Original Article

Glucose Transporters in Human Renal Proximal Tubular Cells Isolated From the Urine of Patients With Non–Insulin-Dependent Diabetes

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The bulk of glucose that is filtered by the renal glomerulus is reabsorbed by the glucose transporters of the proximal convoluted tubular epithelium. However, it has been difficult to investigate this in diseases such as type 2 diabetes because of the inability to isolate primary renal cells from patients without a renal biopsy. We report here a method for the immunomagnetic isolation and novel primary culture of human exfoliated proximal tubular epithelial cells (HEPTECs) from fresh urine. The primary isolates are highly enriched and differentiated and express characteristic proximal tubular phenotypic markers. They continue to express the proximal tubular markers CD13/aminopeptidase-N, sodium glucose cotransporter (SGLT) 2, and alkaline phosphatase through up to six subsequent subcultures in a similar way to human proximal cells isolated from renal biopsies. In a hyperglycemic environment, HEPTECs isolated from patients with type 2 diabetes expressed significantly more SGLT2 and the facilitative glucose transporter GLUT2 than cells from healthy individuals. We also demonstrated a markedly increased renal glucose uptake in HEPTECs isolated from patients with type 2 diabetes compared with healthy control subjects. Our findings indicate for the first time in a human cellular model that increased renal glucose transporter expression and activity is associated with type 2 diabetes. *Diabetes* 54:3427–3434, 2005

Diabetes is a leading cause of end-stage renal disease (1). Hyperglycemia increases the filtered load of glucose at the glomerulus, and glomerular hyperfiltration itself is also associated with diabetes (2). The progression of renal dysfunction in type 2 diabetes heavily involves injury of the tubular epithelium as well as the glomerulus (3), and it is thought that increased glucose fluxes through epithelial pathways result in the increased expression and activity of aldose reductase, protein kinase C, and transforming growth factor-β, which have all been implicated in causing diabetic nephropathy (3–4). The bulk of filtered glucose is ~90% reabsorbed by the low-affinity/high-capacity sodium glucose cotransporter (SGLT) 2 located in the S1 segments of proximal tubule (5). Residual glucose is then absorbed by the high-affinity/low-capacity SGLT1 in the S3 segment. Transcellular glucose transport is facilitated by two basolateral membrane glucose transporters: the low-affinity GLUT2 in the S1 segment and the high-affinity GLUT1 in the S3 segment (5). A study in humans with type 1 diabetes showed a significant increase in the renal transport maximum of glucose (6), implying that the total capacity of tubular glucose carriers may increase in this condition. Changes in these transporters may therefore contribute to the extent of tubular injury. Easily accessible cultures of human proximal tubular cells from diabetic patients would greatly facilitate the investigation of these effects.

Over the past 2 decades, animal models have contributed enormously to our understanding of the role of epithelial glucose transporters in the renal dysfunction of diabetes (7–10). SGLT2 is increased in the alloxan-induced diabetic rat (11), and several groups have shown that renal proximal tubular GLUT2 is increased in streptozotocin-induced diabetic rats (12–13). However, although the expression of intestinal SGLT1 and GLUT2 is increased in patients with type 2 diabetes (14), little is known of the renal expression of these glucose carriers in human diabetes. Nevertheless, SGLT inhibitors are being developed as potential antidiabetic agents (15–16) and might offer the prospect of a reno-protective effect by specifically mitigating transcellular epithelial glucose fluxes (17), preventing at least some of the cellular mechanisms that lead to diabetic renal complications (18).

So far, it has been difficult to study glucose transporters in kidney cells from diabetic patients. Primary cultures of human tubular cells can be prepared from renal biopsies by immuno-separation or microdissection (19–20), but renal biopsy is invasive, time consuming, and generates low yields of cells for culture because the biopsy must be used primarily for diagnostic purposes. In contrast, culture of specific renal tubular cells from human urine could offer a noninvasive and novel tool for renal research in humans. However, only 2,000–7,000 renal cells of various types are normally exfoliated each day (21), and this has meant that

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AMG, methyl-α-D-[U^{14}C]-glucopyranoside; ENaCα, epithelial sodium amiloride–sensitive channel-α; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HEPTEC, human exfoliated proximal tubular epithelial cell; PBST, PBS Tween; PPAR, peroxisome proliferator–activated receptor; SGLT, sodium glucose cotransporter; TRITC, tetramethylrhodamine isothiocyanate.

only few groups have successfully cultured mixed renal tubular cells from urine (22–24). To our knowledge, no one has reported the successful isolation and subculture of enriched populations of proximal tubular cells from the urine of healthy and type 2 diabetic subjects to date. Consequently, there is a need for a human cellular model to investigate the renal glucose transport physiopathology associated with diabetes. Here, we report for the first time a method to isolate and subculture highly differentiated and characterized proximal tubular epithelial cells from human urine. This technique, with its variations, is a significant new tool for understanding human renal pathophsiology, and we apply it to investigate the expression and activity of renal glucose transporters in proximal tubular epithelial cells from patients with type 2 diabetes compared with healthy volunteers.

RESEARCH DESIGN AND METHODS

Reagents and equipments. PBS (pH = 7), DMEM-Ham's F12 (DM-F12), fetal bovine serum (FBS), trypsin, superscript first-strand synthesis system, SDS-polyacrylamide gels, and nitrocellulose membranes were obtained from Invitrogen (Paisley, U.K.). Biocoat collagen-I culture dishes were obtained from Becton Dickinson (Oxford, U.K.). Primary human renal proximal tubule epithelial cells, SingleQuot kit, and renal epithelial growth media basal media were purchased from Cambrex Bio Science (Wokingham, U.K.). The CELLection Pan Mouse IgG Kit, CELLection Dynabeads kit, and magnetic particle concentrator (Dynal MPC) were obtained from Dynal Biotech (Bromborough, U.K.). RNeasy Mini kits were from Qiagen (Crawley, U.K.). Moviol was obtained from Calbiochem (Nottingham, U.K.).

