Characterization of Glucose Transporter in Cultured Human Retinal Pigment Epithelial Cells: Gene Expression and Effect of Growth Factors

Hitoshi Takagi,* Hidenobu Tanihara,* Yutaka Seino,† and Nagahisa Yoshimura*


Methods. Glucose transport activity was analyzed by [3H]2-deoxyglucose uptake studies. Gene and protein expression of glucose transporter isoforms were analyzed by polymerase chain reaction, Northern blot analysis, and Western blot analysis.

Results. Polymerase chain reaction, nucleotide sequencing, and Southern blot analyses revealed that the retinal pigment epithelial cells express GLUT-1, -3, and -5 genes. Northern and Western blot analysis detected only GLUT-1 transcripts and protein. A 24-hour exposure to fetal bovine serum (15%), basic fibroblast growth factor (50 ng/ml), platelet-derived growth factor (10 ng/ml), epidermal growth factor (50 ng/ml), and insulin-like growth factor-1 (50 ng/ml) significantly stimulated [3H]2-deoxyglucose uptake in cultured human retinal pigment epithelial cells. Western blot analysis showed that serum and platelet-derived growth factor induced an increase of GLUT-1 protein in the membrane preparation in the cells. Serum, fibroblast growth factor, platelet-derived growth factor, and insulin-like growth factor-1 did not increase GLUT-1 gene expression to an appreciable level, as shown by Northern blot analysis.


Retinal pigment epithelial (RPE) cells are believed to play an important role in a number of ocular diseases including proliferative vitreoretinopathy, and to have some role in the development of macular edema in diabetic retinopathy. In these conditions RPE cells are exposed to growth factors, which may be leaked out through a deficient blood–ocular barrier or produced by various types of retinal cells or by the RPE cells themselves. Although growth factor-induced stimulation of glucose uptake activity in other tissues is reported, the glucose transport by RPE cells under such conditions is not well understood.

Five distinct isoforms of mammalian facilitative glucose transporters, which differ in their tissue distribution and functional properties, have been cloned. Each member shares 40% to 60% overall amino acid homology, with the putative transmembrane α-helical domains having the highest homology. These proteins have been designated as GLUT-1/erythrocyte, GLUT-2/liver, GLUT-3/brain, GLUT-4/muscle/fat, and GLUT-5/small intestine. As to the glucose transport in RPE, facilitated diffusion has been observed in isolated RPE–choroid preparations and in cultured pigment epithelial cells. Previous immunocytochemical studies and Western blotting us-

From the Departments of *Ophthalmology and †Metabolism and Clinical Nutrition, Kyoto University, Faculty of Medicine, Kyoto, Japan. Supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of the Japanese Government. Submitted for publication January 14, 1993; revised June 4, 1993; accepted June 23, 1993.

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Reprint requests: Nagahisa Yoshimura, Department of Ophthalmology, Kyoto University Faculty of Medicine, Kyoto 606, Japan.
ing isolated RPE24-26 showed that rat RPE cells contain GLUT-1 protein, but the other types of glucose transporters have not yet been identified.

Herein we demonstrate the dominant expression of GLUT-1 gene and protein with minor expression of GLUT-3 and -5 genes in cultured human RPE cells. We also describe much higher basal glucose transport activity of the cells with modest increase by growth factors compared to that of fibroblasts.

MATERIALS AND METHODS

Cell Culture

The research was approved by the institutional human experimental committee, and methods for securing human tissue were humane, included proper consent and approval, and complied with the tenets of the Declaration of Helsinki. Cultured human RPE cells were established as described previously.27 The cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS, M.A. Bioproducts, Wakersville, MD) and antibiotics and incubated at 37°C in a humidified atmosphere of 5% CO2 and 95% air. Cells from the third through the fifth passage were used in the experiments.

