Table 2 Transcript level of endothelial, non-endothelial markers, and transporters in retinal vascular endothelial cell (RVEC) and non-RVEC fractions

Target mRNA	Target mRNA/β-actin mRNA (× 10^{-4})		Enrichment in the RVEC fraction
	RVEC fraction	Non-RVEC fraction	
Endothelial markers			
CD31 (PECAM-1)	38.7 ± 6.5	0.143 ± 0.018	270
Tie-2	15.4 ± 2.0	N.D.	> 221
Claudin-5	1570 ± 140	0.555 ± 0.148	2830
Occludin	15.0 ± 2.8	0.142 ± 0.024	106
Jam-1	56.2 ± 10.5	0.442 ± 0.070	127
Non-endothelial markers			
S-100β	4.02 ± 0.46	133 ± 16.2	0.0302
Glutamine synthetase	49.9 ± 9.8	645 ± 83	0.0774
Neurofilament heavy chain	0.294 ± 0.036	2.15 ± 0.29	0.137
α-Smooth muscle actin	5.06 ± 1.55	0.223 ± 0.052	22.7
Transporters			
mdr1a (Abcb1a)	38.6 ± 5.63	N.D.	> 526
mdr1b (Abcb1b)	0.198 ± 0.032	N.D.	> 2.71
mdr2 (Abcb4)	1.72 ± 0.22	0.222 ± 0.023	7.73
GLUT1 (Slc2a1)	120 ± 42	65.4 ± 4.3	1.83
GLUT3 (Slc2a3)	N.D.	0.313 ± 0.019	–
GLUT4 (Slc2a4)	0.747 ± 0.149	0.267 ± 0.063	2.80
MCT1 (Slc16a1)	133 ± 34	425 ± 28	0.312
MCT2 (Slc16a7)	1.16 ± 0.15	0.877 ± 0.165	1.32
Oatp1 (Slc21a1)	N.D.	N.D.	–
Oatp2 (Slc21a5)	36.5 ± 15.7	N.D.	> 242
Oatp3 (Slc21a7)	N.D.	N.D.	–
Oatp14 (Slc21a14)	51.5 ± 8.0	1.28 ± 0.06	40.2
CRT (Slc6a8)	7.08 ± 0.45	41.9 ± 10.1	0.169

Enrichment in the RVEC fraction is the ratio of the target mRNA between the RVEC and non-RVEC fractions (RVEC fraction/non-RVEC fraction). When the target gene is not detected in the non-RVEC fraction, enrichment in the RVEC fraction is the ratio of the target mRNA level in the RVEC fraction and the lower limit of detection of each gene. Each value represents the mean ± SEM of at least three different samples. N.D., not detected.

of glial cells, some cells are associated with the vasculature. Thus, it is suggested that a part of the vasculature-associated glial cells is captured along with the endothelial cells.

The transcript level of α-smooth muscle (SM) actin, which is exclusively expressed in perivascular cells like pericytes and SM cells, was 22-fold greater in the RVEC fraction than in the non-RVEC fraction (Table 2), demonstrating that some perivascular cells were also captured along with the endothelial cells. Nevertheless, it is also indicated that the transcript level of α-SM actin in the RVEC fraction is 2000-fold less than that of β-actin (5.06 versus 10^4). Retinal perivascular cells express α-SM actin more abundantly than β-actin (Bandopadhyay *et al.* 2001), and the transcript level of α-SM actin in cultured brain pericytes is 1.3-fold greater than that of β- and γ-actin (Boado and Pardridge 1994). The current results and previous report support the idea that the content of perivascular cells in the RVEC fraction is also very low. From these observations, although the RVEC fraction isolated using magnetic beads coated with anti-rat CD31 antibodies represents RVEC as far as the mRNA expression levels of endothelial markers are concerned, the RVEC fraction is contaminated with a small amount of non-RVEC cells like perivascular, glial, and neuronal cells. We have not analyzed markers of retinal pigment epithelial (RPE) cells as the retinas were separated from the RPE cell layer.

Transporters

Although multidrug resistance protein (mdr; P-glycoprotein) expression at the inner BRB has been recognized since 1992 (Greenwood 1992; Holash and Stewart 1993), the corresponding gene has not yet been identified. The transcript levels of three mdr isoforms, mdr1a (Abcb1a), mdr1b (Abcb1b), and mdr2 (Abcb4), were examined in the RVEC and non-RVEC fractions (Table 2). As the data were represented as the copy number of target mRNA in the samples relative to that of β-actin, it is possible to compare the mRNA expression levels among genes. The expression of mdr1a was 200- and 24-fold greater than that of mdr1b and mdr2, respectively, in the RVEC fraction. No expression of mdr1a and 1b mRNA was detected in the non-RVEC fraction. Thus, this is the first evidence that mdr1a is predominantly expressed at the inner BRB as well as the blood–brain barrier (Schinkel *et al.* 1994). Quinidine and cyclosporine A, which are substrate of P-glycoprotein, undergo limited distribution to retinal tissue from the circulating blood (BenEzra and Maftzir 1990; Duvvuri *et al.* 2003). In light of these findings, mdr1a-encoding P-glycoprotein may prevent accumulation of substrate drugs in the retina.

Organic anion transporter(s) at the inner BRB may play a major role in transporting neurotransmitters and their metabolites in the retina. The transcript levels of organic anion transporting polypeptides (oatp), such as oatp1 (Slc21a1), oatp2 (Slc21a5), oatp3 (Slc21a7), and oatp14 (Slc21a14), were examined in the RVEC and non-RVEC fractions (Table 2). Oatp2 and 14 were detected in the RVEC fraction, whereas oatp1 and 3 were not. Oatp2 and 14 were predominantly expressed at the inner BRB. In the case of oatp2, our result is consistent with a previous immunohistochemical study (Gao *et al.* 2002). On the other hand, oatp14 has been identified as a blood–brain barrier specific anion transporter (Li *et al.* 2001). The present study revealed, for the first time, that oatp14 is also expressed at the inner BRB and may play an important role in transporting the neurosteroid conjugate of estradiol-D-17β glucuronide and thyroid hormones to the neural retina as well as oatp2 (Sugiyama *et al.* 2003). Oatp3 was not detected in either the

RVEC or non-RVEC fractions. The lack of agreement between our result and the previous report needs an explanation. Although oatp3 was cloned from the retina, poly(A)$^+$ RNA is necessary to detect the expression of oatp3 in the retina by northern blot and RT-PCR analyses because of the low level of expression (Ito *et al.* 2002).

Facilitative glucose transporter at the inner BRB mediates the transport of D-glucose and dehydroascorbic acid, which is an oxidized form of vitamin C, from the circulating blood to the retina (Takata *et al.* 1992; Hosoya *et al.* 2004). Among the GLUT family transporters, GLUT1 (Slc2a1), GLUT3 (Slc2a3), and GLUT4 (Slc2a4) are capable of transporting D-glucose and dehydroascorbic acid as substrates. Although it has been believed for a long time that GLUT1 has a major role in transporting D-glucose at the inner BRB (Takata *et al.* 1992), there are some arguments in favor of GLUT3 being expressed at the inner BRB and playing a role there (Knott *et al.* 1996; Hosoya *et al.* 2004). GLUT1 expression was the largest of three GLUTs, whereas GLUT3 was not detected in the RVEC fraction. The expression of GLUT4 was 100-fold less than that of GLUT1 (Table 2). Therefore, GLUT1 is responsible for the transport of D-glucose and dehydroascorbic acid at the rat inner BRB. The expression of GLUT1 in the non-RVEC fraction is half that in the RVEC fraction. Nevertheless, this evidence is in good agreement with the localization of GLUT1 in retinal glial and neuronal cells as well as the inner BRB (Kumagai *et al.* 1994).

The expression of monocarboxylate transporter-1 (MCT1/Slc16a1) in the RVEC fraction was 100-fold greater than that of MCT2 (Slc16a7). However, the transcript level of MCT1 in the RVEC fraction was 3-fold less than that in the non-RVEC fraction (Table 2), supporting the hypothesis that MCT1 is not concentrated at the inner BRB. In addition to this evidence of the mRNA level, Gerhart *et al.* (1999) used immunoelectron microscopy to provide morphological evidence that MCT1 was widely distributed in the retina including the inner BRB and MCT2 was largely expressed in glial cells of the retina. Moreover, we have reported that L-lactate transport was mediated by MCT1 in a conditionally immortalized rat retinal capillary endothelial cell line (Hosoya *et al.* 2001). From these observations, it appears that MCT1 plays a key role in monocarboxylate transport at the inner BRB.

We have recently reported that a creatine transporter (CRT/Slc6a8) at the inner BRB is responsible for the blood-to-retina transport of creatine (Nakashima *et al.* 2004). Although CRT mRNA was expressed in the RVEC fraction, the CRT expression level in the RVEC fraction was sixfold less than that in the non-RVEC fraction (Table 2). This result implies that CRT is also distributed to other retinal tissues in addition to the inner BRB as is MCT1. CRT may also play an important role in transporting creatine to photoreceptors as creatine appears to be concentrated in photoreceptors (Wallimann *et al.* 1986).

