Antisense GLUT-1 protects mesangial cells from glucose induction of GLUT-1 and fibronectin expression

CHARLES W. HEILIG,1–3 JEFFREY I. KREISBERG,4 SVEND FREYTAG,3 TAKASHI MURAKAMI,5 YOUSUKE EBINA,5 LIRONG GUO,2 KATHLEEN HEILIG,1,3 ROBERT LOBERG,6 XUAN QU,6 YING JIN,1 DOUGLAS HENRY,7 AND FRANK C. BROSIUS III6

1Division of Nephrology, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205; 2Nephrology Unit, University of Rochester Medical Center, Rochester, New York 14642; 3Division of Molecular Biology, Henry Ford Hospital, Detroit 48202; 4Division of Nephrology, Department of Medicine and Veterans Affairs Medical Center, University of Michigan, Ann Arbor 48109; 5Department of Physiology, College of Human Medicine, Michigan State University, East Lansing, Michigan 48824; 6Department of Surgery, University of Texas Health Science Center and the Audie Murphy Veterans Administration Hospital, San Antonio, Texas 78284; and 7School of Medicine, Tokushima University, Tokushima 770, Japan

Received 1 March 2000; accepted in final form 6 December 2000

Heilig, Charles W., Jeffrey I. Kreisberg, Svend Freytag, Takashi Murakami, Yousuoke Ebina, Lirong Guo, Kathleen Heilig, Robert Loberg, Xuan Qu, Ying Jin, Douglas Henry, and Frank C. Brosius III. Antisense GLUT-1 protects mesangial cells from glucose induction of GLUT-1 and fibronectin expression. Am J Physiol Renal Physiol 280: F657–F666, 2001.—A stable clone of rat mesangial cells expressing antisense GLUT-1 (i.e., MCGT1AS cells) was developed to protect them from high glucose exposure. GLUT-1 protein was reduced 50%, and the 2-deoxy-3H]glucose uptake rate was reduced 33% in MCGT1AS. MCLacZ control cells and MCGT1 GLUT-1-overexpressing cells were used for comparisons. In MCLacZ, 20 mM D-glucose increased GLUT-1 transcription 90% vs. no increase in MCGT1AS. Glucose (8 mM) and 12 mM xylitol [a hexose increased GLUT-1 transcription 90% vs. no increase in MCGT1AS. D-Glucose (20 mM) increased fibronectin expression, thereby demonstrating a protective effect that could be beneficial in the setting of diabetes.

Substantial progress has been made over the last 10 years toward understanding the processes that mediate diabetic nephropathy. A strong case has developed for hyperglycemia per se as the major factor responsible for development of diabetic tissue complications, including diabetic nephropathy (2, 7). The key process mediating diabetic renal disease is believed to be excessive mesangial synthesis and release of extracellular matrix (ECM) molecules that accumulate in and expand the mesangium, thereby scarring the glomerulus and impairing renal function (25, 28). The hypothesis that hyperglycemia plays a key role in development of renal disease was greatly strengthened by results from the Diabetes Control and Complications Research Group (7) in 1993 that demonstrated tight control of blood glucose concentrations prevented or delayed diabetic renal failure in humans. The precise cellular mechanisms involved in mediating glucose-induced mesangial cell ECM synthesis are now the focus of intense research.

In 1992, Dimitrakoudis et al. (8) reported increased translocation of GLUT-1 to the sarcolemma of diabetic muscle. GLUT-4 levels were decreased and caused overall GLUT expression in skeletal muscle to be decreased; however, GLUT-1 increased in the sarcolemma. This led the authors to speculate that tissues that do not require insulin for glucose uptake might have substantial quantities of GLUT-1 and that, in diabetes, the increased availability of GLUT-1 could be expected to lead to excessive glucose uptake and tissue damage in the eye, kidney, and nerves (8). This theory went untested, however, until our discovery of D-glucose-induced GLUT-1 expression in mesangial cells (12, 13, 15) and the description by Kumagai et al. (21) of the GLUT-1 response in retinal endothelial cells.
Both of these cell types are from classic diabetes target organs. The in vivo correlates of excess mesangial cell ECM synthesis are glomerulosclerosis and renal failure (25, 28). Previous studies have focused heavily on the aldose reductase (or polyol) pathway (3, 19, 26, 31), the protein kinase C pathway (10, 11, 29), and growth factors such as transforming growth factor-β1, insulin-like growth factor I, and platelet-derived growth factor (3, 20, 24, 30, 33, 34) in addition to advanced glycosylation end products (4, 9, 30). Common to all four pathways is their stimulation by high extracellular glucose concentrations (4, 5, 12, 13, 15, 20, 35, 36). We have previously demonstrated that GLUT-1 is a major glucose transporter of the glomerulus (14) and of mesangial cells in vitro (12, 13). Our recent work in mesangial cells demonstrated that high glucose in the diabetic range (20 mM) is a potent stimulus to increased mesangial cell GLUT-1 expression and glucose uptake (13). Furthermore, overexpression of GLUT-1 in mesangial cells stimulates ECM production (12). The Michaelis constant for GLUT-1 is low (0.5–3.7 mM) in mesangial cells, as it is in most tissues, and it is at or near saturation at physiological glucose concentrations (12, 13, 15). Therefore, GLUT-1 represents a site for regulation of ECM synthesis by factors that regulate GLUT-1 expression, translocation, and/or activity (13, 16, 18). Rates of ECM synthesis may be directly proportional to levels of plasmalemmal GLUT-1 expression. Subsequently, in the present study, we investigated the potential protective effect of antisense GLUT-1 against β-glucose-induced GLUT-1 and ECM (fibronectin, FN) expression.

