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Research Paper

Vascular Endothelium–Selective Gene Induction by Tie2 Promoter/Enhancer in the Brain and Retina of a Transgenic Rat

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Purpose. To produce a transgenic rat harboring the Tie2 promoter/enhancer linked green fluorescence protein (GFP) gene and investigate the blood-brain barrier (BBB)- and inner blood-retinal barrier (iBRB)-selective GFP expression in the brain and retina.
Methods. Transgenic rats were produced by the microinjection of mouse Tie2/GFP gene into fertilized oocytes of Wistar rats. The expression of GFP was observed in tissue slices by confocal microscopy.
Results. One of the obtained transgenic lines showed intense GFP expression in vascular endothelial cells throughout the brain. After double immunostaining with glucose transporter 1, the GFP was found to be localized in the cytoplasmic compartment of brain capillary endothelial cells. In contrast, no fluorescence was observed in neural cells. In the retina of transgenic rats, intense GFP expression was detected in retinal capillary endothelial cells. No fluorescence was detected in other cells. In kidney and liver, GFP expression was detected in vascular endothelial cells, whereas the expressed region was limited.
Conclusions. Mouse Tie2 promoter/enhancer has the ability to induce gene expression selectively in vascular endothelial cells in the brain and retina of transgenic rats.

KEY WORDS: blood-brain barrier; green fluorescence protein; inner blood-retinal barrier; Tie2 promoter/enhancer; transgenic rats.

INTRODUCTION

The capillary endothelial cells in the brain and retina form the blood-brain barrier (BBB) and inner blood-retinal barrier (iBRB), respectively. To date, a number of transporters expressed in the brain and retinal capillary endothelial cells have been identified as playing roles in drug distribution to the brain and retina (1–3). For further studies of transport function, the genetic manipulation of BBB and iBRB function is a key method. Indeed, many studies have been reported concerning the contribution of mdr1a to drug efflux transport at the BBB using mdr1a/1b knockout mice (4).

The knockout mouse is a useful tool for clarifying the contribution of transporters to the transport system at the BBB and iBRB. However, in the knockout mouse, the target gene is disrupted throughout the whole body, resulting in changes in drug transport and metabolism in all the other tissues expressing the target gene. This makes it difficult to clarify the role of the target gene product at the BBB and iBRB. Furthermore, the knockout strategy is only available for the mouse, and analysis using rats is necessary for research into drug transport because of the large amount of data on this species and method limitations.

The transgenic technique linked with selective promoters and enhancers can induce gene expression with regional and/or temporal control. Therefore, when the transgene is linked with promoter/enhancer, which enhances the gene expression in capillary endothelial cells, the gene is selectively expressed in capillary endothelial cells in the brain and retina of transgenic animals. The transgenic technique is now used for the various species of animals, including rats, rabbits, and pigs (5–7), and the transgene can be used not only for gene overexpression but also for gene silencing by means of anti-sense and RNAi methods.

Motoike *et al*. have reported that the promoter/enhancer of Tie2, a vascular endothelial-specific receptor tyrosine kinase, induces linked genes [green fluorescent protein (GFP) and β-galactosidase] specifically in vascular endothelial cells in the transgenic mouse (8,9). However, there is no information regarding gene induction in rats and in retinal capillary endothelial cells. The purpose of this study was to investigate the vascular endothelial cell-selective expression of GFP in rat brain and retina by creating a transgenic rat harboring the Tie2 promoter/enhancer linked GFP gene (Tie2/GFP gene).

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ABBREVIATIONS: BBB, blood-brain barrier; GFP, green fluorescence protein; GLUT1, glucose transporter 1; iBRB, inner blood-retinal barrier; oBRB, outer blood-retinal barrier; PI, propidium iodide; Tie2, vascular endothelial-specific receptor tyrosine kinase.