In conclusion, application of magnetically isolated RVEC allows the determination of transporter mRNA levels at the inner BRB *in vivo*. Mdr1a, oatp2, oatp14, GLUT1, and MCT1 are the respective family members predominantly expressed at the inner BRB. These transporters appear to play a physiologically significant role in transporting their substrates at the inner BRB. To the best of our knowledge, this is the first report to determine the transcript level of transporters at the inner BRB *in vivo*. These findings regarding the transporter gene levels at the inner BRB offer important information that will increase our understanding of the dominant gene for each transport system and their role in regulating the transport at the inner BRB.

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Review

Advances in the Cell Biology of Transport *via* the Inner Blood-Retinal Barrier: Establishment of Cell Lines and Transport Functions

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The retinal capillary endothelial cells are connected to each other by tight junctions that play a key role in permeability as the inner blood-retinal barrier (inner BRB). Thus, understanding the inner BRB transport mechanism is an important step towards drug targeting of the retina. Nevertheless, inner BRB transport studies have been very limited in number since it is not easy to use the retinal capillaries, which are very small in size, for *in vitro* transport studies. Conditionally immortalized rat retinal capillary endothelial cells (TR-iBRB), pericytes (TR-rPCT) and Müller cell lines (TR-MUL) have been established from transgenic rats harboring the temperature-sensitive simian virus 40 large T-antigen gene. These cell lines posses respective cell type markers and maintain certain *in vivo* functions. Using a combination of newly developed cell lines and *in vivo* studies, we have elucidated the mechanism whereby vitamin C, L-cystine, and creatine are supplied to the retina. TR-iBRB cells are also able to identify transporters and apply to study regulation of transporters under pathophysiological conditions. Furthermore, these cell lines permit the investigation of cell-to-cell interactions and the expression of inner BRB-specific genes between TR-iBRB and other cell lines.

Key words inner blood-retinal barrier; blood-ocular barrier; transport function; transporter; conditionally immortalized cell line; cell-to-cell interaction

The retina, which is a highly differentiated tissue playing a key role in vision, has a blood-retinal barrier (BRB) to maintain a constant milieu and shield the neural retina from the circulating blood. The BRB forms complex tight junctions of retinal capillary endothelial cells (inner BRB) and retinal pigment epithelial cells (RPE; outer BRB).[1,2] The inner two thirds of the human retina is nourished by retinal capillaries and the remainder is covered by choriocapillaris *via* the outer BRB.[3] In addition to the BRB, the blood-aqueous barrier, which is formed by epithelial barriers of the cilliary body and by the iridial endothelial cells, is present in the anterior segment of the eye to maintain aqueous humor conditions. Both barriers form the so-called blood-ocular barrier (Fig. 1).[4]

The concept of the BRB was first proposed by Schnaudigel in 1913[5] following the classical work of Ehrlich and Goldman who discovered the blood-brain barrier (BBB).[6,7] The inner BRB is structurally similar to the BBB and the retinal capillary endothelial cells are covered with pericytes and glial cells.[2] Glial Müller cells predominantly support retinal endothelial cells, although glial astrocytes play a major role in supporting endothelial functions at the BBB and also, partly, at the inner BRB (Fig. 1).[8] Many groups have carried out detailed investigations of the transport functions at the BBB and Cornford postulated that the BBB acts as a dynamic regulatory interface.[9—11] Since then, many influx and efflux transporters have been identified and characterized at the BBB.[12,13] It was believed that the transport functions at the inner BRB are the same as those at the BBB. Nevertheless, information about transport functions and transporters at the inner BRB is very limited. Until 1999, only three transporters, *i.e.* facilitative D-glucose transporter (GLUT)1,[14] monocarboxylate transporter (MCT)1,[15] and P-glycoprotein (P-gp),[16] had been identified immunohistochemically at the inner BRB. This lack of interest in this aspect of vision research is somewhat surprising, given that the inner BRB plays important roles in supplying nutrients to the neural retina and is responsible for the efflux of neurotransmitter metabolites from the retina to maintain neural functions. *In vivo* transport studies using the Retinal Uptake Index (RUI) method have been performed to investigate solute transport into the retina.[17—19] Although these have the advantage of being able to estimate the ability to transport solutes from the circulating blood to the retina under physiological conditions, it is difficult to distinguish between substrates that are taken up by the inner BRB and the outer BRB. In order to successfully identify the transporters and transport mechanisms at the inner BRB, we need to develop a good *in vitro* system, which accurately reflects *in vivo* transport functions. The techniques of isolation[20] and primary culture of bovine retinal capillaries[21] have been applied to studies of the inner BRB. However, it is not easy to carry out a series of transport experiments since only 170—250 μg protein (capillary) can be obtained from a single bovine eye[20] and, recently, bovine spongiform encephalopathy has presented a serious social problem.[22] Thus, it is important to develop retinal capillary endothelial cell lines which reflect *in vivo* transport functions and remain reproducible during multiple passages in order to elucidate transport mechanisms and identify transporters at the inner BRB. The results of such studies should provide useful information for the treatment of retinal vascular disease and macular pathology, as well as allowing more specific retinal drug targeting. The loss of pericytes in retinal capillaries is one of the earliest changes in diabetic retinopathy and this causes angiogenesis due to an increase in retinal endothelial cells.[23] Therefore, the growth of endothelial cells is thought to be regulated by pericytes and studies of cell-to-cell interactions between retinal endothelial cells and pericytes are important to elucidate the mechanisms responsible for the onset of diabetic retinopathy.

In this review, we shall focus on the cell characteristics, transport functions, and cell-to-cell interactions of newly developed rat retinal capillary endothelial cells, pericytes, and

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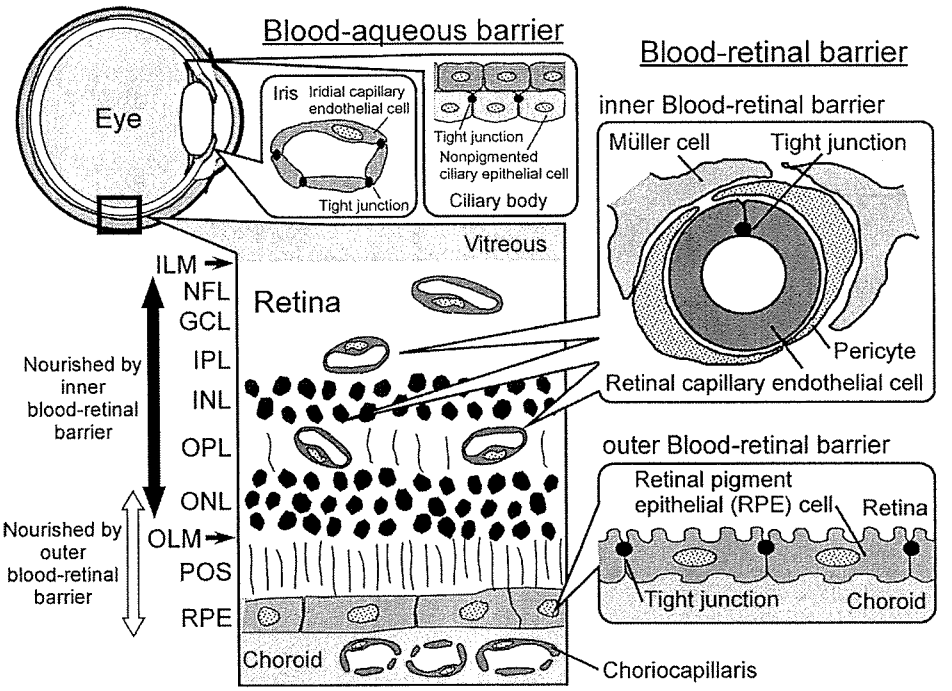

Fig. 1. Schematic Diagram of the Blood-Ocular Barrier

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 enuculeated 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]

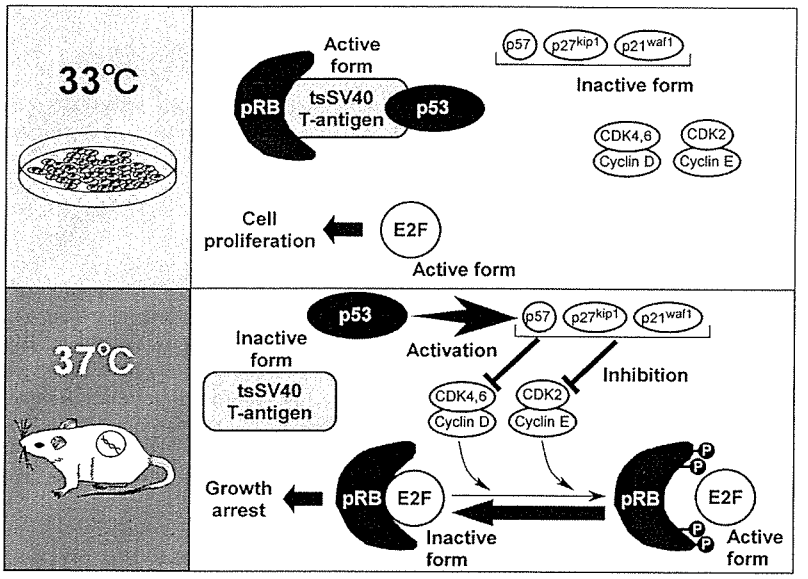

Fig. 2. The Role of Temperature-Sensitive SV40 Large T-Antigen (ts SV40 T-Antigen) in the Activation of Cell Proliferation
The tsSV40 T-antigen gene is ubiquitously expressed in the tsA58 Tg rat. The tsSV40 T-antigen is activated at 33 °C and inactivated at 37 °C.