Isolation of Total RNA

After a 24-hour serum starvation, RPE cells grown in 100 mm culture dishes were incubated with growth factors. Cells were made quiescent and reactivated in Dulbecco’s modified Eagle’s medium containing 0.25% bovine serum albumin. Six hours later, total RNA was isolated from the samples using the acid guanidinium thiocyanate-phenol-chloroform extraction method.28 Growth factors used in the experiment were obtained from several sources: human recombinant epidermal growth factor and human recombinant platelet-derived growth factor-BB (Wako Pure Chemicals, Osaka, Japan); human recombinant basic fibroblast growth factor (Amersham Japan, Tokyo, Japan); human recombinant insulin-like growth factor-1 (IGF-1), donated by Dr. George L. King (Joslin Diabetes Center, Boston, MA).

Polymerase Chain Reaction

First strand complementary DNA (cDNA) was prepared from the total RNA by a first-strand cDNA synthesis kit (Pharmacia-LKB, Uppsala, Sweden). Total RNA of 5 μg was used to synthesize cDNA. Specific primers for GLUT-1 to -5 were synthesized by an oligonucleotide synthesizer (Applied Biophysics, Model 392, Foster City, CA) using previously published sequences so that each sequence would be detected individually and intron sequences that were excised during RNA processing would be included in genomic targets (Table 1).13-17 Polymerase chain reaction (PCR) was performed by the method of Saiki et al29 with a slight modification. The following conditions were used: denaturation at 94°C for 1.5 minutes; annealing, 55 or 65°C for 2 minutes; and polymerization, 72°C for 3 minutes. The reaction was initiated by adding two units of Taq polymerase, after which 35 cycles were carried out. Taq DNA polymerase and reagents for PCR experiments were obtained from Perkin-Elmer Cetus (Norwalk, CT). After the PCR, the reaction products were separated by 1.5% agarose gel electrophoresis and bands of the expected length were extracted. The resultant DNA was subcloned into pBluescript II vector (Stratagene, La Jolla, CA). Before subcloning, the vector was treated by Eco RV and the T vectors were made by Taq polymerase and deoxythymidine triphosphate.30

Sequencing of PCR Products

To confirm that the PCR products were derived from nucleotide sequences corresponding to glucose transporter isoforms, sequencing of subcloned DNA was performed according to the dideoxynucleotide chain termination method31 using a Sequenase version 2.0 DNA sequencing kit (United States Biochemicals, Cleveland, OH). Double-strand template DNA was dena-

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<th>GLUT-3 Oligoprobe</th>
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tured by alkaline treatment and the sequencing reaction was initiated by using T3 and/or T7 primer.

Southern Blot Analysis

For Southern blot analysis, 10 μg of PCR product was separated by 1.5% agarose gel electrophoresis and then transferred to a nylon filter (Hybond-N+, Amersham, Little Chalfont, UK) by capillary transfer with 20X standard saline citrate. The DNA was fixed to the filter by baking at 80°C for 2 hours in a vacuum oven. DNA fragments corresponding to the target sequences were labeled by the enhanced chemoluminescence gene detection kit (Amersham).

Deoxyglucose Uptake Study

Assay of [3H]2-deoxyglucose uptake was performed by the method of Pekala et al.32 Briefly, after being serum starved with Dulbecco’s modified Eagle’s medium containing 0.25% bovine serum albumin for 24 hours, quiescent cultures in 6-well cluster dishes were incubated with growth factors in the same culture media. After three washes with phosphate-buffered saline, each culture was incubated with 1 ml Krebs-Ringer HEPES buffer (HEPES 12 mM, NaCl 121 mM, KCl 4.9 mM, CaCl2 0.33 mM, MgSO4 1.2 mM), pH 7.4, containing 0.1% bovine serum albumin and 2-deoxyglucose (0.1 mM) with 1 μCi of [3H]2-deoxyglucose at 37°C for 10 minutes. After terminating the transport assay with three washes of ice-cold Krebs-Ringer HEPES buffer, each monolayer was solubilized in 1 ml of 1N NaOH. An aliquot of 0.4 ml was removed for determination of radioactivity by scintillation counting and two 0.1 ml aliquots were used for measurement of protein by a BCA Protein Assay Kit (Pierce, Rockford, IL). Measurements were made in triplicate on three different batches of cells. The values were corrected for nonspecific diffusion as determined in the presence of 10 μM cytochalasin B.