All real-time PCRs used the TaqMan 7900HT. MicroAmp Optical 96-well reaction plate, master mix, primers, probes, and assays-on-demand gene expression products (TaqMan MGB probes, FAM dye-labeled) were purchased from Applied Biosystems (Warrington, U.K.).

The following antibodies were used: mouse anti-human CD13/aminopeptidase-N (clone B-F10) (Neomarkers; Lab Vision, Newmarket Suffolk, U.K.), epithelial sodium amiloride–sensitive channel-α (ENaCα) antibody (Calbiochem), anti-human aquaporin-2 antibody (Alomone Labs, Jerusalem, Israel), anti-human peroxisome proliferator–activated receptor (PPAR)γ, anti-human GLUT1 and GLUT2 antibodies (Santa Cruz, CA), anti-human cytokeratin-7 antibody (InnoGenex, San Ramon, CA), anti-human SGLT2 antibody (Alpha Diagnostic, San Antonio, TX), anti-human uroplakin III antibody (Research Diagnostic, Flanders, NJ), human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody and horseradish peroxidase–conjugated secondary antibodies (Abcam, Cambridge, U.K.), anti-human CD90 fibroblast antigen (Ab-1) monoclonal antibody (Oncogene, Boston, MA), anti-human Tamm-Horsfall glycoprotein (Chemicon International, Hampshire, U.K.), anti-human alkaline phosphatase antibody (gift from Dr. J.T. Deng), and fluorescein isothiocyanate (FITC)- or tetramethylrhodamine isothiocyanate (TRITC)-conjugated anti-mouse secondary antibodies (Jackson Immuno-Research Laboratories, Westgrove, PA). Supersignal west femto/dura extended duration substrate was from Perbio Science (Cramlington, U.K.) Calf skin type 1 collagen, TRITC-phalloidin, and sodium hydroxide were from Sigma (Poole, U.K.). Methyl-α-D-[U-^{14}C]-glucopyranoside was from Amersham Life Sciences (Buckinghamshire, U.K.), and paraformaldehyde was from VWR (Leicester, U.K.).

Collection of urine specimens. The study protocol was approved by the Cambridge Local Research Ethics Committee of Addenbrooke's National Health Service Trust, and informed written consent was obtained from all participants. Urine specimens (~100 ml) from 50 male healthy and 50 type 2 diabetic volunteers were collected and prepared for culture within an hour. The type 2 diabetic subjects were on metformin and ACE inhibitor medications, with fasting blood glucose <126 mg/dl (7.0 mml/l) and albumin/creatinin <30 mg/ml.

Tissue culture

Human exfoliated primary cells. The samples were initially centrifuged at 4,000 rpm for 20 min at 4°C, and the cells were washed with iced PBS. The cells were cultured on Biocoat collagen-I culture dishes (Becton Dickinson) in a 5% CO_2 humidified atmosphere at 37°C and in DMEM-Ham's F12 (DM-F12) supplemented with a SingleQuot kit (Cambrex) and 10% FBS. Confluent cells were designated as passage 0 and subcultured using trypsin (0.25% in 1 mmol/l EDTA).

Selective subculture of human exfoliated proximal tubular epithelial cells: immunomagnetic cell separation. CELLection Pan Mouse IgG Kit, CELLection Dynabeads kit, and the magnetic particle concentrator (Dynal MPC) were used to isolate the tubular proximal epithelial cells from the heterogeneous cell population following the manufacturer's instructions. The precoated Dynabeads Pan Mousse IgG were coated with a mouse anti-human CD13/aminopeptidase-N antibody directed against the external epitope of the proximal tubular cells (25). The human exfoliated primary cells between passage 3 or 4 were incubated with Dynabeads-CD13 antibody, isolated by the magnetic particle concentrator as previously described by Baer et al. (20). The human exfoliated proximal tubular epithelial cells (HEPTECs) were seeded into collagen-I culture surfaces in a 0.5% FBS media (DMEM-Ham's-F12/Singlequot kit).

Primary human renal biopsy proximal tubule epithelial cells. Commercially available renal biopsy proximal tubule epithelial cells prepared from human kidney biopsies were used as a comparator for the HEPTECs. Renal proximal tubule epithelial cells were grown in a renal epithelial growth media/Singlequot medium as recommended by the manufacturer (Cambrex).

Quantitative real-time PCR (TaqMan) for mRNA levels. A comparative study (healthy [aged 38 ± 13 years] versus type 2 diabetic [aged 48 ± 3 years] subjects) to assess the gene level of expression of the apical (SGLT2) and basolateral (GLUT1/2) glucose transporters was performed. Total RNA was extracted from the human primary exfoliated cells (passages 1–3) using the RNeasy Mini kit as per manufacturer's instructions (Qiagen). First-strand cDNA was synthesized from the total RNA (1 μg/sample) using the Superscript First-Strand Synthesis System, as per manufacturer's instructions. The mRNA expression was quantified by real-time PCR using a TaqMan 7900HT (Applied Biosystems). Reactions were performed in a MicroAmp Optical 96-well reaction plate. Each reaction contained 1 × master mix, 200 nmol/l forward and reverse primers, and 100 nmol/l probes in a total volume of 50 μl. PCR conditions were 50°C for 2 min, then 95°C for 10 min, and finally 40 cycles of 95°C for 15 s and 60°C for 1 min.