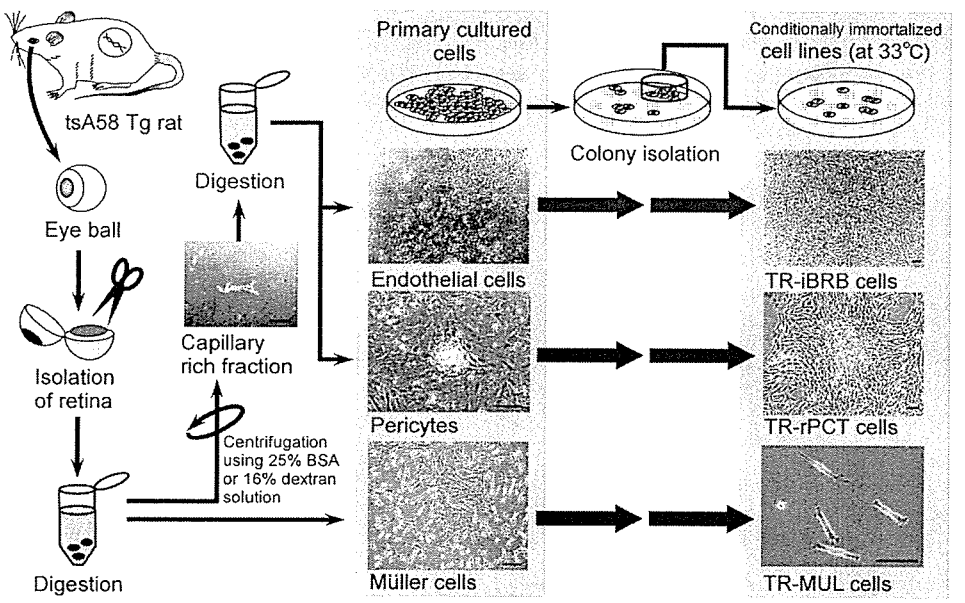

Fig. 3. Establishment of Conditionally Immortalized Inner BRB Cell Lines from the tsA58 Tg Rat
Scale bar=100 μm.

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

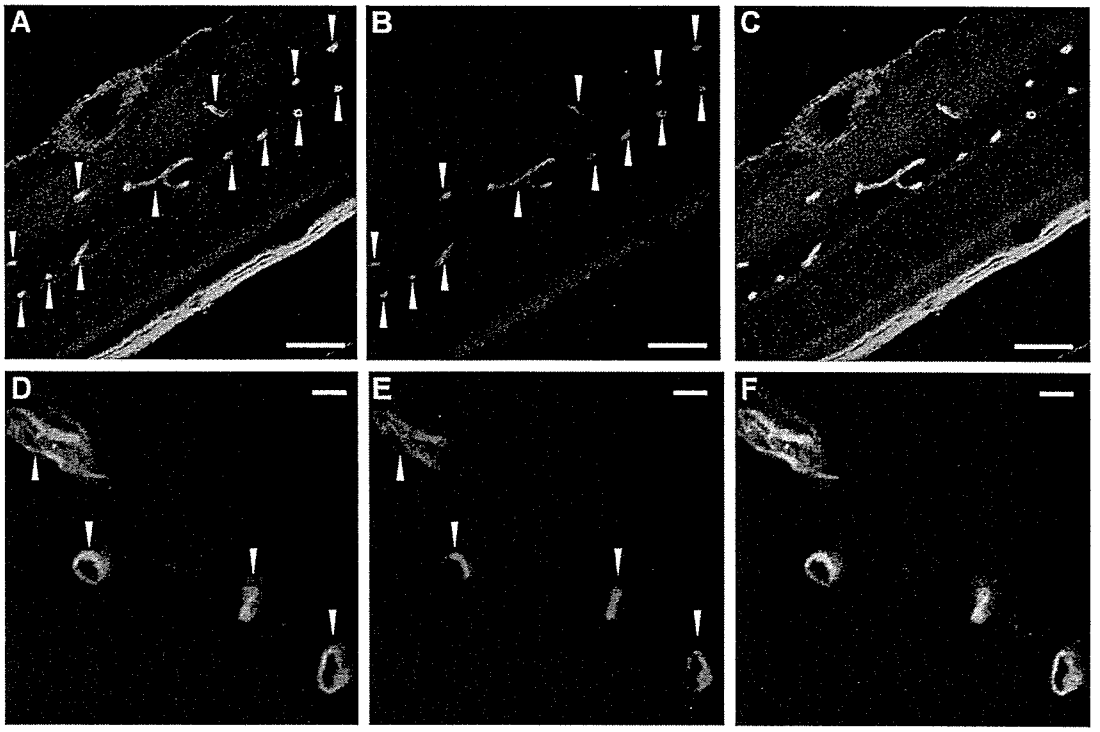

Fig. 4. Confocal Immunofluorescence Microscope Images of Single- and Dual-Labeled Rat Retinal Sections

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 tsA58 Tg 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-

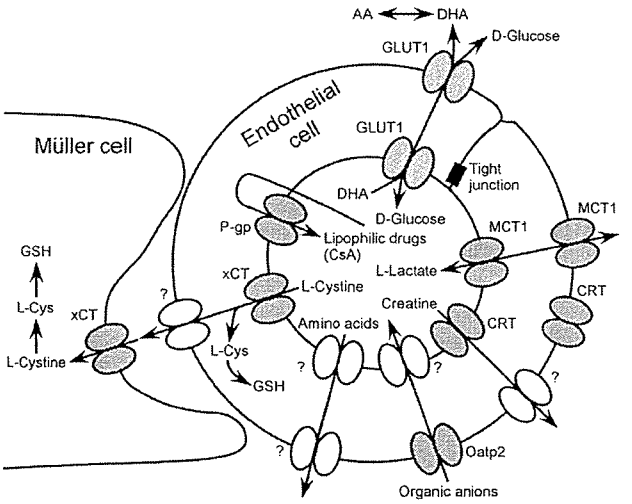

Fig. 5. Putative localization of inner BRB transporters

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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PKC/MAPK Signaling Suppression by Retinal Pericyte Conditioned Medium Prevents Retinal Endothelial Cell Proliferation

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Little is known about the regulation mechanism of endothelial cell proliferation by retinal pericytes. The purpose of this study was to elucidate the suppression mechanism of retinal capillary endothelial cell growth by soluble factors derived from retinal pericytes. Conditioned medium of retinal pericytes (rPCT1-CM) suppressed ischemia-induced retinal neovascularization. The growth and DNA synthesis of TR-iBRB2 cells, a conditionally immortalized rat retinal capillary endothelial cell line, were suppressed in a concentration-dependent manner by concentrated rPCT1-CM. The number of human cultured endothelial cells was also reduced by rPCT1-CM. These results provide the first evidence that CM from the cultivation of pericytes alone can inhibit retinal neovascularization in vivo and in vitro. Although the growth reduction of TR-iBRB2 cells was only partly reversed by treatment of rPCT1-CM with antibodies to transforming growth factor-β1, it was completely lost by heat-treatment of rPCT1-CM, suggesting that anti-angiogenic factors are soluble proteins. The levels of expression of G1/S-phase-related proteins, such as cyclin D1, cyclin-dependent kinase (cdk)4, cdk6, and proliferating cell nuclear antigen, were reduced and a cdk inhibitor, $p21^{Cip1}$, was induced in rPCT1-CM-treated TR-iBRB2 cells. Moreover, phosphorylated p44/42 mitogen-activated protein kinase (p44/42 MAPK) in TR-iBRB2 cells was reduced by rPCT1-CM treatment and phosphorylated protein kinase C (PKC)α/βII, which is upstream of p44/42 MAPK, was also suppressed. In conclusion, CM from retinal pericytes suppresses PKC-p44/42 MAPK signaling, inhibits endothelial cell growth, and prevents retinal neovascularization. Anti-angiogenic factors derived from retinal pericytes are likely to play a critical role in the regulation of retinal endothelial cell growth. J. Cell. Physiol. 203: 378–386, 2005. © 2004 Wiley-Liss, Inc.