MATERIALS AND METHODS

DNA Construction, Preparation of Infective Virus, and Transduction of Rat Mesangial Cells

We previously published the basic method for development of the β-galactosidase-expressing MCLacZ control cells and the GLUT-1-overexpressing MCGT1 cells (12). The same method was used here for development of a GLUT-1-underexpressing MCGT1AS cell clone. The pWZLneo MoMuLV retrovirus vector (Ariad, Cambridge, MA) was employed for all gene transductions (12). Transcription of the dicistronic proviral RNA is driven from the MoMuLV long terminal repeat. pWZLneoLacZ (control), pWZLneoGT1 (GLUT-1 sense), and pWZLneoGT1(AS) (GLUT-1 antisense) DNA constructs were used to transfect packaging cells, produce live virus, and transduce subconfluent mesangial cells. Mesangial cell clones surviving G418 selection were initially stored at −80°C until use. RNA samples (20 μg each) were denatured in glyoxal/DMSO at 55°C for 1 h and then loaded to individual lanes of a 10% sodium phosphate-1% agarose gel inside a circulating buffer electrophoresis gel box (Hoeffer Super Sub; Hoeffer Scientific Instruments, San Francisco, CA). Gels were run at 90 volts overnight. Subsequently, they were stained with ethidium bromide, destained, and photographed. Integrity of the RNA was confirmed by inspection of ribosomal RNA bands. Gels were blotted to Genescreen hybridization membranes (NEN, Boston, MA) for 36 h with 10× saline-sodium citrate using a standard method (27a). Blots were then ultravioletly fixed with a Stratalinker (Stratagene, La Jolla, CA), prehybridized with eronomic (IRES) in the construct allows GLUT-1 and neo to be expressed in a single transcript of size 5.6 kb. This 5.6-kb band is far above the native 2.8-kb GLUT-1 band that allows both to be visualized in the same lane on Northern blots.

Culture of Rat Mesangial Cells

Cells were cultured in a 37°C, 5% CO2 humidified incubator. The media used for culture of the three cloned mesangial cell lines, MCLacZ β-galactosidase-expressing control cells, MCGT1 GLUT-1-overexpressing cells, and MCGT1AS GLUT-1-underexpressing cells, was based on standard RPMI 1640 medium lacking glucose. β-glucose was added to the medium to achieve the desired concentrations. NuSerum IV (Collaborative Biomedical Research, Bedford, MA), a supplemented FCS, was added to the media to achieve a final concentration of 20% by volume. The concentration of insulin in the serum was 25 μg/ml. The final concentration of insulin in our standard 20% serum medium is 0.88 μmol/l. Our previous work has demonstrated that alterations in the medium insulin concentration up to 10 μmol/l do not alter total GLUT-1 protein levels in the mesangial cells (13). Insulin concentration in the medium was not altered in the experiments, except when low serum medium (1%) was required. The final concentration of myo-inositol in the medium was ~60 μM. Cells were fed fresh media every other day unless otherwise noted, and they did not significantly deplete their medium of glucose between feedings (12).