The following assays-on-demand gene expression products (TaqMan MGB probes, FAM dye labeled) were used as per manufacturer's instructions:

- PPARα (gene PPARA accession no. L02932), PPARγ (gene PPARG accession no. P37231), and PPARδ (gene PPARD accession no. L07592).
- SGLT2 (gene SLC5A2 accession no. P31639).
- GLUT1 (gene SLC2A1 from GeneBank accession no. NM_006516.1) and GLUT2 (gene SLC2A2 from GeneBank accession no. P11168).

Expression of SGLT2, GLUT1, and GLUT2 was assessed in HEPTECs, at passages 4–5, cultured from healthy and type 2 diabetic volunteers (n = 4 or 5). The results were expressed as fold change relative to the baseline condition, after normalization relative to GAPDH.

The PCR for the following cellular markers (Table 1) were performed with aquaporin-2 (forward: 5′-AAgAAgggATCAgTCgTTgCA-3′, reverse: 5′-gCTgggAgCCggAACAC-3′); ENaCα (forward: 5′-GGCCCTGGCAAGATTGAA −3′, reverse: 5′-GCTTCCCCTCCACACATCA −3′), ENaCβ (forward: 5′-CTAAATTCCCATCggTAggCATTA-3′, reverse: 5′-CTAAATTCCCATCggTAggCATTA-3′), ENaCγ (forward: 5′-AgTCggTAgCATCATCC-3′, reverse: 5′-AATCCCACCACTTgCTTggA-3′), NaCl cotransporter (forward: 5′-gATggggTTTCACCATgTTg-3′, reverse: 5′-AACATACT ggAggAC-gTggg-3′), and mineralocorticoid receptor (forward: 5′-ggAgACCAAAggCTACCAC-3′, reverse: 5′-ggTggAAgTAgAg-CggCTTg-3′).

Immunoblotting procedure. A comparative study (healthy [aged 38 ± 13 years] versus type 2 diabetic [aged 57 ± 4 years] subjects [n = 4]) to assess the protein level of expression of the apical (SGLT2) and basolateral (GLUT2) glucose transporters was performed. SDS-PAGE was used according to the method of Laemmli (26). The cell lysates were run on a NuPAGE 4–12% Bis-Tris gels and blotted on an XCell II blot module (Invitrogen). Membranes were probed with the following antibodies in PBS Tween (PBST)/5% low-fat milk: anti-human ENaCα antibody, anti-human aquaporin-2 antibody, anti-human PPAR-γ, anti-human GLUT1, anti-human cytokeratin-7 (all 1:500 dilution), anti-human GLUT2 antibody (1:100 dilution), anti-human SGLT2 antibody (1:1,000 dilution), anti-human uroplakin III antibody (1:50 dilution), and anti-human GAPDH antibody (1:1 × 10^6 dilution). The nitrocellulose membranes were washed in PBST and probed for 1 h with horseradish peroxidase–conjugated secondary antibodies (Abcam) at a dilution of 1:10,000 in PBST/5% low-fat milk. The blots were washed in PBST and developed with Supersignal west femto/dura. Images were captured using a Genegenius imaging system (Syngene) and the SGLT2/GLUT2 and GAPDH bands quantitated using Genetools (Syngene). GAPDH was used as an internal loading control, and the results were expressed as fold change relative to baseline condition, after normalization to GAPDH.

Immunofluorescence and confocal imaging. Exfoliated cells (primary isolate or subcultured HEPTECs) were seeded on calf skin type 1 collagen (20 μg/ml)-coated coverslips. The cells were PBS washed and fixed in 4% paraformaldehyde for 20 min, and the nonspecific staining was blocked (45 min, 20°C) by 1% BSA/PBS. All further staining was carried out. Cells were

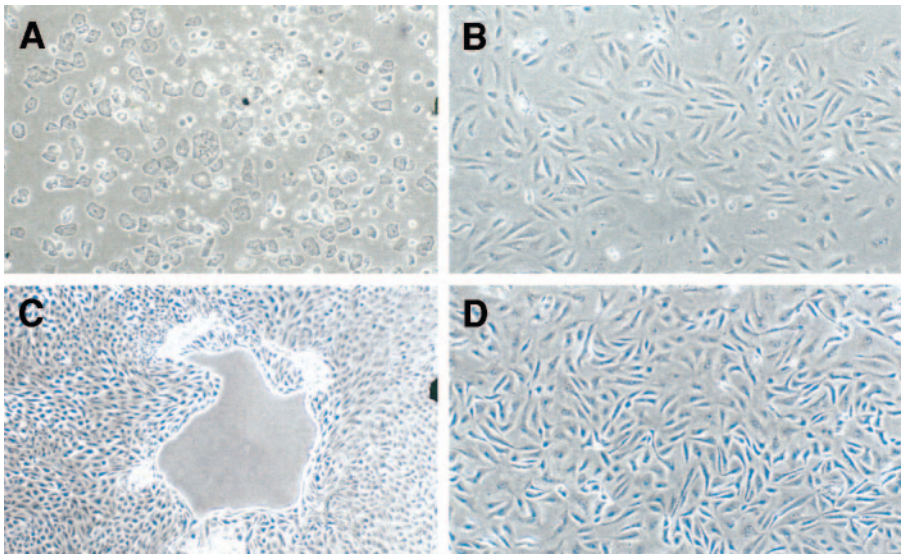

FIG. 1. Phase-contrast light micrograph of human primary cells isolated from fresh urine in primary culture after 24 h in culture (*A*). After, adhering to the collagen after 2–3 days, the cells are adhering to the collagen-I (*B*). Colonies of cells are formed after a week (*C*) and formed a confluent monolayer of cuboidal cells with cobblestone-like appearance. *D*: The cells after passage 2. Original magnification: ×20.