Visual disorders, such as blindness caused by diabetic retinopathy and age-related macular degeneration in adults, and retinopathy of prematurity (ROP) in childhood, lead to a deterioration in the quality of life (Miller et al., 1997; Steinkuller et al., 1999). The common pathological finding of these diseases is proliferation of endothelial cells, namely retinal neovascularization (Campochiaro, 2000). It is very important to elucidate the mechanism of retinal neovascularization, because knowledge of the processes involved may allow the development of new therapies to block retinal angiogenesis.

The pericytes surrounding retinal microvessels are selectively lost in the earliest phase of diabetic retinopathy (Cogan and Kuwabara, 1963). The loss of pericytes induces proliferation of pre-existing retinal capillary endothelial cells and causes formation of abnormal new vessels, in which blood-retinal barrier function is impaired (Hirschi and D'Amore, 1996). Therefore, it appears that retinal pericytes inhibit the irregular growth of endothelial cells. In the processes of retinal neovascularization, endothelial cell growth is mainly stimulated by protein kinase C (PKC)-dependent p44/42 mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) phosphorylation (Shiba et al., 1993; Poulaki et al., 2002). The signaling is activated by vascular endothelial growth factor (VEGF) (Takahashi et al., 1999b). Pigment epithelium-derived factor (PEDF) suppresses ischemia-induced retinal neovascularization due to induction of apoptosis in retinal endothelial cells (Dawson et al., 1999; Stellmach et al., 2001). VEGF and PEDF are expressed in retinal pericytes (Yamagishi et al., 2002). Therefore, this suggests that soluble factors secreted from retinal pericytes play an important role in the regulation of retinal neovascularization. However, it has been reported that conditioned medium (CM) from cultivation of pericytes (or smooth muscle cells) alone failed to inhibit endothelial cell growth (Antonelli-Orlidge et al., 1989; Sato and Rifkin, 1989). This appears to be in conflict with the existence of anti-angiogenic factors, such as PEDF, in retinal pericytes (Martin et al., 2000). Therefore, little is yet known about the mechanism of suppression of endothelial cell proliferation by retinal pericytes.

Although physiological role of retinal pericytes remains largely unknown at present, more information

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for physiological role of brain pericytes is contributed by the use of primary cultured pericytes (Ramsauer et al., 2002) and a conditionally immortalized rat pericyte cell line (Asashima et al., 2002, 2003). Co-culture systems between brain endothelial cells and pericytes afford to elucidate a paracrine interaction between both cells at the blood-brain barrier (Ramsauer et al., 2002; Hori et al., 2004). We have recently established a conditionally immortalized rat retinal endothelial cell line (TR-iBRB2) (Hosoya et al., 2001), and conditionally immortalized rat retinal pericyte cell line (TR-rPCT1) (Kondo et al., 2003) from transgenic rats harboring the temperature-sensitive SV40 large T-antigen gene (tsA58 Tg rats) (Takahashi et al., 1999a). The co-culture system using these conditionally immortalized cell lines could be a useful tool for analyzing the paracrine interaction between capillary endothelial cells and pericytes in the retina. In fact, our published report showed that in contact co-culture with TR-rPCT1 cells, the proliferation of TR-iBRB2 cells was significantly inhibited compared with that of a single culture. However, no such inhibition was observed in non-contact co-culture (Kondo et al., 2003). These results suggest that contact co-culture with TR-rPCT1 cells afforded highly concentrated pericyte-soluble factors in the TR-iBRB2 cells microenvironment. Therefore, we hypothesize that concentration of soluble factors from pericytes is important for the suppression of endothelial cell growth. The suppression of retinal neovascularization by soluble factors in vivo provides important information for the treatment of diabetic retinopathy. The elucidation of the growth mechanism of endothelial cells will increase our understanding of the critical information of physiological role for pericytes and proliferative retinopathy.

The purpose of the present study was to determine whether concentrated CM derived from TR-rPCT1 cells (rPCT1-CM) suppresses retinal neovascularization in vivo, and to elucidate the growth signaling mechanism of TR-iBRB2 cells as indicators of growth regulation by rPCT1-CM.

MATERIALS AND METHODS

Animals

Female C57/BL6J mice at 14 days' gestation were purchased from SLC (Hamamatsu, Japan). All procedures conformed to the provisions of the Animal Care Committee, Graduate School of Pharmaceutical Sciences, Tohoku University and the ARVO Statement on the use of animals in ophthalmic and vision research.

Ischemia-induced retinopathy and subcutaneous injection of rPCT1-CM

A previously described murine model of ischemic-induced retinopathy was set up using dams and neonatal C57/BL6J mice (Smith et al., 1994). Mice in the normal group were maintained under normal room ventilation conditions throughout the experiment. Mice in the ischemic group were exposed with their nursing dams to 75% oxygen from postnatal day 7 (P7) to P12, then removed to room air and injected subcutaneously each day from P12 through P16 with 30 μl Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline [PBS(−)] or 30 μl concentrated rPCT1-CM-dialyzed PBS(−). At P17, all pups were anesthetized and perfused with 1 ml fixative (4% paraformaldehyde/0.1M sodium phosphate buffer, pH 7.4) containing 50 mg 2,000 kDa fluorescein isothiocyanate-labeled dextran (FITC-dextran, Sigma, St. Louis, MO) via the left ventricle of the heart. Eyes were enucleated and placed in fixative. The flat-mounted retinas were viewed under a fluorescence microscope (AxioCam, Zeiss, Thornwood, NY). The ratio of the fluorescence area to total retina area in retinas of mice with ROP was quantified using NIH Image software.

Cell culture

TR-iBRB2, TR-rPCT1, TR-BBB13, and TR-CSFB3 cells were established and characterized as described previously (Hosoya et al., 2000; Hosoya et al., 2001; Kitazawa et al., 2001; Kondo et al., 2003). These rat cell lines were seeded onto rat tail collagen type I-coated tissue culture dishes (BD Biosciences, Bedford, MA). TR-rPCT1 and TR-CSFB3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceuticals, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS; Moregate, Bulimbra, Australia) at 33°C. TR-iBRB2 and TR-BBB13 cells were cultured in DMEM supplemented with 10% FBS and 15 mg/L endothelial cell growth factor (Roche Diagnostics, Mannheim, Germany) at 33°C. The permissive temperature for rat cell lines to be cultured was 33°C, due to the presence of temperature-sensitive SV40 large T-antigen. Human retinal endothelial cells (HREC), human brain microvascular endothelial cells (HBMEC), human umbilical vein endothelial cells (HUVEC), human dermal fibroblasts (HDF), and human skeletal muscle cells (HSkMC) were obtained from Cell Applications (San Diego, CA) or Dainippon Pharmaceutical (Osaka, Japan). These human cells were cultured on rat tail collagen type I-coated tissue culture dishes in basal medium containing growth supplement (Cell Applications) at 37°C. All cells were cultured in a humidified atmosphere of 95% air and 5% CO_2.

Preparation of rPCT1-CM

TR-rPCT1 cells were cultured in 10% FBS DMEM for 48 h. The number of TR-rPCT1 cells was measured, and then 4 ml serum-free DMEM per 1.0×10^6 cells was added to the culture dishes. After 24 h, CM from TR-rPCT1 culture medium was collected and filtered through a 0.22 μm filter. The CM was concentrated up to 20-fold (20×, 10 μg protein/30 μl) using a Centriprep-3 (3 kDa cut-off) (Millipore, Bedford, MA), and then stored at −20°C until required. Normal medium was prepared by the same procedure using serum-free DMEM for control experiments. rPCT1-CM was adjusted to an appropriate concentration by diluting 20× rPCT1-CM with serum-free DMEM for the in vitro study. In the in vivo study, 20× rPCT1-CM was dialyzed against PBS(−) and then concentrated up to 100× (50 μg protein/30 μl) using a Microcon YM-3 (3 kDa cut-off) (Millipore).

Heat treatment and neutralization of transforming growth factor (TGF)-β1 in rPCT1-CM

Heat treatment was carried out by boiling 20× rPCT1-CM for 10 min. The neutralization of TGF-β1 in 20× rPCT1-CM was performed by the addition of 50 μg/ml anti-TGF-β1 neutralizing antibody (R&D systems, Minneapolis, MN) followed by incubation at 4°C for 16 h (Chodon et al., 2000). For experiments, the CM was adjusted to 5× rPCT1-CM containing 10% FBS.

Assay of DNA synthesis activity

TR-iBRB2 cells (1.5×10^3 cells/well) were plated on rat tail collagen type I-coated 96-well plates (BD Biosciences) in 10% FBS DMEM. After 24 h, the culture medium was replaced with rPCT1-CM containing 10% FBS, and the plates were further incubated for 48 h. Then the cells were incubated with 5-bromo-2′-deoxyuridine (BrdU) reagent for 2 h. The BrdU incorporation study was performed by means of Cell Proliferation ELISA (colorimetric) (Roche Diagnostics) according to the manufacturer's protocol.