NORTHERN ANALYSES FOR GLUT-1, FN, AND β-TUBULIN

A standard method was employed for Northern analysis as previously described (12, 13). Cells were seeded at a density of 5 × 10⁶ cells/150-mm culture plate and were grown until 90–100% confluent (7 days). On the final day of growth, total RNA was harvested using a commercial preparation of guanidinium and phenol (RNA STAT-60; Tel-Test, Friendswood, TX). Total RNA from each sample was isolated by following the manufacturer’s instructions (Tel-Test). RNA was resuspended in diethyl pyrocarbonate-treated double-distilled water and was stored at −80°C until use. RNA samples (20 μg each) were denatured in glyoxal/DMSO at 55°C for 1 h and then loaded to individual lanes of a 10 mM sodium phosphate-1% agarose gel inside a circulating buffer electrophoresis gel box (Hoeffer Super Sub; Hoeffer Scientific Instruments, San Francisco, CA). Gels were run at 90 volts overnight. Subsequently, they were stained with ethidium bromide, destained, and photographed. Integrity of the RNA was confirmed by inspection of ribosomal RNA bands. Gels were blotted to Genescreen hybridization membranes (NEN, Boston, MA) for 36 h with 10× saline-sodium citrate using a standard method (27a). Blots were then ultravioletly fixed with a Stratalinker (Stratagene, La Jolla, CA), prehybridized with eronomic (IRES) in the construct allows GLUT-1 and neo to be expressed in a single transcript of size 5.6 kb. This 5.6-kb band is far above the native 2.8-kb GLUT-1 band that allows both to be visualized in the same lane on Northern blots.
were measured at 5 min on the linear portion of the 2DOG uptake curve. Uptake rates have a persistent 33% reduction in glucose uptake rate. Uptake rates were measured at 5 min on the linear portion of the 2DOG uptake curve. *P < 0.01 for MCGT1AS vs. MCLacZ; n = 6 experiments in each group.

The specific cDNAs used here were as follows: 1) a 2.4-kb human GLUT-1 cDNA (gift of M. Mueckler, Washington University); 2) a rat β-tubulin cDNA (gift of P. Marsden, University of Toronto); and 3) a 0.5-kb human FN cDNA (ATCC). Each of these cDNAs has been used successfully for Northern analyses in the past, confirming their sensitivity and specificity for the individual messages (12, 13). In the present investigation, we confirmed their ability to detect the respective transcripts in rat mesangial cells. The [32P]cDNA probes were produced by the Random Hexamer Priming method (PRIME-1 kit; Sigma, St. Louis, MO). Northern blots were exposed to Kodak XAR-5 film for periods of 3–14 days, and autoradiograms were analyzed by optical scanning densitometry, followed by analysis with the National Institutes of Health (NIH) Image gel-plotting software (NIH Image, version 1.52; National Technical Information Service, Springfield, VA). Changes in cell transcript levels were compared after normalization to the 2.1-kb mRNA for the β-tubulin housekeeping gene.

Immunoblotting of GLUT-1 and FN

A chemiluminescent immunoblot assay (ECL Western Blot kit; Amersham Life Sciences, Buckinghamham, UK) was used in which luminol was employed to detect GLUT-1 and FN proteins using specific antibodies. The GLUT-1 antibody was obtained from East Acres Biologicals (Southbridge, MA). It was directed against a unique 13-amino acid carboxy terminal sequence of the protein. The FN antibody was obtained from Chemicon International (Temecula, CA). This monoclonal antibody recognizes rat FN protein on immunoblots. SDS-PAGE (10% gels for GLUT-1 and 6% gels for FN) was used to electrophorese 50 µg of total protein/lane by the method of Laemmli (22). Equal lane loading was further confirmed with Ponceau-S staining or antibody to β-tubulin. GLUT-1 was detected in mesangial cells as a protein of ~48 kDa. Total protein was measured for each sample by the assay of Lowry et al. (23), and exactly 50 µg were loaded to each lane of the SDS-PAGE. FN protein was identified by the size of the single detected band at ~210 kDa. GLUT-1 protein was identified by the 48-kDa size of the single band and was confirmed by preadsorption of GLUT-1 antibody with the specific carboxy terminal antigen.