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MATERIALS AND METHODS

Production of the Transgenic Rat

All experiments were approved by the Animal Care Committee, Graduate School of Pharmaceutical Sciences, Tohoku University. The vector containing the Tie2/GFP gene (pSP14/15.t2hgfpPan5') was a kind gift from Dr. T. N. Sato (The University of Texas Southwestern Medical Center at Dallas) (8). The Tie2/GFP transgene was excised from the plasmid vector by Sal I digestion. Microinjection into fertilized oocytes of Wistar rats and other surgical procedures were performed in the YS Institute (Tochigi, Japan) as described previously (7). Screening of the first offspring and estimation of the numbers of transgene copies per diploid genome were performed by genomic Southern blotting with probing Not I fragment containing the GFP gene. The genotyping of all offspring was analyzed by polymerase chain reaction (PCR) of genomic DNA from the tail using an Extract-N-Amp Tissue PCR kit (sigma; St. Louis, MO, USA). The primers were 5′-ATTCTCGTGGAACTGGATGG-3′ and 5′-GGACAGGTAATGGTTGTCTGG-3′. The PCR was conducted with the following thermal cycle program: 1 cycle of 94°C for 3 min, 40 cycles of 94°C for 30 s, 55°C for 1 min, 72°C for 1 min, and a final elongation of 72°C for 2 min. The amplified band was detected at 567 bp.

Immunohistochemical Analysis

Under deep pentobarbital anesthesia with ketamine and xylazine, rats were perfused transcardially with 4% paraformaldehyde (PFA). After perfusion, the tissues were dissected out and stored overnight in 4% PFA at 4°C. Then, cryostat sections of the brain, eye, liver, and kidney (20 μm in thickness; CM1900, Leica, Heidelberg, Germany) and microslicer sections of the brain (50 μm; VT1000S, Leica) were prepared for immunofluorescence. Because the retinal pigment epithelial cell layer and choroid easily dropped out when the retinal section was isolated, the sections retaining the retinal choroid were selected for histochemical analysis. The remainder of the retinal pigment epithelial cell layer and choroid was also confirmed by GFP expression in the retinal choroid capillary endothelial cells and by nuclear staining. For immunostaining, the sections were reacted with anti-glucose transporter 1 (GLUT1) antibody (0.5 μg/ml), which was a kind gift from Dr. M. Watanabe (Hokkaido University, Japan) and reacted selectively with brain capillary endothelial cells (10,11). This was followed by incubation with TRITC-conjugated secondary antibody (Dako, Glostrup, Denmark). Nuclei were stained with 6.6 μM propidium iodide (PI), and sections were viewed by confocal laser microscopy (TCS SP, Leica).

RESULTS

Production and Characterization of Transgenic Rats Harboring the Tie2/GFP Gene

Two transgenic pups were obtained out of 200 microinjected eggs, and about 30 copies of the transgene per diploid genome were integrated in both cases. The transgenic rats transmitted the transgene to the progeny by mating with Wistar rats, and no obvious abnormalities were observed. The phenotype of the transgenic rats was examined by the GFP expression in the brain. In one of the lines, intense GFP expression was detected with a capillary shape throughout the whole brain (Fig. 1A). In contrast, the other line showed weak GFP expression around the capillary endothelium nuclei in restricted brain regions. Therefore, further studies were conducted using the transgenic rats with a hemizygote, which showed intense GFP expression in the brain.

GFP Expression in the Brain of Transgenic Rats

Ramified fluorescence of GFP was observed uniformly in the cerebrum of transgenic rats (Fig. 1A). The GFP expression (green in Fig. 1B and D) in the brain overlapped that of GLUT1 (red in Fig. 1C and D), which is localized at the plasma membrane of brain capillary endothelial cells (10,11). In the magnified images, a signal for GLUT1 was detected at both the abluminal and luminal membrane of brain capillary endothelial cells (Fig. 1F, arrowheads). On the same section, GFP was detected in the cytosolic compartment between the GLUT1 signals (Fig. 1E and G). These results indicate that GFP is expressed in cerebral capillary endothelial cells. Not only capillary endothelial cells, but also endothelial cells of larger vessels were found to express GFP (Fig. 1A and K).