Western Blot Analysis

After a 24-hour serum starvation, RPE cells grown in 100 mm culture dishes were incubated with growth factors. Cells were washed three times with cold phosphate-buffered saline and solubilized in 10 mM Tris-buffer, pH 6.0, containing 1.25 M NaCl, 15 mM ethylenediaminetetraacetic acid, 0.1 mM phenylmethylsulfonylfluoride, and 5 μg/ml each of protease inhibitors (aprotinin, leupeptin, and pepstatin A). After centrifugation at 6500g for 10 minutes, the supernatant was centrifuged at 100,000g for 30 minutes, and the resulting pellet was suspended in 10 mM Tris-buffer, pH 7.4, containing 1.25 M NaCl, 15 mM CaCl2, 1% Triton X-100, 0.1 mM phenylmethylsulfonylfluoride, and 5 μg/ml each of the protease inhibitors. Five micrograms of each sample was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 10% polyacrylamide gel and electrophoretically transferred to a nitrocellulose filter. The filter was incubated with a 1:300 dilution of rabbit anti-GLUT-1 (rat brain) antibody (Chemicon, Temecula, CA) or rabbit anti-human GLUT-3 antibody (East-Acres Biologicals, Southbridge, MA) for 1 hour. Both antibodies were raised against synthetic peptides in the carboxy terminal region of each protein. The filter was then incubated for 1 hour with goat anti-rabbit immunoglobulin G coupled to peroxidase and stained with the enhanced chemiluminescence Western blotting analysis system (Amersham). The bands for GLUT were quantitated using a scanning densitometer.

Measurement of GLUT-1 Messenger RNA by Northern Blot Analysis

Total RNA (10 μg) of each sample was separated by electrophoresis on a 1.25% agarose-2M formaldehyde gel and transferred to a nylon membrane (GeneScreen Plus, NEN-DuPont, Boston, MA) by capillary method. After transfer, the RNA was fixed to the filter by baking at 80°C for 2 hours in a vacuum oven. Northern blot analysis was done essentially according to the method of Thomas.33 The final washing was with 0.2X standard saline citrate with 0.1% sodium dodecyl sulfate for 45 minutes at 65°C. Autoradiographs were quantitated using a scanning densitometer.

DNA Probes

The cDNA probes used in this study were as follows: GLUT-1, a 1.6-Kb EcoRI-HindIII fragment from hGT2-134 that encodes the HepG2/brain glucose transporter; GLUT-2, a 1.4-Kb EcoRI fragment from RhHTL-1415 that encodes GLUT-2 (liver-type); GLUT-3, a 2.2-Kb EcoRI fragment (phMGT-3113) that encodes GLUT-3 (brain-type); GLUT-4, a 1.7-Kb EcoRI fragment (phJHT-316) encoding GLUT-4 (muscle/fat-type); and GLUT-5, a 1.9-Kb EcoRI fragment (RJHJ3-194) encoding GLUT-5 (small intestine-type).

Statistical Analysis

All values are reported as the mean ± SEM. Statistical analysis was performed with Student’s t test. We compared the 2-deoxyglucose (DOG) uptake in RPE cells stimulated by growth factors with control RPE cells and also compared that in NIH 3T3 cells stimulated by FBS with control NIH 3T3 cells.