Immunoprecipitation of FN Protein to Measure Changes in its Synthesis by Mesangial Cells

Determination of FN protein synthesis was performed using a modification of the procedure described by Ayo et al. (1). Mesangial cells were first grown to confluence and then were assigned to different treatment groups for specified periods of time (up to 4 days). At the end of the incubations under the different treatment regimens, the medium was aspirated, and the cells were washed three times with Hanks' balanced salt solution without Ca2+ or Mg2+ and incubated in methionine- and cysteine-deficient RPMI 1640 medium containing 50 mCi/ml [35S]methionine and [35S]cysteine (ICN Biomedicals, Irvine, CA) for 2 h. The cell layers were solubilized in electrophoresis sample buffer [2.5% SDS, 0.0625 M Tris (pH 6.8), and 10% glycerol] and then were sheered 20 times through a 21-gauge needle. The samples were then diluted 1:1 with immunoprecipitation buffer (20 mM PBS (pH 7.2), 680 mM sucrose, 2% CA-630, 1 mM EDTA, and 2 mM phenylmethylsulfonyl fluoride) and incubated with 20 µg of rabbit IgG (Sigma) for 1 h at 4°C with gentle

Fig. 2. 2-Deoxy-[3H]glucose (2DOG) uptake rates in MCLacZ control cells and in MCGT1AS antisense GLUT-1 cells. The MCGT1AS cells have a persistent 33% reduction in glucose uptake rate. Uptake rates were measured at 5 min on the linear portion of the 2DOG uptake curve. *P < 0.01 for MCGT1AS vs. MCLacZ; n = 6 experiments in each group.

Fig. 3. A: Northern blot of native (2.8 kb) and antisense (5.6 kb) GLUT-1 transcripts in MCLacZ control cells and in MCGT1AS antisense GLUT-1 cells. This exposure allows one to visualize the respective transcripts in rat mesangial cells. The [35S]cDNA probes were produced by the Random Hexamer Priming method (PRIME-1 kit; Sigma, St. Louis, MO). Northern blots were exposed to Kodak XAR-5 film for periods of 3–14 days, and autoradiograms were analyzed by optical scanning densitometry, followed by analysis with the National Institutes of Health (NIH) Image gel-plotting software (NIH Image, version 1.52; National Technical Information Service, Springfield, VA). Changes in cell transcript levels were compared after normalization to the 2.1-kb mRNA for the β-tubulin housekeeping gene.

B: Western blot of GLUT-1 protein in MCLacZ control cells (C), MCGT1 GLUT-1-overexpressing cells (GT1), and MCGT1AS antisense GLUT-1 cells (AS). Total protein (50 µg) was loaded to each lane. GLUT-1 protein was suppressed ~50% in the MCGT1AS cells as shown.
shaking. Protein G agarose (20 μl; Santa Cruz Biotechnology) was added, and the solution was incubated for 4 h at 4°C with gentle shaking. The protein G agarose was then pelleted by centrifugation in a microfuge at 14,000 rpm for 2 min. The supernatants were then incubated overnight with 20 mg of a rabbit polyclonal antiserum to human FN (Cappel, West Chester, PA) at 4°C. After incubation with 20 μl of protein G agarose for 4 h at 4°C, the immunoprecipitates were pelleted by spinning at 14,000 rpm for 1 min. The pellets were washed two times with immunoprecipitation buffer and repelleted. The final pellet was dissolved in 35 μl of electrophoresis buffer plus 5 ml loading buffer [50 mM Tris·HCl (pH 6.8), 3% SDS, 50% glycerol, and 5% β-mercaptoethanol] and was heated at 100°C for 5 min. The samples were separated on 6% SDS-PAGE and then were transferred to modified nitrocellulose membranes. The membranes were exposed in a PhosphorImager, and the FN band intensities were quantitated.

p-D-Glucose (20 mM, High Concentration), 3-O-Methylglucose, or Xylitol Treatment of MCLacZ, MCGT1, and MCGT1AS Cells

MCLacZ, MCGT1, and MCGT1AS cells were chronically adapted to standard 8 mM glucose RPMI 1640 medium with 20% NuSerum plus penicillin/streptomycin. For the majority of experiments described here, the cells were grown in this 8 mM glucose medium. However, some experiments required the use of high extracellular glucose in the diabetic range (e.g., 20 mM glucose = 360 mg/dl), and for these experiments the glucose concentration in the medium was changed from 8 to 20 mM high glucose for periods up to 14 days. We have previously grown mesangial cells in 20 mM glucose media with every-other-day replenishment of the media and regular passaging for periods of 3 mo and longer without apparent loss of viability (13). This was done without excessive depletion of medium glucose concentrations in either 8 or 20 mM glucose-treated groups (12, 13).

Fig. 4. Growth curves are shown for MCLacZ control cells, MCGT1 GLUT-1-overexpressing cells, and MCGT1AS GLUT-1-underexpressing cells. MCGT1AS cells grew slower than either the control cells or the GLUT-1-overexpressing cells. *P < 0.05 for MCGT1 vs. MCLacZ at days 7–15. **P < 0.02 for MCGT1AS vs. MCLacZ at day 9.