After double staining with PI, flat-shaped endothelium nuclei overlapped the GFP fluorescence. In contrast, the nuclei of neural cells did not overlap the GFP fluorescence in the cerebral cortex and hippocampus (Fig. 1K–N). In the cerebellum, GFP fluorescence was also detected in capillary endothelial cells and there was no overlap with the PI fluorescence of neural cells (Fig. 1H and I). In the choroid plexus, GFP expression was detected in capillary endothelial cells and not in epithelial cells (Fig. 1J). Therefore, the Tie2 promoter/enhancer induces GFP expression selectively in vascular endothelial cells in the brain.

GFP Expression in the Retina of Transgenic Rats

GFP fluorescence was detected as a ramified capillary shape throughout the retina of transgenic rats (Fig. 2A, arrowhead). This fluorescence overlapped with the expression of GLUT1, which is localized at the plasma membrane of retinal capillary endothelial cells (12) (Fig. 2D–F). Weak fluorescence was observed in the rod and cone layer (Fig. 2A, asterisk), and this fluorescence was also detected in normal rats (data not shown), suggesting that this is not GFP fluorescence. After double staining with PI, GFP fluorescence did not overlap the nuclei except in the case of the retinal capillaries (Fig. 2B and C). No GFP expression was detected in the retinal pigmented epithelial cells that form a layer outside the rod and cone layer (Fig. 2A). This result indicates that GFP is selectively localized in the capillary endothelial cells in the retina.

GFP Expression in the Kidney and Liver of Transgenic Rats

GFP fluorescence was detected in limited regions of the liver and kidney. In the kidney, GFP fluorescence was