Western blot analysis of cell cycle-related proteins

TR-iBRB2 cells were cultured in 10% FBS DMEM or 5× rPCT1-CM containing 10% FBS for 48 h. Whole cell lysates were obtained by dissolving cells in lysis buffer containing 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 on ice for 30 min, followed by centrifugation for 10 min at 4°C

and 15,000*g*. Supernatants were separated and used as whole cell extracts. The protein (50 μg) was electrophoresed on an SDS–polyacrylamide gel and electrotransferred to polyvinylidene fluoride membranes. The membranes were incubated with primary antibodies at 4°C for 16 h using blocking agent solution (Block Ace; Dainippon Pharmaceutical). The membranes were washed with 0.1% Tween 20/PBS(−) and incubated with horseradish peroxidase-conjugated secondary antibodies. The bands were visualized with an enhanced chemiluminescence kit (SuperSignal; Pierce, Rockford, IL). Primary antibodies such as cdk2 (1:500), cdk4 (1:250), cyclin D1 (1:250), and $p21^{Cip1}$ (1:500) were from BD Biosciences. Antibodies to cyclin E (1:500) and proliferating cell nuclear antigen (PCNA) (1:1,000) were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-large T-antigen antibody (1:100) was from Oncogene Research Products (Cambridge, MA) and anti-cdk6 (1:2,500) and anti-β-actin (1:2,000) antibodies were from Sigma.

Phosphorylated status of signal transduction proteins

TR-iBRB2 cells were cultured in 10% FBS DMEM for 24 h. The culture medium was replaced with serum-free DMEM or 5× rPCT1-CM, and then culture was continued for 24 h. Serum-starved TR-iBRB2 cells were treated with serum and then the phosphorylation of signaling proteins was measured by Western blot analysis using the same procedure as in the case of cell cycle-related proteins. Primary antibodies such as total or phosphorylated p44/42 MAPK (1:1,000) and protein kinase B (Akt) (1:1,000), and phosphorylated PKCα/βII (1:1,000) were obtained from Cell Signaling Technology (Beverly, MA). Anti-Ras antibody (1:1,000) was from Upstate Biotechnology (Lake Placid, NY).

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 group means.

RESULTS

Effect of rPCT1-CM on ischemia-induced retinal neovascularization

To assess the anti-angiogenic effect of rPCT1-CM in vivo, ischemia-induced retinopathy mice were perfused with FITC-dextran, and flat-mounted retinas were examined using fluorescence microscopy. Leakage of FITC-dextran was seen in whole mounted retinas when PBS(−) or 20× rPCT1-CM (10 μg protein/30 μl) was subcutaneously administered to pups under ischemic conditions (Fig. 1B or C). In contrast, this leakage was reduced when 100× rPCT1-CM (50 μg protein/30 μl) was subcutaneously administered to pups under ischemic conditions (Fig. 1D). These results show that retinal neovascularization is suppressed in an rPCT1-CM dose-dependent manner. The fluorescence intensity ratio in whole retinas was measured using NIH Image software (Fig. 1E). Although the fluorescence intensity ratio in PBS(−)-treated pups under ischemic conditions was increased 1.89-fold compared with the control, this increase was reduced by 8.05% and 49.4% when 20 and 100× rPCT1-CM were administered to pups under ischemic conditions, respectively. These results support the hypothesis that rPCT1-CM has the ability to suppress ischemia-induced neovascularization in the retina in vivo.

TR-iBRB2 cell growth in rPCT1-CM

The growth of TR-iBRB2 cells was measured in DMEM containing 10% FBS or rPCT1-CM containing 10% FBS (Fig. 2). Following 1× rPCT1-CM treatment, the number of TR-iBRB2 cells was the same as that in 1× normal medium. In contrast, the growth of TR-iBRB2 cells was suppressed in concentrated rPCT1-CM. The number of TR-iBRB2 cells was significantly reduced by 41.0% and 69.1% after 6 days in 2× rPCT1-CM and 5× rPCT1-CM, respectively. The proliferation of TR-iBRB2 cells was suppressed in an rPCT1-CM concentration-dependent manner, suggesting that some cell growth-suppressing factors are secreted from TR-rPCT1 cells. In the following experiments, 5× rPCT1-CM was mainly used to investigate the mechanism of cell growth suppression.

Effect of rPCT1-CM on growth of several types of cultured cells

TR-iBRB2 cells are a conditionally immortalized cell line derived from a tsA58 Tg rat (Hosoya et al., 2001). It is possible that the cell growth suppression in Figure 2 is a typical phenomenon for large T-antigen expressing cells or endothelial cells. Therefore, several types of cells were investigated, and the results are summarized in Table 1. The growth of rat endothelial cell lines TR-iBRB2 and TR-BBB13 and that of human cultured endothelial cells HREC, HBMEC, and HUVEC following 5× rPCT1-CM treatment was suppressed by 65.2%, 62.5%, 61.0%, 50.2%, and 69.8%, respectively. In addition, the cell numbers of a rat epithelial cell line TR-CSFB3, human cultured fibroblasts HDF and skeletal muscle cells HSkMC following 5× rPCT1-CM treatment were reduced by 59.9%, 52.0%, and 24.9%, respectively. However, the number of COS7 cells, transformed by SV40 large T-antigen (Gluzman, 1981), following the 5× rPCT1-CM treatment was not much different from that in normal medium. These results show that soluble factors from TR-rPCT1 cells suppress the growth of several types of cells, that is, an immortalized cell line, endothelial cells from human, epithelial cells, and fibroblasts. Nevertheless, endothelial cells have a greater ability for cell growth suppression by rPCT1-CM than other cell types. This supports the idea that the cell suppression mechanism is not related to the promoter of cell growth in the cell line.

Treatment with TGF-β1-neutralizing antibody or heated rPCT1-CM

TGF-β1 derived from pericytes plays an important role in regulating endothelial cell growth in vitro. Therefore, the number of TR-iBRB2 cells was measured in anti-TGF-β1-antibody-treated rPCT1-CM (Table 2). The growth of TR-iBRB2 cells in 5× rPCT1-CM and in antibody-treated 5× rPCT1-CM was reduced by 71.4% and by 59.4%, respectively. These results suggest that TGF-β1 is one of the anti-angiogenic factors in rPCT1-CM. However, growth only partly recovered in TGF-β1-antibody-treated rPCT-CM. In contrast, the number of TR-iBRB2 cells fully recovered in heated 5× rPCT1-CM culture (Table 2), suggesting that other proteinaceous anti-angiogenic factor(s) exist in rPCT1-CM. Moreover, the TR-iBRB2 cells following rPCT1-CM treatment did not exhibit apoptotic morphology (data not shown), suggesting that the anti-angiogenic effect of rPCT1-CM involved a reduction in cell growth, but not induction of cell death.

rPCT1-CM concentration-dependence of BrdU incorporation

BrdU incorporation by TR-iBRB2 cells was measured at several concentrations of rPCT1-CM (Fig. 3). BrdU incorporation was markedly reduced in an rPCT1-CM