Experiments with 3-O-methylglucose. The majority (87.5%) of the standard culture medium 8 mM D-glucose was replaced by nonmetabolizable 3-O-methylglucose (3OMG), and cells were exposed to 3OMG for the same number of days as was used in 20 mM glucose experiments, before GLUT-1-chloramphenicol acetyltransferase (CAT) assay analysis. Replacement of D-glucose was achieved by preparing media with 1 mM D-glucose plus 7 mM 3OMG.

Experiments with xylitol. Culture media was prepared with the standard 8 mM D-glucose plus 12 mM xylitol, and its effect on mesangial cell GLUT-1 transcription was compared with the effects of 8 and 20 mM D-glucose media. Cells were exposed to xylitol for the same duration as was

Fig. 5. Map of the pGT1–1.3CAT reporter construct carrying the mouse GLUT-1 promoter (black) in a 1.3-kb 5′-flank sequence. This construct was transfected into mesangial cells to investigate the GLUT-1 promoter response to altered glucose concentration and GLUT-1 expression.

Fig. 6. CAT assay for GLUT-1 transcription (A) in MCLacZ control cells and in MCGT1 GLUT-1-overexpressing cells. Lanes 1, 3, and 5 are from MCLacZ cells transfected with the pGT1–1.3CAT reporter construct. Lanes 2, 4, and 6 are from MCGT1 GLUT-1-overexpressing cells transfected with this same construct. Data from experiments with MCLacZ cells in standard 8 mM glucose, MCLacZ cells treated with 20 mM high glucose, and MCGT1 cells in the standard 8 mM glucose medium are summarized (B). *P < 0.01 for 20 mM glucose MCLacZ vs. 8 mM glucose MCLacZ. **P < 0.02 for 8 mM glucose MCGT1 vs. 20 mM glucose MCLacZ.
used for 20mM glucose exposure, before GLUT-1-CAT assay analysis.

**Transient Transfection of Three Different Transduced Rat Mesangial Cell Lines for CAT Assay Detection of GLUT-1 Promoter Activation**

The Lipofectamine reagent (Bethesda Research Laboratories) was used for transfection of cultured, transduced rat mesangial cells with a GLUT-1-CAT reporter construct containing a 1.3-kb fragment (promoter) of the murine GLUT-1 gene. The cloned, transduced mesangial cell lines undergoing transient transfection for the CAT assay were as follows: MCLacZ cells, which express the control reporter gene β-galactosidase; MCGT1 cells, which overexpress GLUT-1 10-fold; and MCGT1AS cells, which underexpress GLUT-1 by 50%. The effects of altered GLUT-1 expression or altered media composition on the cells were determined by CAT assay analysis with the Fast CAT system from Molecular Probes (Eugene, OR). This assay produces a single acetylated product that is detected and semiquantitated by fluorescence at 545 nm (Bio-Image-Analyzer BAS-2000 fluorescence detector). Cells ($7 \times 10^5$) were seeded to 90-mm-diameter culture dishes 48 h before transfection. Transfections were carried out by lipofection (Lipofectamine reagent; Bethesda Research Laboratories; see Ref. 32). One microgram of either the GLUT-1-CAT gene recombinant plasmid DNA or pCAT3 control DNA plasmid (GIBCO-BRL) was diluted in 100 μl of water, and 80 μg of Lipofectamine reagent were also diluted to 100 μl in water. Next, 100 μl of DNA sample and 100 μl of the Lipofectamine reagent dilution were combined in 15-ml polystyrene tubes, mixed, and left to stand at room temperature for 15 min. In addition, 6.5 ml of serum-free 8 mM glucose RPMI 1640 medium were added, and the preparation was mixed. Three milliliters of the medium containing the DNA-Lipofectamine complex were added to two dishes of mesangial cells previously washed with serum-free 8 mM glucose RPMI 1640 medium. The cells were incubated for 7 h, and then 1 ml of medium supplemented with 2% NuSerum was added. This preparation was incubated for 10 h, and the old medium was replaced with new medium containing 0.5% NuSerum. Incubation was then allowed to proceed for 38 h. Cells were scraped off, and the products were isolated. Semi-quantitation of the single band acetylated substrate on the TLC gels was performed using a Bio-Image Analyzer BAS-2000 fluorescence detector. CAT activity was normalized for transfection efficiency, dividing GLUT-1-CAT activity by the pCAT3 control CAT activity from parallel wells of identically treated cells.