incubated with primary antibodies, in 0.25% BSA/PBS, for extracellular epitopes (1–10 μg/ml) of human CD13/aminopeptidase-N, human CD90, and human Tamm-Horsfall glycoprotein at concentrations between 1 and 10 μg/ml for 1 h at 20°C. After washing, cells were incubated with FITC- or TRITC-conjugated anti-mouse secondary antibody (10 μg/ml, 1 h at 20°C). Cells were washed in PBS/0.25% BSA and refixed in 4% paraformaldehyde (20 min, 20°C), permeabilized (6 min, 0.2% Triton X-100), and then incubated with TRITC-phalloidin (1 μg/ml, 45 min) to stain actin filaments, or anti-human cytokeratin-7. FITC-conjugated anti-rabbit secondary antibody was used to visualize cytokeratin-7 staining. Coverslips were mounted in Moviol. Confocal laser scanning microscopy (Leica TCS SP2 mounted over a DM-RXA microscope) was conducted. For positive cell counting, five fields on each of two separate coverslips were randomly selected and counted for CD13, CD90, cytokeratin-7, or alkaline phosphatase (27) positively expressing cells. Counts were averaged and converted to percentages (Fig. 4).

Glucose transport studies. A differential transport of the methyl-D-glucoside, a nonmetabolized model substrate, by the three SGLTs transport is sodium dependent (15). α-AMG (methyl-α-D-[$U^{14}C$]-glucopyranoside) was used to assess HEPTEC glucose transport in healthy (aged 31 ± 5 years) and type 2 diabetic (aged 56 ± 7 years) subjects as previously described (28). Briefly, HEPTECs between passages 4 and 5, isolated from healthy and type 2 diabetic subjects (n = 3), were PBS washed (37°C), and radiolabeled α-AMG at 10 μmol/l (2 μCi/ml) was added to the HEPTECs for 30 min at 37°C. Cells were washed three times with iced PBS then solubilized with 1 ml 0.1 N NaOH + 1% SDS and incubated at 37°C for at least 2 h. Scintillation cocktail (Microscint 2; Packard Bioscience) was added to the cell lysate, and the cellular accumulation of radioactive α-AMG was measured by a β-counter (Packard Bioscience).

Statistical analysis. All values are given as means ± SE. Differences between mean values were analyzed by the Student's t test. P values <0.05 were considered significant.

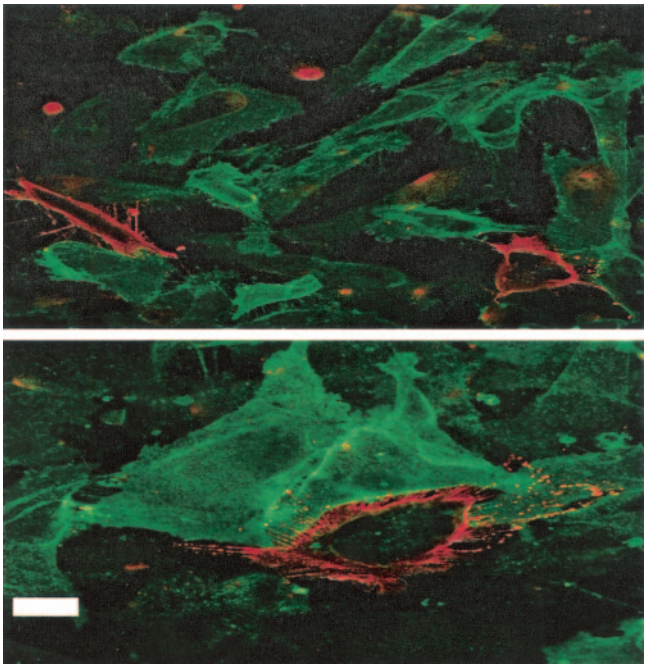

FIG. 2. Confocal microscopy of the human exfoliated primary cells (bar = 10 μm). Proximal cell marker: CD13-aminopeptidase-N (green) and distal and loop of Henle cell marker: Tamm-Horsfall protein (red). These markers indicate the presence of distal and proximal cells in the exfoliated primary cell culture.

RESULTS

Human urinary tract and renal tubular cells in primary culture. We successfully established primary cultures of human urinary urethelial and kidney cells from healthy and type 2 diabetic volunteers (Fig. 1). Typically, we found 20–50 cells per 100 ml of fresh urine. These cells divided within 24 h when seeded into culture (Fig. 1*A*) and readily adhered to the collagen-I–coated plates (Fig. 1*B*). After a week in culture, small islets of cells had formed (Fig. 1*C*), and these cells were trypsinized and subcultured (Fig. 1*D*). Two types of colonies arose: type 1 colonies, with irregular contours, and type 2 colonies, with smooth edges, were both observed as described by Dorrenhaus et al. (24). The cells were characterized by Western blotting/Taqman RT-PCR and confocal microscopy (Table 1) and proved to be a heterogeneous population as also found by Inoue et al. (23), who grew mixed primary cultures of distal and proximal cells from voided urine on glass coverslips. In our hands (Table 1), the cultures included urothelial and distal tubular cells, identified by the marker cytokeratin-7 (29); proximal tubular cells, identified by the selective marker CD13/aminopeptidase-N (25); and CD90-positive fibroblast-like cells (30).