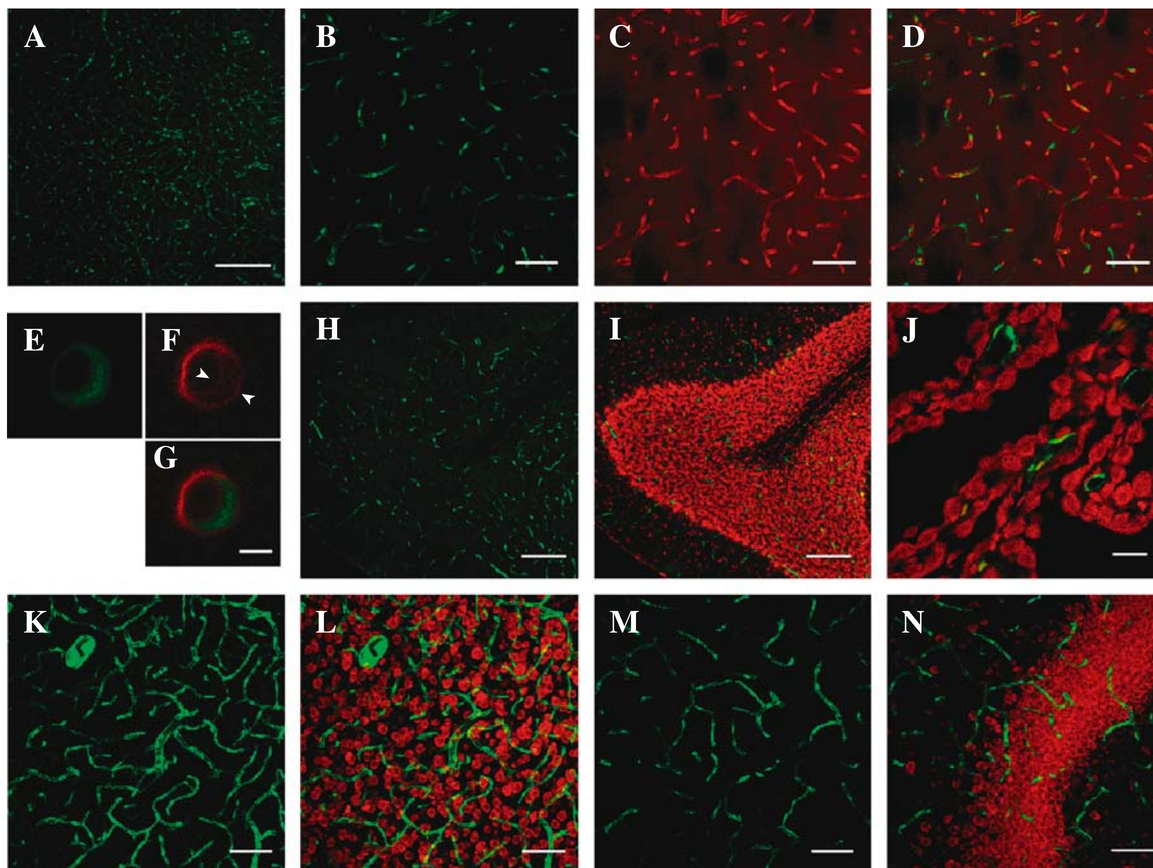

Fig. 1. GFP expression in the brain of the Tie2/GFP transgenic rat. (A) Low-magnified image of GFP expression in the cerebral cortex. (B–D) Double fluorescence images of GFP (B, green) and GLUT1 (C, red). D is an overlaid image of B and C. (E–G) Localization of GFP (E) and GLUT1 (F) in the capillary endothelial cells. G is an overlaid image of E and F. Arrowheads indicate the abluminal and luminal membrane localization of GLUT1. (H and I) Double fluorescence images of GFP (H and I, green) and nuclei with PI (I, red) in the cerebellum. (J) Double fluorescence images of GFP (green) and nuclei with PI (red) in the choroid plexus. (K–N) Double fluorescence images of GFP (green) and nuclei with PI (L and N, red) in the cerebral cortex (K and L) and hippocampus (M and N). Scale bars: 200 μm (A), 80 μm (B–D, K–N), 4 μm (G), 160 μm (H, I), 20 μm (J).

detected at the capillaries in the renal glomerulus and around the renal tubules (Fig. 3A and B). In the liver, GFP fluorescence was detected at the sinusoidal capillaries (Fig. 3C and D).

DISCUSSION

In this study, mouse Tie2 promoter/enhancer was found to induce gene expression selectively in vascular endothelial cells in the brain and retina of transgenic rats. Motoike *et al.* have shown that there is selective GFP expression in vascular endothelial cells in the brain of transgenic mice, using a single fluorescent microscopic image of a single brain region (8). In this study, the GFP expression in capillary endothelial cells was evaluated by double immunostaining with GLUT1. As shown in Fig. 1D and G, GFP was selectively expressed in the cytosolic compartment of brain capillary endothelial cells. Furthermore, this study investigated the GFP expression in the cerebellum and choroid plexus of transgenic rats. In the cerebellum, GFP was selectively expressed in vascular endothelial cells as in the cerebrum (Fig. 1I). Therefore, mouse Tie2 promoter/enhancer induces gene expression in the cells forming the BBB in the cerebrum and cerebellum. Choroid plexus epithelial cells form the blood-cerebrospinal fluid barrier (13). As shown in Fig. 1J, GFP expression was not detected in choroid plexus epithelial cells but in capillary endothelial cells. Because choroid plexus capillaries have fenestrae like the peripheral capillaries, gene induction by Tie2 promoter/enhancer would not affect the function of the blood-cerebrospinal fluid barrier.