**Statistical Analyses**

The Student's t-test was used for analysis of differences between two groups. ANOVA was employed to determine F-statistics in experiments that involved more than two groups. The Bonferroni t-test correction was also used in circumstances where multiple comparisons were made. Means ± SE were calculated for individual groups in each experiment. P values of < 0.05 were considered significant for group comparisons.

**Fig. 7.** CAT assay for GLUT-1 transcription (A) in MCLacZ control cells with 8 mM glucose medium, MCLacZ cells with 20 mM high glucose medium, and MCGT1AS (antisense GLUT-1) cells with 20 mM high glucose medium. Glucose (20 mM) exposures were of 4 days’ duration. Glucose (20 mM) stimulated GLUT-1 transcription in the MCLacZ control cells, whereas 20 mM glucose was unable to stimulate GLUT-1 transcription when GLUT-1 expression was suppressed by antisense GLUT-1 (i.e., MCGT1AS cells). Data from experiments are summarized in B. *P < 0.05 for 20 vs. 8 mM glucose in MCLacZ cells. **P < 0.02 for 20 mM glucose MCGT1AS cells vs. 8 mM glucose MCLacZ cells.

**Fig. 8.** CAT activity for GLUT-1 transcription in MCLacZ control cells. In some experiments (left), 87% of 8 mM d-glucose media was replaced with the nonmetabolizable 3-O-methylglucose (3OMG). In other experiments, 12 mM xylitol, a hexose monophosphate (HMP) shunt substrate, was added to 8 mM glucose medium, as opposed to treating with 20 mM glucose. The 3OMG and xylitol exposures were of 4 days duration.
RESULTS

Development of a Clone of Antisense GLUT-1-Transduced Mesangial Cells (MCGT1AS Cells)

The pWZLneoGT1(AS) antisense GLUT-1 expression construct (Fig. 1) was assembled and packaged into retroviruses as described in MATERIALS AND METHODS. These retroviruses were then used to transduce cultured rat mesangial cells (MCs) (16KC2 line) and express proviral RNA of ~5.6 kb containing the antisense GLUT-1 and neo sequences. We then isolated multiple G418-resistant mesangial cell clones that, by definition, also expressed the antisense GLUT-1 RNA. Next, 2DOG-uptake rates were performed on individual cell clones to identify a clone we labeled MCGT1AS, exhibiting a stable 33% reduction in the 2DOG uptake rate (Fig. 2). This clone had an approximately 50% reduction in native GLUT-1 mRNA and protein levels, as determined by Northern analysis and Western analysis, respectively (Fig. 3). It also grew slower than the MCGT1 and MCLacZ cells (Fig. 4). This clone maintained its stable phenotype even after many months in culture.

GLUT-1-CAT Assays in Mesangial Cell Clones

The effect of GLUT-1 overexpression on GLUT-1 gene transcription was assessed in mesangial cells by CAT assay analysis. MCLacZ and MCGT1 cells were grown in their standard 8 mM glucose RPMI 1640 medium with 20% NuSerum for 3 days and then were switched to 8 or 20 mM glucose medium for another 4 days. At that time, the cells were trypsinized and transferred to 35-mm wells of six-well culture plates for the CAT assay. The GLUT-1-CAT construct con...

Fig. 9. A: fibronectin (FN) mRNA in MCLacZ control cells and in MCGT1AS antisense GLUT-1 cells in response to 20 mM high-glucose exposure for 3 days and 2 wk. The FN mRNA level increased 47% in MCLacZ cells at 2 wk of high glucose treatment, whereas it did not increase in the MCGT1AS cells. B: FN mRNA levels in MCLacZ and MCGT1AS cells in response to 2-wk treatment with 8 or 20 mM glucose. Glucose (20 mM) stimulation of FN mRNA was inhibited in the MCGT1AS cells. O.D., optical density. *P < 0.05 for 20 vs. 8 mM glucose in MCLacZ cells. **P < 0.01 for 20 mM glucose in MCGT1AS vs. 20 mM glucose in MCLacZ cells.