Selective subculture and characterization of HEPTECs. The selectivity of CD13/aminopeptidase-N for proximal tubular epithelium (25) was used to isolate the enriched HEPTECs by immunomagnetic cell separation as described by Baer et al. (20). Prior to separation, the human exfoliated primary cells expressing heterogeneous cell population (Table 1), the proximal and distal tubular cells, were present throughout the passages CD13/aminopeptidase-N and Tamm-Horsfall glycoprotein, respectively

TABLE 1
Expression of relevant cellular markers in human exfoliated primary cells and HEPTECs

Cellular marker	mRNA*	Protein*	Cell type
Cytokeratin-7	ND	+++	High in distal cells and bladder cells and low in proximal cells
Aquaporin-2	ND	++	Collecting duct
ENaCα	+++	+++	Collecting duct and distal cells
ENaCβ/γ	++	ND	Collecting duct and distal cells
PPARγ/α/δ	+++	PPARγ only	Collecting duct and distal cells/proximal cells
NaCl cotransporter	+++	ND	Proximal cells/connecting tubules
Aquaporin-2	ND	+++	Collecting duct
L1-CAM (cell adhesion molecule)	ND	+++	Collecting duct
Aminopeptidase-N (CD13)	ND	+++	Proximal cells
Sodium-glucose transporters (SGLT2, GLUT1, and GLUT2)	+++	+++	Proximal cells
Alkaline phosphatase	ND	+++	Proximal cells
Uroplakin III	ND	+++	Bladder cells
Mineralocortcoid receptor	+++	ND	Connecting tubules/distal cells/collecting duct

Expression of cellular markers of human primary exfoliated cells: *1*) bladder/urethra (uroplakin III/cytokeratin 7), *2*) proximal tubular cells (CD13 aminopepetidase-N/SGLT2, *3*) renal distal tubular/collecting duct cells (aldosterone receptor, cytokeratin-7, aquaporin-2, and ENaCα) by PCR and/or immunoblotting. Marker level of presence (+ + +). *mRNA (Taqman) and proteins were measured and expressed as relative abundance to gapdh. +, weak expression; ++ high expression; +++ very high expression; ND, not been done.

(Fig. 2). Approximately 90% of all cells isolated immunomagnetically expressed CD13 (Fig. 3). The HEPTECs also expressed alkaline phosphatase, another marker of proximal tubular epithelium (27). CD90-expressing cells decreased from 20% of the total prior separation to ~5% in the HEPTECs (Fig. 3*C* and *D*). Furthermore, confocal microscopy confirmed the proximal tubular marker alkaline phosphatase, and CD13 was maintained up to at least passage 6 (Fig. 4*B*), suggesting that HEPTECs remain differentiated on subculturing. These characteristics of HEPTECs were similar to those of the renal tubular proximal epithelial cells, commercially available, grown from human renal biopsies (data not shown). The survival as well as the replication rate of urinary tract/renal tubular cells and the HEPTECs isolated from healthy and type 2 diabetic subjects were similar once established in culture and was mainly dependent on the state/nature of the shedded cells.

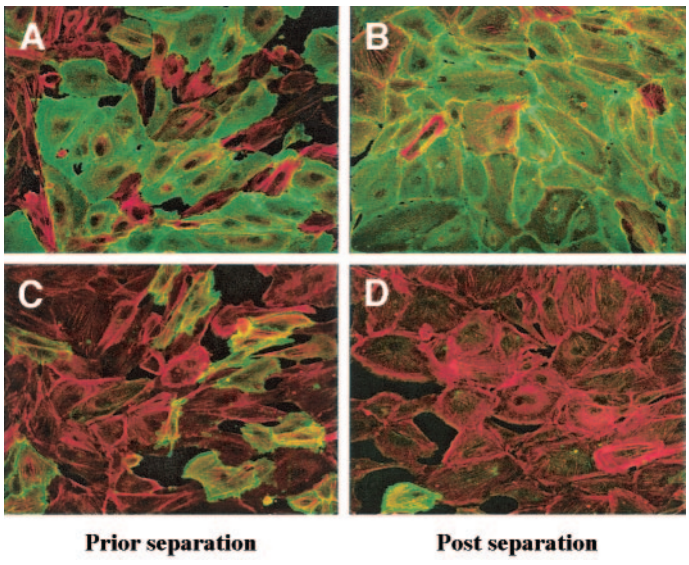

FIG. 3. HEPTEC immunocychemistry characterization prior (*A* and *C*) and post (*B* and *D*) immuno-magnetic cell separation. TRITC-phalloidin (red) staining actin filaments (*A–D*) and CD13-aminopeptidase-N (green) enrichment 50% (*A*) to 90% (*B*). CD90-FITC (green) a fibroblast-like expression decreasing from ~10% (*C*) to <5% post immunomagnetic cell separation (*D*).

Renal glucose transporter expression. At passages 4 and 5, the SGLT2 mRNA level in HEPTECs isolated from type 2 diabetic versus healthy volunteers (Fig. 5*A*) was elevated ($P < 0.05$). Densitometry of Western blots (Fig. 6) confirmed a significantly increased 75-kDa band in HEPTECs from diabetic patients ($P < 0.05$); 75 kDa is the predicted molecular weight of native SGLT2 (15). GLUT2 mRNA levels were also increased in HEPTECs from type 2 diabetic patients ($P < 0.05$) (Fig. 5*B*), and GLUT2 protein, represented by a 55-kDa band (31), was also correspondingly higher ($P < 0.05$) (Fig. 6). Interestingly, GLUT1 mRNA levels were reduced in the diabetic HEPTECs ($P < 0.05$) (Fig. 5*C*), a result analogous to the decreased renal GLUT1 expression in sreptozotocin-induced diabetic rats (9–10). Our results therefore suggest that there are differences in the expression of specific renal glucose transporters in HEPTECs isolated from type 2 diabetic patients compared with healthy control subjects and that these differences remain between cells that have been subcultured up to five times under identical conditions.

