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Metallothionein isoform 3 and proximal tubule vectorial active transport

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Metallothionein isoform 3 and proximal tubule vectorial active transport.

Background. Metallothionein isoform 3 (MT-3) is expressed in the proximal tubule cells of the human kidney. The goal of the present study was to further characterize the basal expression of MT-3 in the proximal tubule and to determine if MT-3 participates in the maintenance of proximal tubule cell function.

Methods. Expression of MT-3 mRNA was determined in the intact proximal tubule using microdissection and reverse transcription-polymerase chain reaction (RT-PCR). Basal expression of MT-3 mRNA and protein was determined in cultured human proximal tubule (HPT) cells and an immortalized proximal tubular cell line, HK-2 cells, using RT-PCR and immunoblotting. The *MT-3* gene was stably transfected into the HK-2 cell line using the pcDNA3.1/Hygro (+) vector.

Results. MT-3 mRNA was detected in the proximal tubule of the in situ kidney with relative expression in excess to that of the β-actin housekeeping gene. The mortal HPT cells were shown to express both MT-3 mRNA and protein and to form domes, while immortal HK-2 cells were shown to have no expression of MT-3 mRNA and protein nor to form domes. The stable transfection of MT-3 in HK-2 restored MT-3 expression and dome formation to the HK-2 cells.

Conclusions. MT-3 mRNA is present in the human proximal tubule, and MT-3 expression is involved in the transport function of a human renal cell line that retains properties of the proximal tubule.

In both mice and humans, there are four classes of very similar metallothionein (MT) proteins designated MT-1 through -4 that are distinguished by small differences in sequence and charge characteristics [1]. All members of the MT gene family are cysteine-rich, low molecular weight (6 kD), intracellular proteins that bind transition metals [1–4]. From the extensive studies on the MT-1 and MT-2 isoforms, the MTs are believed to serve an important role in the homeostasis of essential metals such as Zn^{+2} and Cu^{+2} during growth and development as well as in the detoxification of heavy metals such as Cd^{+2} and Hg^{+2}, rendering the MTs important mediators and attenuators of heavy metal-induced toxicity, particularly hepato- and nephrotoxicity [1–6]. The MT-1 and MT-2 isoforms exhibit a ubiquitous pattern of tissue expression and are highly inducible by a wide spectrum of stimuli [1, 3, 5]. The MT-3 and MT-4 family members have received far less study, owing to their more recent discovery in 1992 and 1994, respectively [7, 8]. The MT-4 isoform was shown to have a very limited tissue distribution and has not been studied further since its initial isolation [8]. The MT-3 isoform has received comparatively more interest due to the fact that it was first identified in a search for a neuronal growth factor whose dysregulation might be involved in the inappropriate formation of neurofibrillary tangles in brains of individuals with Alzheimer's disease [9, 10]. This protein, which was originally called growth inhibitory factor (GIF), was shown to retain all the characteristic features of the traditional MTs and was renamed MT-3 [7]. Based on studies in the mouse, MT-3 was designated as a brain-specific MT with expression limited to cells of neural origin [7]. In addition to a limited tissue distribution, there are other features of MT-3 that are unique compared to the highly characterized MT-1 and MT-2 isoforms. At the level of transcription, MT-3 is not inducible by most of the stimuli that normally increase MT-1 and MT-2 gene transcription in liver and brain [7, 11–14]. There also is evidence that the regulation of tissue-specific MT-3 expression does not involve a repressor and that the metal response elements (MREs) present in the promoter region of the *MT-3* gene are nonfunctional [15]. Structurally, the MT-3 isoform possesses seven additional amino acids that are not present in any other member of the *MT* gene family, a 6 amino acid C-terminal sequence and a Thr in the N-terminal region [7, 9, 10]. Functionally, MT-3 has been shown to possess a neuronal cell growth inhibitory activity that is not duplicated by the other human MT classes

Key words: proximal tubule, MT-3, dome formation, cell culture, growth inhibitory factor, HK-2 cells.

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[9, 16]. This non-duplication of function occurs despite a 63 to 69% homology in amino acid sequence among MT-3 and the other human MT isoforms [17]. There also is evidence to suggest that the interactions with zinc can be different between MT-3 and the other MT isoforms [18, 19].