RESULTS

PCR, Nucleotide Sequencing, and Southern Blot Analysis

To ascertain the type of glucose transporter isoforms that are expressed in the RPE cells, PCR amplification
using specific primers was performed. Figure 1 indicates that the PCR products of the expected length were detected for GLUT-1, -3, and -5 (Fig. 1, lanes 2, 7, and 12, respectively), although those for GLUT-3 are multiple (Fig. 1, lane 7). PCR for GLUT-2 and -4 was negative (Fig. 1, lanes 5 and 10). The resultant PCR products of GLUT-1, -3, and -5, which have expected lengths, were isolated and subcloned into the pBluescript II vector and sequenced. All nucleotide sequences of the subcloned PCR products revealed that they corresponded to sequences of GLUT-1, -3, and -5. We also sequenced the extra bands of PCR products for GLUT-3 and database analyses showed these products were not homologous to any type of GLUT isoforms. Negative control (PCR done on templates made without reverse transcription) experiment revealed no positive bands (Fig. 1, lanes 3, 8, and 13). Southern blot analysis confirmed that the bands from cDNA of the RPE cells were derived from GLUT-1, -3, and -5 (Fig. 2).

Stimulation of Glucose Uptake by Growth Factors and Serum

To examine the effect of growth factors on the glucose transport activity in human RPE cells, DOG uptake was determined. In the time course study, 50 ng/ml of epidermal growth factor, fibroblast growth factor, IGF-1, and 10 ng/ml of platelet-derived growth factor (PDGF) increased DOG uptake in the RPE cells as early as 6 hours and stimulated maximally at 12 hours and sustained the peak value until 24 hours (graphic data not shown). There was no additional increase after 24 hours, so this time period was used to obtain the growth factor dose dependency relationship for evaluating DOG uptake. The results shown in Figure 3A indicate that the EC50 for increasing DOG uptake was about 1 to 10 nM for these growth factors and PDGF was the most effective. Figure 3B indicate that glucose transport in RPE cells was stimulated significantly by a 24-hour exposure to epidermal growth factor (50 ng/ml), fibroblast growth factor (50 ng/ml), PDGF (10 ng/ml), IGF-1 (50 ng/ml), and FBS (15%) compared to the control. RPE cells without stimulation exhibited DOG uptake of 1.5 ± 0.03 nmol/min/mg of protein, and a 24-hour exposure to 15% FBS increased the uptake by about 81%. To compare DOG uptake of RPE cells to that of fibroblasts, DOG uptake of NIH 3T3 cells were measured. Control 3T3 cells exhibited 60 ± 10 pmol/min/mg of protein (n = 3, mean ± SEM), and a 24-hour exposure to FBS (15%) increased the rate by about 20-fold (Fig. 3B). However, even with 15% FBS, DOG uptake of the 3T3 cells was smaller than that of control RPE cells.

Expression of GLUT protein

To investigate effect of growth factors on GLUT protein expression, membrane protein of cultured human RPE cells was subjected to Western blot analysis. A 24-hour exposure to PDGF (10 ng/ml) and FBS (15%) was chosen because these growth factors stimulated glucose transport activity of the cells more than other stimulants. The results (Fig. 4) show that anti-GLUT-1 antibody labeled broad band of 40 to 43 kD. The ex-
induced increase in the GLUT-1 gene expression, total RNA isolated after a 6-hour exposure to epidermal growth factor (50 ng/ml), fibroblast growth factor (50 ng/ml), PDGF (10 ng/ml), IGF-1 (50 ng/ml), and FBS (15%) were subjected to Northern blot analysis. The results indicate that a 6-hour exposure to growth factors did not increase GLUT-1 gene expression to an appreciable level (Fig. 5A). For any of these growth factors, the average ratio compared to control are below 1.4 (Fig. 5B).

DISCUSSION

The current study demonstrates that cultured human RPE cells dominantly express GLUT-1 gene and protein and also suggests the expression of GLUT-3 and -5 messenger RNA in the cells. A cDNA probe for GLUT-1 hybridized to the 2.8 kb messenger RNA by Northern blotting (Fig. 5A), which agrees with previous reports. Western blotting using anti-GLUT-1 antibody revealed the molecular weight of GLUT-1 protein in human RPE cells to be about 40 to 43 kD.