Glucose transport. To measure glucose uptake by the isolated HEPTECs, the glucose analogue methyl-α-D-[U-^{14}C]-glucopyranoside (AMG) was used. HEPTECs isolated from type 2 diabetic patients showed a significant increase in the glucose uptake, up to threefold, compared with those from healthy volunteers (Fig. 7).

DISCUSSION

We describe here, for the first time, the isolation and culture of well-enriched and characterized exfoliated proximal tubular epithelial cells from healthy and type 2 diabetic subjects. The cultured HEPTECs showed similar characteristics to the renal proximal tubular grown from human biopsies as well as the proximal tubular markers such as the CD13, alkaline phosphatase, and SGLT2 (19–20). Although, distal tubular cells are known to transdifferentiate to a more proximal phenotype (32), HEPTECs showed stable levels of expression of proximal tubular markers from the time of their first passage (Figs. 2–4). We therefore believe it is unlikely that our HEPTECs arose

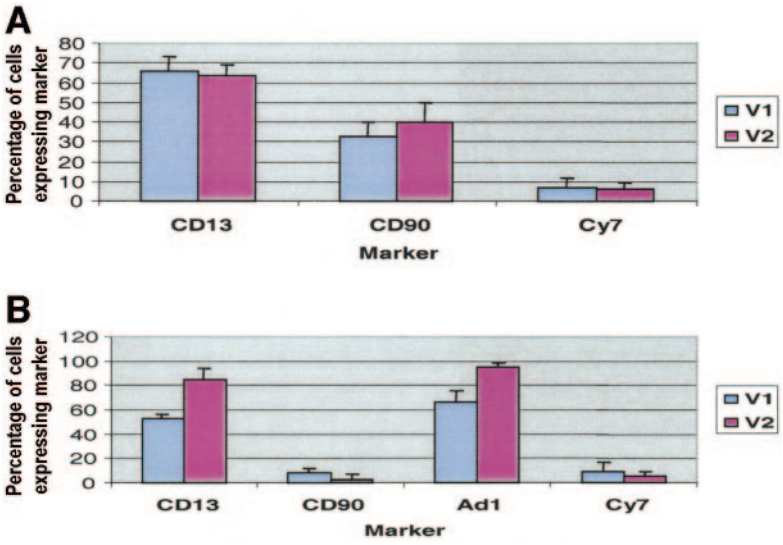

FIG. 4. Typical human exfoliated primary cells of two healthy volunteers (V1 and V2). Positive cell counting by confocal microscopy at passage 4 prior (*A*) to CD13-immunomagnetic cell separation and post separation at passage 6 (*B*). Five fields on each of two separate coverslips were randomly selected and counted. An average of 30.2 cells per field were counted (range from 19 to 54 cells), and the counts were averaged and converted to percentages.

through gradual transdifferentiation from distal tubular cells. This conclusion is supported by the fact that the HEPTEC level of CD13 did not increase from passage 4 to 6 (Fig. 4). By using low-FBS media (33) and applying the immunomagnetic cell sorting technique, the level of fibroblasts in the cultures was kept very low (<5%), even at later passages (Fig. 3). We believe that this ability to isolate and subculture phenotypically stable human exfoliated proximal tubular cells is a significant new tool for understanding of the renal pathophysiology of human diseases such as diabetes.

Proximal tubular cells not only reabsorb filtered glucose but are also thought themselves to play a part in the glucotoxicity associated with diabetes such as high intracellular accumulation, formation of advanced glycation end products (34), and an increased expression of collagen I/IV (35). Fanconi-Bickel syndrome (36), which is associated with a proximal tubulopathy and glycogen accumulation, at least suggests that the transcellular glucose flux within the proximal tubular cells contribute to the development of a diabetic-like nephropathy.

Most mechanistic studies of renal glucose transport in diabetes have been performed in animal models (7–12). The proximal tubular high-capacity and low-affinity SGLT2 plays a key role in renal apical glucose reabsorption (5), and a mutation of the SGLT2 gene is associated with glucosuria (37). We found that, in a hyperglycemic environment, SGLT2 mRNA and protein levels were only increased in the HEPTECs isolated from type 2 diabetic patients compared with cells from normal subjects and most interestingly that this difference was sustained despite subculturing the cells through successive identical passages. An increase in SGLT2 mRNA in vivo has been demonstrated in renal sections of rat alloxan-induced diabetes (11). However, increased levels of SGLT2 protein have not been found in the proximal tubules of rats with streptozotocin-induced diabetes, perhaps because of the lack of suitable anti-SGLT antibodies (12). However, it is also likely that differences in the expression levels of renal glucose transporters, such as SGLT2, between human type 2 diabetes and various animal models depend on different pathogenic milieu and/or the effect of streptozotocin on the animal (38), including genetic predisposition in humans (39). The sustained elevation of SGLT2 in HEPTECs from patients with type 2 diabetes, compared with HEPTEC from healthy control subjects, through successive passages in identical culture media, begs the question of whether this is the part of the genetic milieu of type 2 diabetes or the result of sustained changes acquired in vivo by the patients during their illness.