Our interest in the role of MT-3 in the renal system comes from the unexpected finding that MT-3 mRNA was present in total RNA isolated from human kidney tissue and cell cultures derived from the human proximal tubule [20]. That MT-3 protein was expressed in the in situ human kidney was subsequently confirmed by immunolocalization using an antibody directed against the human MT-3 protein [21]. Using this antibody and immunohistochemistry, it was demonstrated that MT-3 was variably expressed in the epithelial cells comprising the glomerular and tubular elements of the adult and developing human kidney. In the glomerulus, moderate cytoplasmic staining for MT-3 was demonstrated in parietal epithelial cells of Bowman's capsule and in visceral epithelial cells of the glomerular tuft. Proximal convoluted tubule cells exhibited moderate cytoplasmic MT-3 immunoreactivity. Distal tubules showed strong cytoplasmic staining for MT-3, particularly in the medullary rays. In the medulla, MT-3 staining was the most variable, with weak to moderate staining in the medullary collecting ducts and a general absence of staining in the thin loops of Henle and in the transitional epithelium of the renal pelvis. The goal of the present study was to further characterize the basal expression of MT-3 in the proximal tubule and to determine if MT-3 participates in the maintenance of proximal tubule cell function.

METHODS

RNA isolation from proximal tubules microdissected from paraffin sections and RT-PCR determination of MT-3 mRNA expression

The PixCell II™ LCM System (Arcturus Engineering, Mountain View, CA, USA) was used for laser capture. Five-micrometer thick sections were cut from formalin-fixed, paraffin embedded tissue blocks of human kidney obtained from the pathology archives and mounted on plain glass slides. The slides were stained with hematoxylin and eosin (H&E). Total RNA was extracted from samples using the micro RNA isolation kit (Catalog No. 200344; Stratagene, La Jolla, CA, USA) and reverse transcription-polymerase chain reaction (RT-PCR) preformed as described previously [22, 23]. Total RNA (1 μL) was reverse transcribed in a 20 μL reaction mixture using murine leukemia virus (MLV) reverse transcriptase (50 units) in 1 × PCR buffer (50 mmol/L KCl and 10 mmol/L Tris-HCl, pH 8.3), 5 mmol/L $MgCl_2$ solution, 20 units RNase inhibitor, 1 mmol/L each of the deoxynucleoside triphosphate (dNTP) and 2.5 μmol/L random hexanucleotide primers. The samples were reverse transcribed for 20 minutes at 42°C, followed by a five-minute step at 99°C to inactivate the reverse transcriptase using a GeneAmp 9600 thermocycler (Perkin-Elmer-Cetus 9600; Perkin Elmer, Foster City, CA, USA). The resulting cDNA was amplified in a 100 μL reaction mixture containing 2 mmol/L $MgCl_2$, 1 × PCR buffer, 2.5 units of AmpliTaq DNA polymerase and 0.10 μmol/L of the respective primers. The primers for MT-3 and β-actin were developed using the Oligo 5.0 software (National Biosciences Inc., Plymouth, MN, USA). The primers for MT-3 were: upper 5′-CCGTTCACCGCC TCCAG-3′ and lower 5′-CACCAGCCACACTTCAC CACA-3′ (product size, 325 bp) and for β-actin: outer primer upper 5′-ATGGATGATGATATCGCCGCG-3′ and lower 5′-CTCCATGTCGTCCCAGTTGGT-3′ (product size, 249 bp). Controls for each PCR reaction included a no-template control in which 1 μL water was added instead of the RNA and a no-reverse-transcriptase control in which 1 μL of water was added instead of the enzyme. A single step PCR was sufficient to detect MT-3 mRNA.