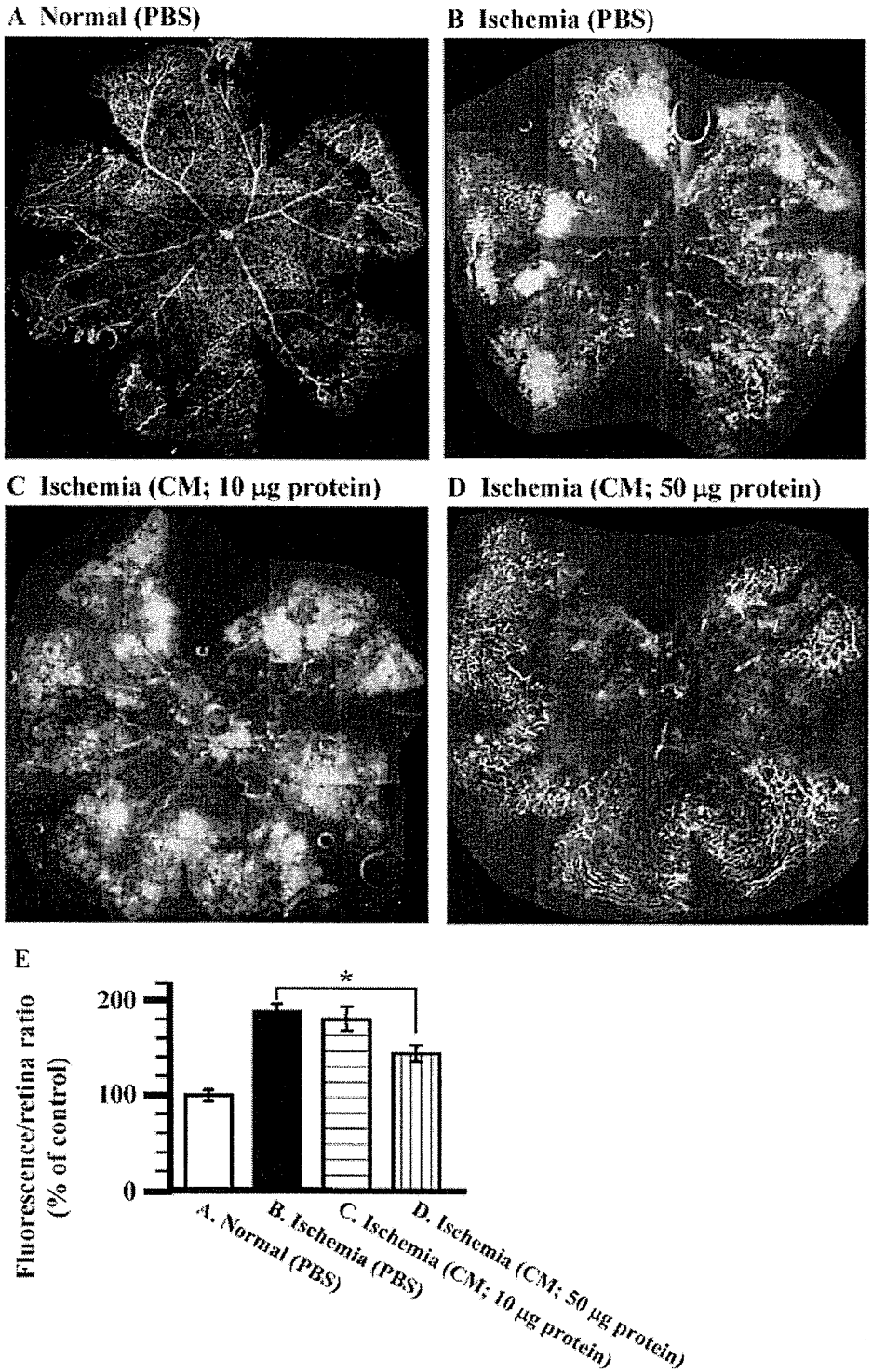

Fig. 1. Effect of rPCT1-CM on retinal neovascularization. **A**: Mouse pups at P17, kept in a normal room environment, were perfused with 1 ml FITC-dextran solution. **B–D**: Retinal neovascularization was induced and PBS or rPCT1-CM injected as described under Materials and Methods. B: 30 μl PBS (Ischemia (PBS)), (C) 30 μl 20× rPCT1-CM (10 μg protein)-dialyzed PBS (Ischemia (CM; 10 μg protein)), and (D) 30 μl 100× rPCT1 (50 μg protein)-CM-dialyzed PBS (Ischemia (CM; 50 μg protein)). At P17, the pups were perfused with 1 ml FITC-dextran solution. **E**: The ratio of the fluorescence area to total retina area in retinas of mice with ROP was quantified. Each column represents the mean ± SEM (n = 6~9). *, $P < 0.05$ significantly different from PBS administration in ischemia.

concentration-dependent manner. BrdU incorporation at 5, 10, and 20× rPCT1-CM was reduced by 22.7%, 42.2%, and 48.8%, respectively. Therefore, rPCT1-CM appears to suppress DNA synthesis and to delay the cell cycle progression in TR-iBRB2 cells.

Expression of cell cycle-related proteins

The G1/S-phase expression of cdks, cyclins, and inhibitors in TR-iBRB2 cells was determined by Western blot analysis (Fig. 4). The 5× rPCT1-CM reduced

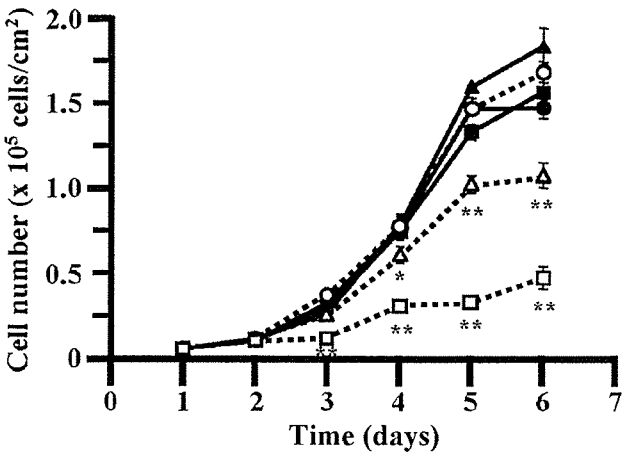

Fig. 2. Comparison of TR-iBRB2 cell growth between normal medium and rPCT1-CM. rPCT1-CM was concentrated using a Centriprep-3 (Millipore). TR-iBRB2 cells were cultured in 1× (closed circles), 2× (closed triangles), and 5× (closed squares) normal medium containing 10% FBS; and 1× (open circles), 2× (open triangles), and 5× (open squares) rPCT1-CM containing 10% FBS. Each point represents the mean ± SEM (n = 3). *, $P < 0.05$ and **, $P < 0.01$, significantly different from the cell number in normal medium.

the expression of cdk4 and cdk6, but not cdk2. The expression of cyclin D1, which forms complexes with cdk4 and cdk6, was reduced by 5× rPCT1-CM. Moreover, cyclin E and PCNA expression was reduced by 5× rPCT1-CM. These results indicate that the expression of G1/S-phase-accelerating proteins in TR-iBRB2 cells is reduced by rPCT1-CM. On the other hand, the expression of $p21^{Cip1}$, a cdk inhibitor, was increased by 5× rPCT1-CM. The expression of large T-antigen was constant in the presence or absence of 5× rPCT1-CM. These results suggest that $p21^{Cip1}$ suppresses the growth of TR-iBRB2 cells in rPCT1-CM, but does not affect SV40 large T-antigen.

Suppression of MAPK and Akt activity by rPCT1-CM

The phosphorylation of p44/42 MAPK and Akt was detected by Western blot analysis when serum-starved TR-iBRB2 cells were cultured with serum. p44/42 MAPK and Akt function as major pathways of cell growth (Dhanasekaran and Reddy, 1998; Vanhaesebroeck and Alessi, 2000). The expression of total and phosphorylated p44/42 MAPK in TR-iBRB2 cells remained at a high level in normal medium and 5× rPCT1-CM up to 120 min after serum stimulation (data not shown). However, phosphorylated p44/42 MAPK was reduced from 3 h after serum stimulation in comparison with TR-iBRB2 cells cultured in normal medium (Fig. 5). Although total Akt protein in TR-iBRB2 cells in 5× rPCT1-CM tended to be reduced in a time-dependent manner, little phosphorylated Akt was detected in normal medium or 5× rPCT1-CM (data not shown). The rPCT1-CM appears to suppress p44/42 MAPK signaling in TR-iBRB2 cells.

TABLE 1. Inhibitory effect of rPCT1-CM on proliferation of several types of cultured cells

Cell types	Percentage of control (%)
Endothelial cells	
TR-iBRB2	34.8 ± 2.4**
TR-BBB13	37.5 ± 1.5**
HREC	39.0 ± 4.0**
HBMEC	49.8 ± 2.5**
HUVEC	30.2 ± 0.4**
Other cells	
TR-CSFB3	40.1 ± 3.8**
HDF	48.0 ± 2.4**
HSkMC	75.1 ± 3.9*
COS7	88.3 ± 13.2

The cells were incubated in 10% FBS DMEM or 5× rPCT1-CM containing 10% FBS for 48 h. Each value represents the mean ± SEM (n = 3).
*, $P < 0.05$ and **, $P < 0.01$ significantly different from control (10% FBS DMEM).

TABLE 2. Effect of TGF-β1-neutralizing antibody and heat treatment of rPCT1-CM on proliferation of TR-iBRB2 cells

Conditions	Percentage of control (%)
DMEM	
Absence of anti-TGF-β1 antibody (control)	100 ± 3
Presence of anti-TGF-β1 antibody	102 ± 7
Heat treatment	86.5 ± 11.6
5× rPCT1-CM	
Absence of anti-TGF-β1 antibody	28.6 ± 3.3*
Presence of anti-TGF-β1 antibody	40.6 ± 1.3*
Heat treatment	127 ± 11

The cells were incubated under each set of conditions for 48 h. Each value represents the mean ± SEM (n = 3).
*, $P < 0.01$ significantly different from control.

Up-stream target of rPCT1-CM for cell growth inhibition

p44/42 MAPK is known to be activated via the PKC or Ras pathway (Yashima et al., 2001). The expression of the phosphorylated form of PKCα/βII or Ras in TR-iBRB2 cells was determined by Western blot analysis. Phosphorylated PKCα/βII in TR-iBRB2 cells was reduced in 5× rPCT1-CM compared with that in normal medium (Fig. 5). On the other hand, Ras was detected in similar amounts in normal medium and 5× rPCT1-CM (Fig. 5). Therefore, inactivation of PKCα/βII-mediated p44/42 MAPK signaling is suggested to cause the suppression of TR-iBRB2 cell growth.