Expression of GLUT Messenger RNA

The expression of messenger RNA for GLUT-1 to -5 in human RPE cells was examined by Northern blot analysis. Transcripts for GLUT-1 were detected (Fig. 5A), and the size was 2.8 kbp. Transcripts of GLUT-2 to -5 were not detected by Northern blot analysis (data not shown). To investigate a possible growth-factor-induced increase in the GLUT-1 gene expression, total RNA isolated after a 6-hour exposure to epidermal growth factor (50 ng/ml), fibroblast growth factor (50 ng/ml), PDGF (10 ng/ml), IGF-1 (50 ng/ml), and FBS (15%) were subjected to Northern blot analysis. The results indicate that a 6-hour exposure to growth factors did not increase GLUT-1 gene expression to an appreciable level (Fig. 5A). For any of these growth factors, the average ratio compared to control are below 1.4 (Fig. 5B).

FIGURE 3. Effect of serum and growth factors on 2-deoxyglucose uptake by human RPE cells. The [3H]deoxyglucose uptake by the human RPE cells and NIH 3T3 cells was determined after a 24-hour incubation with epidermal growth factor (50 ng/ml), fibroblast growth factor (50 ng/ml), IGF-1 (50 ng/ml), PDGF (10 ng/ml), FBS (15%), or without any growth factors (control). Cells were made quiescent and reactivated in Dulbecco's modified Eagle's medium containing 0.25% bovine serum albumin. The data are presented as mean ± SEM of three experiments each of which was conducted in triplicate. Statistical analyses indicate epidermal growth factor, fibroblast growth factor, IGF-1, 15% FBS (*P < 0.01), and fibroblast growth factor (**P < 0.05) significantly stimulated the DOG uptake in RPE cells compared to the control (RPE). In NIH 3T3 cells, 15% FBS statistically significantly stimulated DOG uptake (**P < 0.01) much more than that in RPE cells (3T3).

FIGURE 4. Effect of serum and PDGF on the expression of GLUT-1 protein. Membrane protein was harvested from cultured human RPE cells after 24 hours of exposure to 10 ng/ml PDGF, 15% FBS, or no growth factor (control). Cells were made quiescent and reactivated in Dulbecco's modified Eagle's medium containing 0.25% bovine serum albumin. Five micrograms of protein was subjected to Western blot analysis using anti-GLUT-1 antibody. The band of 40 to 43 kD is GLUT-1 protein. The exposure to PDGF and FBS increased the GLUT-1 protein by 0.6- and 0.4-fold, respectively. Anti-GLUT-3 antibody did not detect protein using blots prepared in the same way.

FIGURE 5. Effect of serum and growth factors on 2-deoxyglucose uptake by human RPE cells. The [3H]deoxyglucose uptake by the human RPE cells and NIH 3T3 cells was determined after a 24-hour incubation with epidermal growth factor (50 ng/ml), fibroblast growth factor (50 ng/ml), PDGF (10 ng/ml), IGF-1 (50 ng/ml), or without any growth factors (control). Cells were made quiescent and reactivated in Dulbecco's modified Eagle's medium containing 0.25% bovine serum albumin.
Glucose Transporter in RPE Cells

PCRs, followed by nucleotide sequencing, and Southern blot analysis were very sensitive methods of detecting messenger RNA expression of GLUT-3 and -5 genes in cultured human RPE cells. The gene expression of GLUT-3 and -5 isoforms is believed to be very small, because Northern and Western blot analyses failed to depict transcripts and protein of the isoforms. GLUT-3 is reported to be expressed in most tissues in which GLUT-1 is expressed, but is most abundant in the brain, where this transporter is reported to be located in the cytoplasm of both pyramidal cells and microvascular endothelial cells. GLUT-5 is reported to be a fructose transporter in human spermatozoa and small intestine. These two types of glucose transporter are believed to have little, if any, functional significance in the RPE cells because these gene expressions are very small. Our cells are cultured rather than intact and are of fetal rather than adult origin. These conditions of the cells may affect the pattern of GLUT gene isoform expression.

