2 mRNA level were significantly increased, whereas conditioned medium of TR-PCT1 cells had no effect. These results suggest that rat brain capillaries express functional rABCG2 protein and that the transport activity of the protein is up-regulated by astrocyte-derived soluble factor(s) concomitantly with the induction of rABCG2 mRNA.

Keywords: ABCG2, astrocyte, blood–brain barrier, *in vitro* BBB model.

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ABCG2 (BCRP/MXR/ABCP1), a member of the ATP-binding cassette (ABC) superfamily, was originally isolated from cancer cells resistant to anticancer drugs (Doyle *et al.* 1998). ABCG2 is also expressed in normal tissues, such as small intestine, liver and placenta (Maliepaard *et al.* 2001), and presumed to play a protective role against toxic substances and metabolites.

It has been reported recently that ABCG2 is expressed in isolated porcine brain capillaries (Eisenblatter and Galla 2002; Eisenblatter *et al.* 2003). Moreover, ABCG2 is reported to be localized on the luminal side of human brain capillaries (Cooray *et al.* 2002). Brain capillary endothelial cells (BCECs) constitute the blood–brain

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Abbreviations used: ABC, ATP-binding cassette; AST, (PCT)-CM, conditioned medium of TR-AST (TR-PCT) cells; BBB, blood–brain barrier; BCEC, brain capillary endothelial cell; P-gp, P-glycoprotein; TR-AST, conditionally immortalized rat astrocyte cell line; TR-BBB, conditionally immortalized rat brain capillary endothelial cell line; TR-PCT, conditionally immortalized rat pericyte cell line.

barrier (BBB), which prevents non-specific entry of compounds into the brain. Therefore, ABCG2 present in BCECs may act to restrict the penetration of xenobiotics into the brain and to pump out potential toxins or metabolites from the brain. ABCG2 transports sulfated conjugates of drugs and sterols (Suzuki *et al.* 2003), whereas P-glycoprotein (P-gp), a well-characterized efflux transporter at the BBB, preferentially transports more hydrophobic compounds. Therefore, it is conceivable that ABCG2 functions as a new efflux transporter at the BBB. However, the brain penetration of a dietary carcinogen, which is known to be a substrate of ABCG2, was not significantly increased, whereas the intestinal uptake and the fetal penetration of ABCG2 substrate drugs were increased in ABCG2 knockout mice (Jonker *et al.* 2002; van Herwaarden *et al.* 2003). The reason for this apparent discrepancy is still unknown.

ABCG2 functions as a homodimer and its transport activity was lost when dimer formation was blocked in cDNA-transfected cells (Kage *et al.* 2002). Single amino acid substitutions of ABCG2 in the transmembrane (TM) domain of ABCG2 alter the substrate specificity (Miwa *et al.* 2003). In normal human tissues, a splicing variant of ABCG2 has been identified in the placenta (Imai *et al.* 2002). It is also possible that ABCG2 dimerizes with other ABCG subtypes, and that the resulting heterodimers transport different substrates compared with those transported by the homodimer *in vivo*, as shown in the case of the *Drosophila* homologue of ABCG1 (Ewart *et al.* 1994). It is therefore important to clarify whether the sequence of ABCG2 is functional and the protein forms a functional dimer at the BBB.

The up-regulation of ABCG2 expression in brain vessels has been detected in brain tumors (Zhang *et al.* 2003). Therefore, the functional expression of ABCG2 at the BBB is presumably inducible. Indeed, our recent studies have shown that the transport activities of taurine transporter and xCT were regulated by external stimuli (Hosoya *et al.* 2002; Kang *et al.* 2002) and that astrocytes and pericytes surrounding brain capillaries induced the expression of tight-junction protein (Hori *et al.* 2004). An understanding of the functional regulation of ABCG2 should throw light on the physiological function of ABCG2 at the BBB.

The purpose of this study was to investigate ABCG2 protein expression and modification, as well as the transport properties using ABCG2-overexpressing cells (transduced with cDNA isolated from rat brain capillary fraction), and a conditionally immortalized rat BCEC cell line in order to clarify the functional expression of ABCG2 at the rat BBB. Furthermore, we investigated the effect of astrocyte- and pericyte-derived factors on ABCG2 function by using conditionally immortalized BBB cell lines of the same maturational stage, strain, and genetic background (Terasaki *et al.* 2003).

Materials and methods

Animals

Male Wistar rats, weighing 150–250 g, were purchased from Charles River (Yokohama, Japan). The investigations using rats described in this report conformed to the guidelines established by the Animal Care Committee, Graduate School of Pharmaceutical Sciences, Tohoku University.

Reagents

Endothelial cell growth factor (ECGF) was purchased from Boehringer Mannheim (Mannheim, Germany); benzylpenicillin potassium and streptomycin sulfate were purchased from Wako Pure Chemical Industries (Osaka, Japan). Ko143 was a generous gift from Dr A Schinkel (Netherlands Cancer Institute, Amsterdam, the Netherlands) (Allen et al. 2002). All other chemicals were commercial products of analytical grade.

Cell cultures

TR-BBB13, TR-AST4 and TR-PCT1 cells are conditionally immortalized BCEC, astrocyte and pericyte cell lines (Hosoya *et al.* 2000b; Tetsuka *et al.* 2001; Asashima *et al.* 2002) that have been used as an *in vitro* BBB model (Terasaki *et al.* 2003). The cells were grown in Dulbecco's modified Eagle's medium (DMEM, Nissui Pharmaceutical, Tokyo, Japan) supplemented with 20 mM sodium bicarbonate, 15 ng/mL ECGF, 100 U/mL benzylpenicillin potassium, 100 μg/mL streptomycin sulfate and 10% fetal bovine serum (Moregate, Bulimba, Australia) (culture-medium A). TR-AST4 cells, TR-PCT1 cells and HEK293 cells (American Type Culture Collection, Rockville, MD, USA) were cultured in culture-medium A without ECGF (culture-medium B). TR-BBB13 cells were seeded onto rat-tail collagen type I-coated tissue culture dishes (BD Biosciences, Franklin Lakes, NJ, USA). The BBB cell lines were maintained at 33°C, which is a permissive temperature at which temperature-sensitive SV40 large T-antigen is activated, in a humidified atmosphere of 95% air and 5% CO_2. HEK293 cells were cultured at 37°C under 5% CO_2/air.

Quantitative real-time PCR analysis

Under deep anesthesia induced with ketamine and xylazine, adult rats were transcardially perfused with phosphate-buffered saline (PBS) to remove blood. Rat tissues were obtained after this perfusion. Total RNA was extracted from rat tissues and TR-BBB13 cells with TRIZOL reagent (Life Technologies, Grand Island, NY, USA) and RNeasy kit (Qiagen, Tokyo, Japan) according to the manufacturer's protocol, respectively. RNA integrity was checked by electrophoresis on an agarose gel. Single-stranded cDNA was prepared from 1 μg total RNA by RT (ReverTraAce, Toyobo, Osaka, Japan) using oligo (dT) primer. Quantitative real-time PCR analysis was performed using an ABI PRISM 7700 sequence detector system (PE Applied Biosystems, Foster City, CA, USA) with 2 X SYBR Green PCR Master Mix (PE Applied Biosystems) as per the manufacturer's protocol. To quantify the amount of specific mRNA in the samples, a standard curve was generated for each run using pGEM-T Easy vector (Promega, Madison, WI, USA) containing ABCG2 or β-actin (dilution ranging from 0.1 fg/μL to 1 ng/μL). This enabled standardization of the initial mRNA content