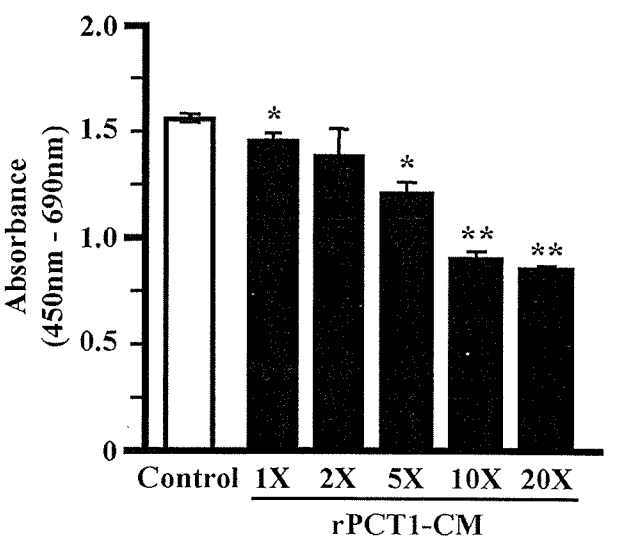

Fig. 3. BrdU incorporation of TR-iBRB2 cells treated with rPCT1-CM. TR-iBRB2 cells were incubated with normal medium (control) or rPCT1-CM for 48 h and then BrdU was incorporated for 2 h. Each column represents the mean ± SEM (n = 3). *, $P < 0.05$ and **, $P < 0.01$ significantly different from the control.

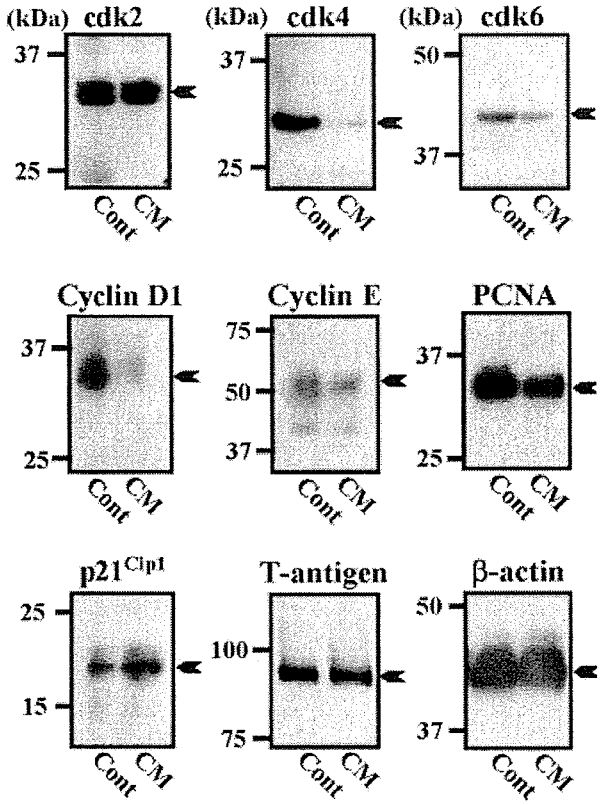

Fig. 4. Western blot analysis of G1 cyclins, cdks, and inhibitors in TR-iBRB2 cells. TR-iBRB2 cells were cultured in DMEM containing 10% FBS until 20% confluence, and then fed DMEM containing 10% FBS (Cont) or 5× rPCT1-CM containing 10% FBS (CM) for 48 h. The arrow on the right-hand side in each picture indicates the expected molecular size. One representative Western blot analysis from at least three independent experiments is shown.

DISCUSSION

The present study demonstrates that soluble factors derived from retinal pericytes suppress ischemia-induced retinal neovascularization. The mechanism of TR-iBRB2 cell growth suppression is the inactivation of the PKCα/βII-mediated p44/42 MAPK signaling pathway and reduction of the expression of cyclin D1, cdk4, and cdk6 (Fig. 6). This is the first report to demonstrate the mechanism of endothelial cell growth suppression by CM derived from retinal pericytes alone.

In the in vivo study, rPCT1-CM administration suppressed retinal neovascularization in a mouse model of ischemia-induced retinopathy (Fig. 1). The diameter of microvessels and leakage of FITC-dextran were reduced following rPCT1-CM administration. In the in vitro study, the number of TR-iBRB2 cells was also reduced following rPCT1-CM (Fig. 2). These suppression effects were observed in an rPCT1-CM concentration-dependent manner. Past reports did not show that CM derived from pericytes alone influenced endothelial cell growth (Antonelli-Orlidge et al., 1989; Sato and Rifkin, 1989). This is probably because of the fact that non-concentrated CM was used. This suggests that concentrated soluble factors from retinal pericytes are important for the suppression of retinal endothelial cell growth. The osmolality of rPCT1-CM (1, 2, and 5×) was not different from the normal medium (1, 2, and 5×) (osmolality: 280 ± 5 mOsm/kg). Therefore, we consider that the suppression of TR-iBRB2 cell growth by rPCT1-CM is due to pericyte-derived anti-angiogenic factors, but not a difference in medium composition. This is the first evidence that soluble factors in rPCT1-CM not only suppress cell growth in vitro, but also play a critical role in controlling retinal neovascularization in vivo.

SV40 large T-antigen, which binds to p53 or retinoblastoma protein (Ray et al., 1996), promotes cell growth. However, cell growth reduction was observed in TR-BBB13 and TR-CSFB3 cells, but not in COS7 cells (Table 1), and the expression of SV40 large T-antigen in TR-iBRB2 cells was the same under normal medium and rPCT1-CM culture conditions (Fig. 4). In addition, the anti-angiogenic effect of rPCT1-CM was also seen with HREC, HBMEC, and HUVEC, but hardly at all with HSkMC (Table 1). This suggests that large T-antigen makes only a minor contribution to the cell growth suppression by rPCT1-CM. Moreover, cell growth suppression by rPCT1-CM appears to exhibit a certain specificity for endothelial cells rather than other cells.

In past reports, latent TGF-β, which is bound to the plasma membrane of pericytes, was activated to TGF-β by plasmin or thrombospondin-1 on the plasma membrane of endothelial cells (Sato and Rifkin, 1989; Crawford et al., 1998). The active TGF-β then inhibited the growth of endothelial cells in vitro. The growth reduction of TR-iBRB2 cells was only partly reversed by treatment of rPCT1-CM with antibodies to TGF-β1, but was completely lost following heat-treatment of rPCT1-CM (Table 2). Although a contribution of TGF-β to cell growth suppression of TR-iBRB2 cells cannot be ruled out, anti-angiogenic soluble proteins (long-chain peptides) are predominantly involved in this suppression. Moreover, rPCT1-CM does not induce cell death as judged from the morphology and unchanged expression of Fas and Fas ligand (FasL) mRNA observed in a cDNA microarray (Atlas Rat Toxicology 1.2 Array, BD Biosciences) in TR-iBRB2 cells (data not shown). Therefore, PEDF, which induces apoptosis via the Fas/FasL pathway (Volpert et al., 2002), may not play a key role in the cell growth-suppressing effect of rPCT1-CM. This suggests that the anti-angiogenic mechanism of rPCT1-CM is related to a reduction in the growth rate of existing endothelial cells in retinal neovascularization (Fig. 1). Moreover, Joussen et al. reported that leukocyte-mediated Fas/FasL-dependent endothelial cell apoptosis led to the breakdown of the blood-retinal barrier in early diabetes (Joussen et al., 2003). If cell growth suppression by rPCT1-CM is not involved in the Fas/FasL pathway, the compound would be a candidate for the treatment of diabetic retinopathy. It remains to be seen, which proteins suppressed cell growth. However, it is also very important to elucidate the growth suppression mechanism involving signal transduction by rPCT1-CM.

The levels of expression of cyclin D1, cdk4, and cdk6 in TR-iBRB2 cells were markedly reduced following rPCT1-CM treatment (Fig. 4). It is known that formation of complexes of cyclin D with cdk4 and cdk6 is particularly important for G1-phase progression of the cell cycle and that the expression of cyclin D1 is induced by growth stimuli via p44/42 MAPK-mediated signaling (Sherr, 1993; Lavoie et al., 1996). Activation of p44/42 MAPK in TR-iBRB2 cells was reduced following rPCT1-CM treatment (Fig. 5), suggesting that the reduction of cyclin D1 expression in TR-iBRB2 cells is also caused by the inactivation of p44/42 MAPK signaling. A recent report indicated that the movement of cyclin D1/cdk4 complex from the cytoplasm to the nuclei induces