(Data normalized to cyclophilin transcript)
sisted of the murine GLUT-1 promoter (1.3 kb) connected to the bacterial CAT gene in the low background pSVOOCAT vector (27; Fig. 5). This GLUT-1-CAT construct was then transfected into mesangial cells with Lipofectamine, as described in MATERIALS AND METHODS. We found that GLUT-1-CAT activity was detectable in MCLacZ control cells at a relatively low level and that GLUT-1-CAT activity was 280% higher in MCGT1 cells \((P < 0.001; \text{Fig. 6})\), indicating a much higher transcriptional rate in the latter cells. When MCLacZ control cells were switched from 8 to 20 mM high-glucose medium for 96 h, we observed a 90% increase in GLUT-1-CAT activity in the 20 mM glucose-treated cells \((P < 0.05; \text{Fig. 6})\), consistent with the response observed in MCGT1 cells, although of lesser magnitude. In contrast, GLUT-1-CAT activity in MCGT1AS cells treated with 20 mM high-glucose medium for the same duration was not increased above GLUT-1-CAT activity in 8 mM glucose-treated MCLacZ control cells (Fig. 7), i.e., the response was completely blocked by antisense GLUT-1.

The near-total (87%) replacement of 8 mM D-glucose in the culture medium of MCLacZ control cells with nonmetabolizable 3OMG led to an 80% reduction in baseline GLUT-1-CAT activity (Fig. 8). This result suggested that metabolism of D-glucose was required to maintain baseline GLUT-1 transcription. However, GLUT-1-CAT activity was maintained in MCGT1 cells by a similar replacement of D-glucose with 3OMG, presumably due to their persistent fivefold higher glucose uptake rate. When the MCLacZ control cells were treated with 8 mM glucose, 20 mM glucose, or 8 mM glucose plus 12 mM xylitol [precursor to xylulose 5-phosphate via the hexose monophosphate (HMP) shunt], there was no stimulation of GLUT-1-CAT activity by the supplemental xylitol \([P < 0.1 \text{ (not significant); Fig. 8}]\). This suggested that metabolism of D-glucose via the HMP shunt was not a likely pathway for 20 mM D-glucose stimulation of GLUT-1-CAT activity in the mesangial cells and, in addition, that hyperosmolality was not a stimulus for increased GLUT-1 transcription.

**FN mRNA Levels, Protein Levels, and Protein Synthesis in Mesangial Cells With Altered GLUT-1 Expression**

We previously reported that the FN mRNA level is increased in MCGT1 cells vs. MCLacZ cells (12). Here, the potential for D-glucose to stimulate FN mRNA...
levels in MCLacZ control cells and MCGT1AS cells was assessed in response to 3 and 14 days of 8 vs. 20 mM D-glucose exposure (Fig. 9). By 2 wk of 20 mM glucose treatment, the FN mRNA level in MCLacZ cells was 47% higher than it was at 3 days ($P < 0.05$). In contrast, the FN mRNA level in MCGT1AS cells did not increase. We next investigated the response of FN protein levels to 20 mM high glucose in MCLacZ, MCGT1, and MCGT1AS cells. Cells were seeded and grown for 3 days in 8 mM glucose medium and then were switched to 8 or 20 mM glucose medium for 14 more days. Total protein was then harvested from each sample. FN protein levels were assessed on immunoblots by optical scanning densitometry. FN protein increased 100% in MCLacZ cells to a level significantly higher than that of MCGT1AS cells after exposure to 20 mM high glucose ($P < 0.0001$; Fig. 10). In fact, the FN protein level in MCGT1AS cells was not significantly increased by 20 mM high glucose exposure ($P > 0.7$). The FN protein level in MCGT1 cells in 8 mM glucose was 161% higher than the FN level in MCLacZ cells in 8 mM glucose ($P < 0.004$). In addition, when MCGT1 cells were exposed to 20 mM high glucose, the FN protein level was 285% higher than in similarly treated MCGT1AS cells ($P < 0.0001$).

We subsequently investigated the potential for GLUT-1 expression to regulate FN protein synthesis in MCLacZ, MCGT1, and MCGT1AS cells. FN synthesis was analyzed by immunoprecipitation of the $^{35}$S-labeled protein and quantitation of the radiolabel as described in MATERIALS AND METHODS. All three cell lines were grown in 8 mM glucose medium. We found that FN synthesis was elevated 48% in MCGT1 cells ($P < 0.025$) and reduced 44% in MCGT1AS cells ($P < 0.01$, Fig. 11). Therefore, FN synthesis was tightly regulated by GLUT-1 expression in mesangial cells, analogous to the GLUT-1 regulation of GLUT-1 transcription, and not by extracellular glucose concentration per se or by osmolality.