Basolateral renal glucose transport occurs mainly through GLUT2. Fanconi-Bickel syndrome (36) is caused

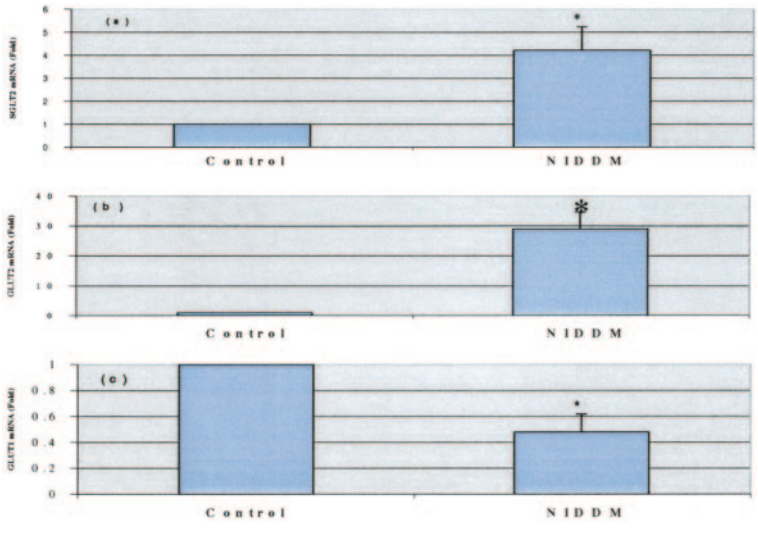

FIG. 5. Glucose transporter mRNA expression in HEPTECs isolated from healthy and type 2 diabetic subjects. *A*: SGLT2 ($n = 4$), *B*: GLUT2 ($n = 4$), and *C*: GLUT1 ($n = 5$). The glucose transporters were analyzed by real-time RT-PCR and normalized to GAPDH. Values are means ± SE (*$P < 0.05$).

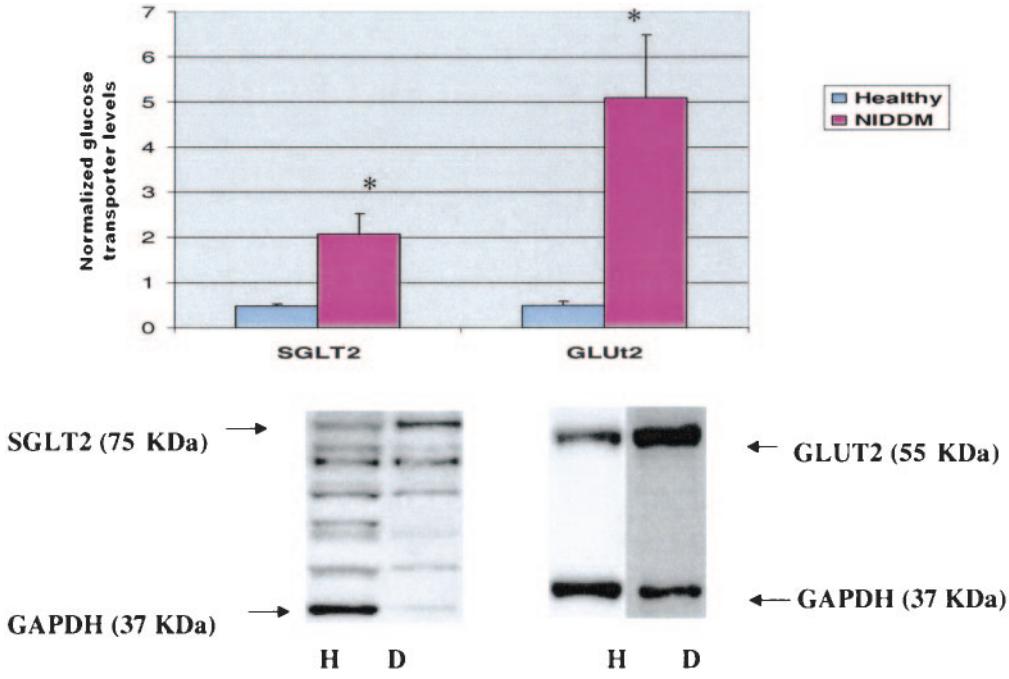

FIG. 6. Immunoblot representing SGLT2 and GLUT2 protein expression in HEPTECs isolated from healthy (H) and type 2 diabetic (D) volunteers' fresh urine (n = 4). The predicted band of 75-kDa molecular weight representing the SGLT2 level of protein expression in HEPTECs isolated from type 2 diabetes increases up to three times compared with healthy volunteers. GLUT2 (55 kDa) level of protein expression in HEPTECs isolated from type 2 diabetes increases up to 10 times compared with healthy volunteers. The level of glucose transporter protein expression is determined from densitometry relative to GAPDH. Values are means ± SE (n = 4; *P < 0.05).