Tie2 is expressed in retinal capillary endothelial cells (14,15), raising the possibility that gene induction by mouse Tie2 promoter/enhancer occurs in the retinal capillary endothelial cells. This study is the first report that the Tie2 promoter/enhancer has the ability to induce the gene selectively in capillary endothelial cells in the retina. The retinal capillary endothelial cells form the iBRB, and the retinal pigmented epithelial cells form the outer blood-retinal barrier (oBRB) (16). Unlike the GFP expression in retinal capillary endothelial cells, no GFP expression was detected in the retinal pigmented epithelial cells (Fig. 2A). Therefore, Tie2 promoter/enhancer induces gene expression selectively in the iBRB and not in the oBRB.

Motoike *et al.* have reported uniform GFP expression in the kidney and liver of transgenic mice (8). The capillary endothelial cells in rat liver and kidney have been reported to express Tie2 in similar levels to those in rat brain (17). Therefore, it is conceivable that mouse Tie2 promoter/

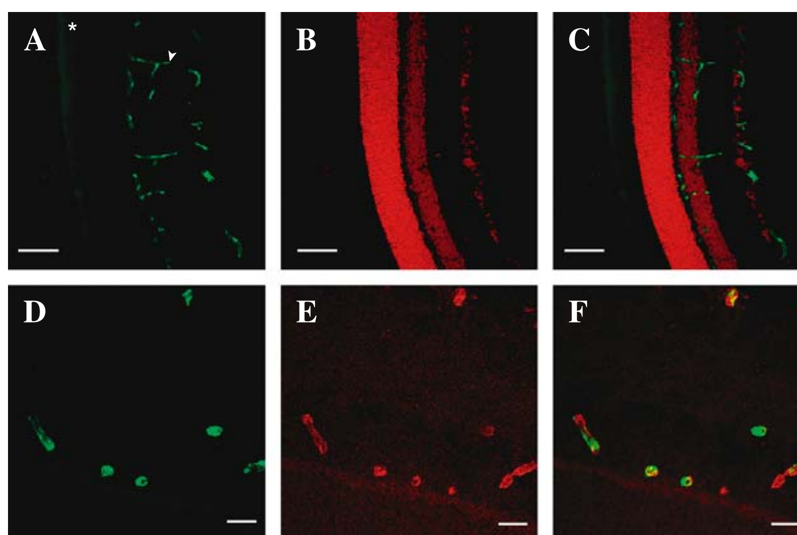

Fig. 2. GFP expression in the retina of the Tie2/GFP transgenic rat. (A–C) Double fluorescence images of GFP (A, green) and nuclei with PI (B, red) in the retina. C represents overlaid images of the corresponding fluorescence images of GFP and nuclei. (D–F) Double fluorescence images of GFP (D, green) and GLUT1 (E, red). F is an overlaid image of D and E. Arrowheads, and asterisks indicate the retinal capillaries, and the rod and cone layer, respectively. Scale bars: 80 μm (A–C), 20 μm (D–F).

enhancer induces gene expression in vascular endothelial cells in the liver and kidney of transgenic rats. Indeed, GFP was expressed selectively in vascular endothelial cells in those tissues (Fig. 3). However, the expression of GFP was not detected uniformly but in limited regions. One possible explanation of this is that the transcriptional regulation by Tie2 promoter/enhancer is affected by other regulatory elements around the insertion point, resulting in heterogeneous expression in the liver and kidney of transgenic rats. Another possible explanation is that, in contrast to the mouse, Tie2 promoter/enhancer does not have the ability to induce uniform gene expression in rat kidney and liver. To clarify this issue, a larger number of transgenic rats will need to be produced. As far as drug transport is concerned, epithelial cells of renal tubules and hepatocytes make a major contribution (18). Therefore, when the transporter expression in endothelial cells is modulated by Tie2 promoter/enhancer, the effect of gene induction on the drug transport function in the kidney and liver would be small.