Growth factors such as epidermal growth factor, fibroblast growth factor, IGF-1, PDGF, and FBS stimulate glucose transport activity in cultured human RPE cells. The growth factor-induced stimulation of glucose transport activity in human RPE cells was less than 1-fold above the basal level, whereas FBS induced a greater than 20-fold increase in 3T3 cells. The glucose uptake in cultured human RPE cells under basal conditions was 1.5 ± 0.03 nmol/min/mg protein, and Vmax of glucose transport in cultured chick and rat pigment epithelial cells is reported to be 22 and 12.2 nM/mg protein/min, respectively. These rates are much higher than those of fibroblasts measured in this study and those reported in previous studies, 60 ± 10 and 60 to 300 pmol/mg protein/min, respectively. These data suggest that cultured human RPE cells have a much higher capacity for glucose transport, which is less prominently upregulated by growth factors than fibroblasts. Dose response studies show that PDGF is the most effective of these four growth factors, which is consistent with the rank order in inducing intracellular calcium transients and protein phosphorylation reported from our laboratory.

PDGF, IGF-1, and insulin are reported to increase GLUT-1 protein in mouse fibroblasts, young rat brain astrocytes, and L6 myocytes. In our study, FBS and PDGF increased the GLUT-1 protein in the membrane protein of human RPE cells. Previous studies indicated that the stimulatory effects of growth factors on glucose transport activity in fibroblasts are mainly due to upregulation of GLUT-1 gene expression. In our study, growth factors did not stimulate GLUT-1 gene expression to an appreciable level as is found in fibroblasts. There is a possibility that some posttranslational mechanisms such as translocation from intracellular vesicle to plasma membrane and activation of glucose transporters could be involved. FBS and PDGF increased the GLUT-1 protein in the membrane protein of human RPE cells. Previous studies indicated that the stimulatory effects of growth factors on glucose transport activity in fibroblasts are mainly due to upregulation of GLUT-1 gene expression. In our study, growth factors did not stimulate GLUT-1 gene expression to an appreciable level as is found in fibroblasts. There is a possibility that some posttranslational mechanisms such as translocation from intracellular vesicle to plasma membrane and activation of glucose transporters could be involved.

FIGURE 5. Effect of serum and growth factors on the expression of GLUT-1 gene. (A) Northern blot analysis of GLUT-1 gene. Total RNA was harvested from cultured human RPE cells after a 6-hour exposure to various growth factors. Total RNA (10 µg) of each sample was separated by electrophoresis on agarose-formaldehyde gel and transferred to a nylon membrane and hybridized to GLUT-1 or β-actin probe. Lane 1, control (0.25% bovine serum albumin); lane 2, 50 ng/ml epidermal growth factor; lane 3, 50 ng/ml fibroblast growth factor; lane 4, 50 ng/ml IGF-1; lane 5, 10 ng/ml PDGF; and lane 6, 15% FBS. Cells were made quiescent and reactivated in Dulbecco's modified Eagle's medium containing 0.25% bovine serum albumin. (B) Autoradiographic bands from five independent experiments were quantitated using a scanning densitometer. Densities were normalized by densities of β-actin of same blots and the average of the ratio compared to control in five experiments is shown. Data are presented as means ± SEM.

(Fig. 4), which also corresponds to previous reports using isolated rat RPE. GLUT-1 is the most widely distributed of the five transporters, and is reported to be concentrated in those cells that form interendothelial or interepithelial occluding junctions, thus constituting blood–tissue barriers. The abundance of GLUT-1 in RPE cells is consistent with this distribution.
tion of intrinsic activity\textsuperscript{45} may play a role in growth factor–dependent stimulation of glucose transport activities in RPE cells. The mechanism is to be further investigated.

In conclusion, human RPE cells dominantly express GLUT-1 gene and protein and also express GLUT-3 and -5 gene. They have a much better ability for glucose transport under basal conditions, which is stimulated by growth factors although in a relatively moderate manner compared to fibroblasts and thought to contribute the maintenance of photoreceptor cell function.

**Key Words**

retinal pigment epithelial cells, glucose transporter, growth factors, gene expression, polymerase chain reaction

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