by an impaired function of the GLUT2 protein, which leads to a generalized proximal tubule dysfunction with severe glucosuria. GLUT2 expression is increased in the rat with streptozotocin-induced diabetes (7–10,13), but Marks et al. (12) have reported that this upregulation disappears after an overnight fast, suggesting that proximal tubular GLUT2 is acutely modulated in response to ambient levels of glucose. Here, we show in humans that renal GLUT2 shows sustained elevations in HEPTECs subcultured through multiple passages from patients with type 2 diabetes. Interestingly, intestinal GLUT2 expression is also increased in vivo in type 2 diabetic subjects (14), suggesting the possibility of a more general regulatory modulation of GLUT2 in diabetes. The changes we found in SGLT2 and GLUT2, both carriers for glucose in the S1 segment of the proximal tubule, were specific because we also found GLUT1 mRNA levels to be decreased in HEPTECs from patients with type 2 diabetes. This finding for GLUT1, which is important for basolateral glucose import into the proximal cells (11) in the S3 but not the S1/2 segment of the proximal tubule, is consistent with the studies in chemically induced diabetic rat model (10–11). Moreover, as discussed by Vestri et al. (11), GLUT1 protein level is decreased in hyperglycemic media and increased by glucose deprivation, whereas GLUT2 protein seems to be inversely regulated, which together forms a biological response to compensate for the lack of the intracellular glucose. However, although we used a hyperglycemic cell culture media (17 mmol/l), this cannot be a complete explanation of the passage-resistant disease-related differences in GLUT1 and GLUT2 as we always grew HEPTECs from patients with type 2 diabetes and from healthy control subjects in the same medium. Thus, preliminary data has also indicated that hyperglycemia (passage from 5 to 15 mmol/l glucose media) contributes up to a 20% increase in GLUT2 mRNA level of expression seen in type 2 diabetes (Fig. 5, up to 20 times), while SGLT2 mRNA level of expression was unaffected by hyperglycemia. The characteristics of the renal cells were unchanged as the relative levels of mRNA expression of GLUT2 and SGLT2 were generally stable with the passage number (data not shown).

Hyperglycemia is also known to be associated with an increase in renal glucose uptake through transcellular glucose transporters (3). The increase in the SGLT2/GLUT2 level of expression in the type 2 diabetic HEPTECs

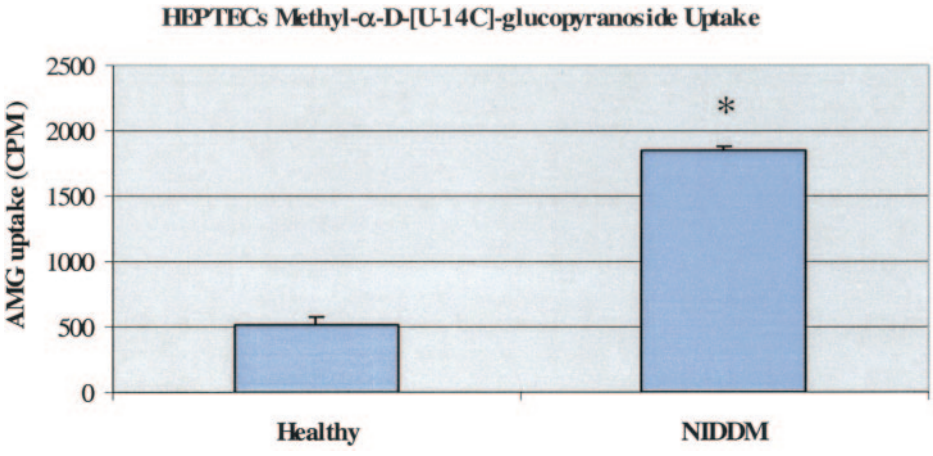

FIG. 7. Glucose uptake assay using the AMG uptake, in triplicates, as a tracer to assess HEPTEC glucose transport in healthy and type 2 diabetic subjects (n = 3) as described by Takamotoa et al. (28). AMG uptake HEPTECs isolated from type 2 diabetes increases up to threefold compared with healthy volunteers. AMG cellular accumulation was presented as means ± SE (n = 3; *P < 0.05)

was also accompanied by a threefold increase in glucose uptake. Members of the SGLT family differ markedly in their affinities to substrates. Hence, neither SGLT2 nor SGLT3 transports D-galactose efficiently, and SGLT1 does not discriminate among α-AMG, glucose, or galactose (15). We found that SGLT1/3 mRNA was not present at detectable levels in our HEPTEC cultures (data not shown). This suggests that the majority of AMG transport in our cells is through SGLT2. The increased glucose uptake, marked by the tracer amounts of α-AMG, we found in the HEPTECs isolated from type 2 diabetic patients is consistent with our finding of upregulated SGLT2 and is supported by a human clinical study demonstrating the increase in renal glucose uptake (40).

Although age may influence the increased renal glucose transporters expression/activity seen in our sex-matched study, a preliminary age-matched study (healthy versus type 2 diabetic [$n = 4$] subjects) of the HEPTECs has shown that renal glucose transporter gene expression is also increased as demonstrated in Fig. 5. A body of evidence has demonstrated that during aging, the renal sodium-dependent glucose transport is decreased (41) or unchanged (42). Therefore, the results obtained here strongly suggest that the increased renal glucose transport is associated with diabetes rather than the aging process. As proximal tubular glucose uptake is insulin independent and hyperglycemia dependent (43), different pathways are thought to augment the development of chronic renal complications associated with the hyperglycemic environment in diabetes, such as advanced glycosylation end products (44), sorbitol accumulation (45), and gluconeogenesis, and the increased flux through the hexoamine pathway (46). Further mechanistic studies are needed to elucidate the basis of the sustained differences in glucose transporters between HEPTECs from patients with type 2 diabetes and healthy control subjects, who our results suggest are resistant to successive passages through identical conditions of subculture.

In conclusion, we have described a method for the selection and culture of HEPTECs from human urine that is a novel tool to *1*) investigate human renal glucose transport regulation in health and disease, *2*) perform mechanistic studies on important and potentially renoprotective new oral antidiabetic agents, such as T-1095, that target SGLTs (16,46), and *3*) access tubular cells to study other renal aspects of diabetes and other diseases.

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