BBB- and iBRB-selective gene expression in rats has advantages for drug transport analysis compared with that in mice. One advantage is the material for isolating capillary endothelial cells. Because the amount of material was limited, most of the studies were conducted with isolated brain capillaries or primary cultured brain capillary endothelial cells from rat or bovine brain (19). GFP is a good marker for brain capillary endothelial cells in Tie2/GFP rats. Therefore, using the cell sorting method, fresh capillary endothelial cells can be isolated with high purity. This solves the problems of contamination of neural cells in the isolated capillary fraction and altered gene expression in cultured brain capillary endothelial cells (19).

Inserting the expression cassette of anti-sense RNA or shRNA instead of GFP gene, it is possible to obtain BBB- and iBRB-selective transporter silencing in the brain and retina of transgenic rats (20). The transgenic rats exhibited intense GFP expression in the brain capillary endothelial cells similar to the levels in transgenic mice (8) (data not shown), and vascular endothelium selective exogenous gene expression and conditional knock down using Cre recombinase has been reported in transgenic mice using the Tie2 promoter/enhancer (21,22). Therefore, the Tie2 promoter/enhancer is likely to have the ability to induce enough gene expression for gene silencing at the BBB and iBRB of the transgenic rats. This BBB- and iBRB-selective gene silencing would be a useful strategy to investigate the contribution of

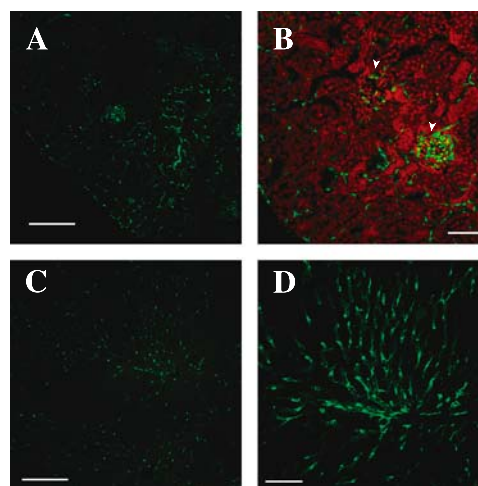

Fig. 3. GFP expression in the kidney and liver of Tie2/GFP transgenic rats. (A and B) Fluorescence image of GFP (green) in the kidney. B is the magnified image of A with nuclei staining (red), and arrowheads indicate the renal glomerulus. (C and D) Fluorescence image of GFP (green) in the liver. D is the magnified image of C. Scale bars: 200 μm (A and C), 80 μm (B and D).

transporters to drug transport across the BBB and iBRB. Brain-to-blood and retina-to-blood efflux transport plays an important role in limiting drug distribution into the brain and retina (1,23), and some of the methods for analyzing efflux transport can be used in rats; for example, the brain efflux method for efflux transport across the BBB (24) and the microdialysis method for efflux transport across the iBRB.

Gene manipulation is also a useful technique for analyzing the function of the BBB and iBRB. Highly purified brain and retinal capillary endothelial cells from rats are necessary to analyze the function of the barriers under conditions associated with a variety of diseases. Furthermore, the molecular mechanism governing functional regulation at the BBB and iBRB has been analyzed using cultured cells (25–27). To clarify these molecular mechanisms *in vivo*, BBB- and iBRB-selective gene induction and silencing are essential. Therefore, this BBB- and iBRB-selective gene manipulation will be an important strategy not only for analyzing drug transport but also for analyzing the different types of barrier function, including tight junctions and signal transduction at the BBB and iBRB.

In conclusion, mouse Tie2 promoter/enhancer induces selective gene expression in vascular endothelial cells in the brain and retina of transgenic rats. To date, the knockout mouse is the only available animal for analyzing the contribution of transporters at the BBB and iBRB *in vivo*. The Tie2 promoter/enhancer now makes it possible to modulate gene expression selectively at the BBB and iBRB in the brain and retina of transgenic rats, respectively. Therefore, gene manipulation using the Tie2 promoter/enhancer will be an important method for future BBB and iBRB research.

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