DISCUSSION

We have previously demonstrated that D-glucose is a stimulus for GLUT-1 expression in rat mesangial cells (13). Here we investigated the roles of GLUT-1 and of high extracellular glucose exposure in regulating GLUT-1 gene transcription, by CAT assay analysis. We found that both overexpression of GLUT-1 and exposure to high extracellular glucose in the diabetic range led to stimulation of GLUT-1 transcription in mesangial cells. Overexpression of GLUT-1 in mesangial cells grown in our normal glucose medium (8 mM) was more potent in stimulating the GLUT-1 promoter than was 20 mM high glucose exposure of MCLacZ control cells. Furthermore, we found that, by suppressing GLUT-1 with an antisense method (i.e., MCGT1AS cell line), we could protect the mesangial cells from 20 mM high glucose induction of GLUT-1 transcription, which would otherwise lead to enhanced glucose uptake and metabolism, with potential adverse consequences such as excess ECM production (12, 13).

The baseline level of GLUT-1 transcription was impaired by partial replacement of culture medium D-glucose with nonmetabolizable 3OMG. This suggested that glucose uptake and metabolism were important to the maintenance of GLUT-1 gene transcription in the control MCLacZ cells grown in their normal 8 mM glucose medium. In contrast, the baseline GLUT-1-CAT activity in the MCGT1 cells, which overexpress GLUT-1 protein 10-fold and which exhibit a 5-fold higher glucose uptake rate than MCLacZ control cells, was not suppressed by the partial (87%) replacement of culture medium D-glucose with 3OMG. Therefore, overexpression of GLUT-1 allowed the mesangial cells to maintain GLUT-1 gene transcription in the face of a low extracellular D-glucose concentration. This is consistent with our previous report of MCGT1 cells, which demonstrated that they develop the diabetic mesangial cell phenotype in the absence of high extracellular glucose (12).

The mechanism by which diabetes leads to glomerulosclerosis and renal failure in approximately one-third of the patients is the subject of intense investi-
gation. We recently described a new mechanism by which elevated glucose concentrations in the diabetic range could stimulate mesangial cell ECM synthesis, i.e., by glucose-induced expression of the GLUT-1 facilitative glucose transporter in these cells (12, 13). We also demonstrated that isolated overexpression of GLUT-1 in mesangial cells to mimic the effect of high glucose reproduces the diabetic mesangial cell phenotype in the absence of high extracellular glucose exposure. In the current study, we described a mechanism by which d-glucose stimulates expression of its own transporter (i.e., increased transcription). We demonstrated that either enhanced glucose transport alone or exposure to high glucose stimulated GLUT-1 transcription via the promoter, suggesting the presence of glucose response elements (17) in the promoter. Suppression of glucose uptake by antisense GLUT-1 treatment was effective in blocking high d-glucose induction of GLUT-1 transcription, indicating glucose uptake was important for the response. Treatment of mesangial cells with xylitol in place of high extracellular d-glucose did not stimulate GLUT-1 transcription, suggesting glucose metabolism via the HMP shunt was not the pathway by which glucose mediated its effect. Antisense GLUT-1 protection of mesangial cells from the adverse effects of d-glucose was also demonstrated by prevention of 20 mM high glucose stimulation of FN mRNA and protein levels in the MCGT1AS cells. In addition, we found FN synthesis to be suppressed in these cells. The expression of other ECM genes in MCGT1AS cells, such as collagens I and IV and laminin, may be examined in future studies. In summary, we have demonstrated that d-glucose and GLUT-1 expression regulate GLUT-1 gene transcription in a positive feedback mechanism. In addition, antisense GLUT-1 treatment prevented high glucose stimulation of GLUT-1 transcription and FN expression, indicating a protective effect. In future studies we may examine potential mechanisms by which d-glucose mediates GLUT-1 transcription, including an investigation for transcription factors and examination of the GLUT-1 gene for potential glucose response elements.

Experiments for these studies were supported by National Institutes of Diabetes and Digestive and Kidney Diseases Grants 1-K08-DK-01953 (C. W. Heilig), 1 RO1 DK-54507–01 (C. W. Heilig), and Physician Scientist Award K11-DK-02193 (D. Henry); the American Diabetes Association (C. W. Heilig); the National Kidney Foundation of Upstate New York (C. W. Heilig); Juvenile Diabetes Foundation Grants 1–1998–174 (F. C. Brosius) and 195044 (D. Henry); the National Kidney Foundation of Michigan (F. C. Brosius); and a Veterans Affairs (VA) Merit Review Award (J. I. Kreisberg) and a VA Career Scientist Award (J. I. Kreisberg) from the South Texas Veterans Health Care Division, Research Division, Audie Murphy VA Hospital.

REFERENCES