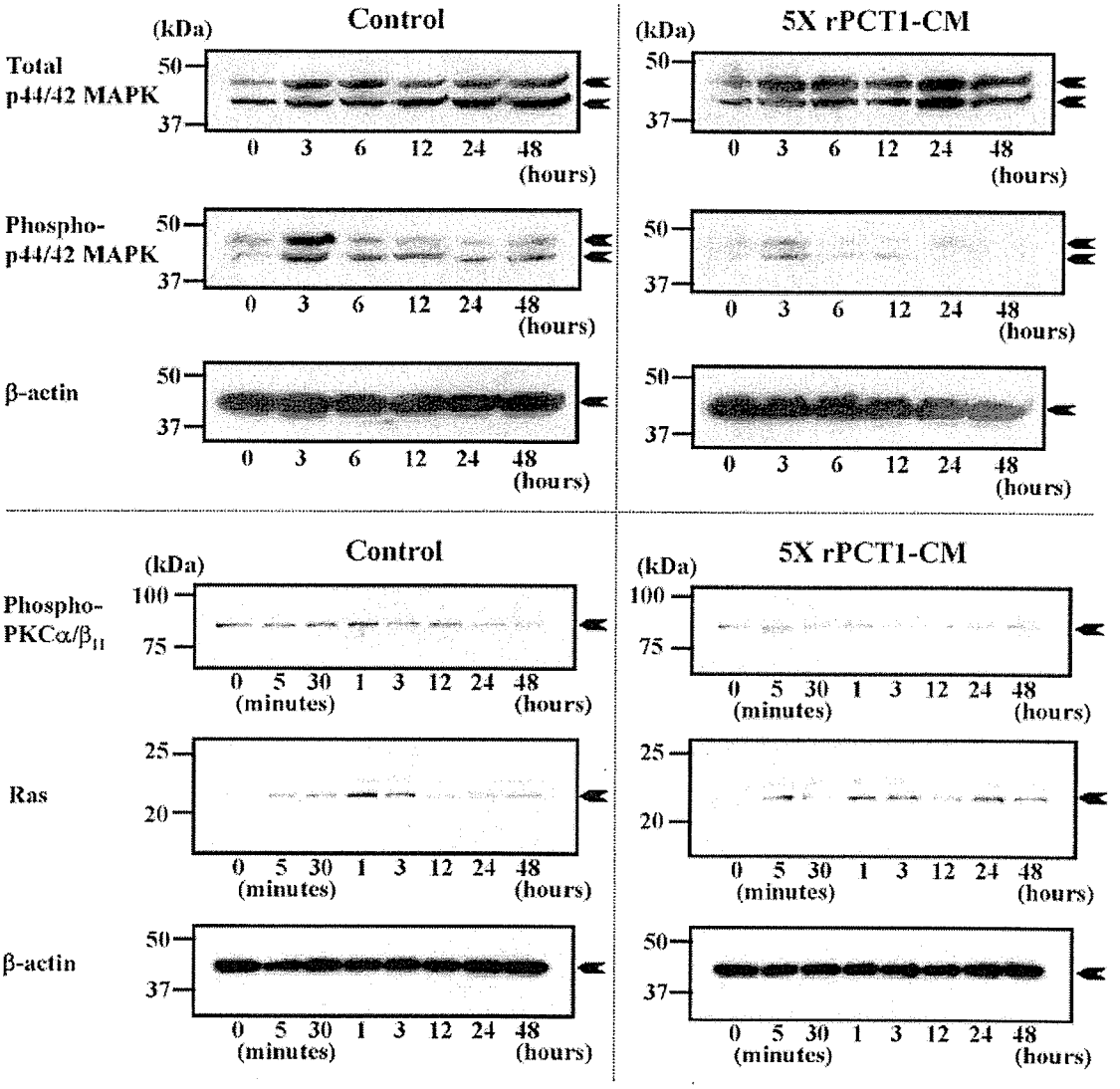

Fig. 5. Time-dependent effects of rPCT1-CM on p44/42 MAPK and PKCα/βII phosphorylation status and Ras expression profile in TR-iBRB2 cells detected by Western blotting analyses. TR-iBRB2 cells were cultured in DMEM containing 10% FBS until 20% confluence and then fed serum-free DMEM or serum-free 5× rPCT1-CM for 24 h. To determine the effect of rPCT1-CM on FBS-stimulated phosphorylation, DMEM containing 10% FBS or 5× rPCT1-CM containing 10% FBS was added to TR-iBRB2 cells after serum-starvation for 24 h. One representative Western blot analysis from at least three independent experiments is shown.

proliferation in rat cardiomyocytes (Tamamori-Adachi et al., 2003). One possibility is that soluble factors in rPCT1-CM not only suppress cell cycle-related proteins, but also reduce the transfer of cyclin D1/cdk4 complex to the nuclei in TR-iBRB2 cells as happens in the case of cardiomyocytes.

The expression of $p21^{Cip1}$ was increased following rPCT1-CM treatment in TR-iBRB2 cells (Fig. 4). It is known that $p21^{Cip1}$ forms a complex with cdk4/6 to inhibit cell growth and is induced by p53 (El-Deiry et al., 1993; Xiong et al., 1993). The p53 in TR-iBRB2 and COS7 cells would be inactivated because of introduction of large T-antigen gene (Gluzman, 1981; Hosoya et al., 2001). However, rPCT1-CM did not suppress COS7 cell growth (Table 1). Therefore, it appears that induction of $p21^{Cip1}$ expression by rPCT1-CM occurs via a p53-independent pathway and prevents cell cycle progression in TR-iBRB2 cells. On the other hand, Rössig et al. reported that Akt-dependent phosphorylation in $p21^{Cip1}$ promotes cell growth because of induction of PCNA and cdk4, which activates DNA polymerase δ and increases the formation of cyclin D/cdk4 complex, respectively (Rössig et al., 2001). The phosphorylated form of Akt was not detected in TR-iBRB2 cells (data not shown). Therefore, it is unlikely that $p21^{Cip1}$ functions as a cell growth accelerator in TR-iBRB2 cells. The expression of PCNA was reduced following rPCT1-CM treatment in TR-iBRB2 cells (Fig. 4). Moreover, the reduction in BrdU incorporation in TR-iBRB2 cells was rPCT1-CM concentration-dependent (Fig. 3). This suggests that the reduction of PCNA activity by rPCT1-CM leads to suppression of DNA synthesis and proliferation. Osuga et al. reported that flavopiridol, a cdk inhibitor, prevents neuronal death by ischemia in rat brain, suggesting that cdks are useful therapeutic targets for neurodegenerative disorders (Osuga et al., 2000). rPCT1-CM down-regulates cdk4/6 and up-regulates $p21^{Cip1}$ (Fig. 4), which suggests that soluble factors from retinal

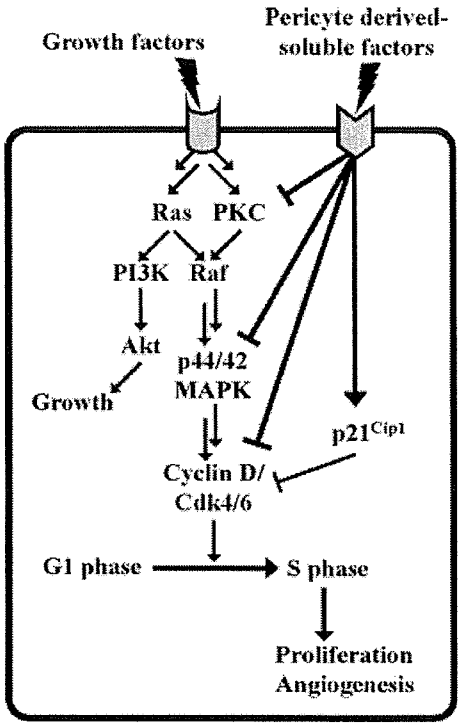

Fig. 6. Putative mechanisms underlying signaling suppression of endothelial cell proliferation involving retinal neovascularization by soluble factors derived from retinal pericytes.

pericytes offer partial neuroprotection for retinal nerve cells.

The reduction of phosphorylated p44/42 MAPK in TR-iBRB2 cells treated with rPCT1-CM was time-dependent, but the activated form of Akt was not detected (Fig. 5), suggesting that p44/42 MAPK-mediated signaling is reduced for a short time following rPCT1-CM and that this leads to suppression of endothelial cell growth. The signal transduction of p44/42 MAPK via PKC or Ras is dependent on the origin of the endothelial cells (Yashima et al., 2001). The phosphorylation of PKCα/βII was suppressed by rPCT1-CM in TR-iBRB2 cells, although the Ras expression was similar to that of control medium (Fig. 5). Persistent hyperglycemia in diabetes stimulates production of diacylglycerol and induces ischemia, resulting in activation of PKCβII-p44/42 MAPK in retinal endothelial cells, which is also detected in mice with ischemia-induced retinopathy (Aiello et al., 1997; Kampik and Gandorfer, 2000; Poulaki et al., 2002; Suzuma et al., 2002). Moreover, retinal neovascularization was inhibited by oral administration of a PKC inhibitor, CGP 41251 (Seo et al., 1999). This suggests that soluble factors derived from retinal pericytes suppress PKCβII-dependent p44/42 MAPK signaling in diabetic retinopathy and that loss of pericytes leads to stimulation of the signaling in retinal endothelial cells.

In conclusion, this study provides the first evidence that soluble factors from retinal pericytes suppress the activation of PKC-p44/42 MAPK signaling and the expression of cyclin D1, cdk4, and cdk6, inhibit endothelial cell growth in vitro, and prevent retinal neovascularization in vivo (Fig. 6). As the number of diabetic patients is increasing throughout the world, acquired blindness is a serious problem. Soluble factors derived from retinal pericytes may help protect diabetic patients from visual disorders.

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