For the determination of β-actin mRNA, a second round of nested PCR was necessary. Twenty microliters of the product from the first-round of PCR was added to 80 μL of a PCR reaction mixture (2 mmol/L $MgCl_2$, 1 × PCR buffer, 200 μmol/L dNTP, 2.5 units of AmpliTaq DNA polymerase and 0.15 μmol/L of the respective inner nested primers) for a second round of PCR. The inner nested primers for the human β-actin gene were developed using Oligo 5.0 software. The sequences of the inner nested primers along with the product sizes are as follows: upper 5′-CGACAACGGCTCCGGCATGT-3′ and lower 5′-TGCCGTGCTCGATGGGGTACT-3′ (product size, 194 bp). The final PCR products were removed at selected cycles to assure linearity and electrophoresed on a 2% agarose gel containing ethidium bromide (EtBr) along with DNA markers (Gibco BRL, Grand Island, NY, USA) to verify the size of PCR products. The intensity (integrated optical density, IOD) of the PCR product bands was determined on a Dell workstation configured with Kontron KS 400 image analysis software (Zeiss, Thornwood, NY, USA).

Cell culture

Stock cultures of human proximal tubule (HPT) cells for use in experimental protocols were grown using serum-free conditions in 75 cm^2 T-flasks using procedures previously described by this laboratory [24]. Three isolates of HPT cells were used and these isolates were derived from normal cortical tissue obtained from kidneys removed for renal cell carcinoma. The kidneys were from a 72-year-old female, a 63-year-old male, and a 58-year-old female. HPT cells between passages 5 and 7 were used in the present study. Stock cultures of the HK-2 cell line were grown in 75 cm^2 T-flasks using an

identical serum-free growth formulation [25]. The growth formulation consisted of a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 growth medium supplemented with selenium (5 ng/mL), insulin (5 μg/mL), transferrin (5 μg/mL), hydrocortisone (36 ng/mL), triiodothyronine (4 pg/mL), and epidermal growth factor (10 ng/mL). The cells were fed fresh growth medium every three days and were subcultured at confluence (normally 3 to 6 days post-subculture) using trypsin-ethylenediaminetetraacetic acid (EDTA; 0.05 to 0.02%). Cell counts were determined by the automated counting of 4′-diamidino-2-phenylindole · 2HCl (DAPI) stained nuclei as described previously [26].

Isolation of total RNA and RT-PCR

Total RNA was isolated from cultured cells according to the protocol supplied with TRI REAGENT™ (Molecular Research Center, Inc., Cincinnati, OH, USA) as described previously by this laboratory [23]. The concentration and purity of samples were determined using spectrophotometer scan in the ultraviolet (UV) region and EtBr visualization of intact 18S and 28S RNA bands following agarose gel electrophoresis. Total RNA (0.5 μg) was reverse transcribed using MLV reverse transcriptase (50 units) in 1 × PCR buffer (50 mmol/L KCl and 10 mmol/L Tris-HCl, pH 8.3), 5 mmol/L $MgCl_2$, 20 units RNase inhibitor, 1 mmol/L each of the dNTPs, and 2.5 μmol/L random hexanucleotide primers. The samples were reverse transcribed for 20 minutes at 42°C, followed by a five-minute denaturation step at 99°C using a GeneAmp 9600 thermocycler (Perkin Elmer). The reverse transcribed product was used for PCR amplification using the AmpliTaq DNA polymerase enzyme (2.5 units; Perkin Elmer) and the specific upstream and downstream primers. The primers for MT-3 were: upper 5′-CCGTTCAC CGCCTCCAG-3′ and lower 5′-CACCAGCCACACT TCACCACA-3′ (product size, 325 bp). Primers for the determination of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were (upper and lower, respectively): 5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′ and 5′-CATGTGGGCCATGAGGTCCACCAC-3′ (product size, 983 bp; Clontech, Palo Alto, CA, USA).

The thermocycler was programmed to cycle at 95°C for a two-minute initial step, at 95°C for 30 seconds, and at 68°C for 30 seconds. Controls for each PCR included a no-template control where water was added instead of the RNA, and a no-reverse-transcriptase control where water was added instead of the enzyme. Samples were removed at appropriate intervals between 18 and 40 PCR cycles to ensure that the reaction remained in the linear region. The final PCR products were electrophoresed on 2% agarose gels containing EtBr along with DNA markers. The IOD of the PCR product bands was determined on a Dell workstation configured with Kontron KS 400 image analysis software.

Stable transfection of HK-2 cells

The MT-3 coding sequence was cloned from cultured human proximal tubule cell RNA by RT-PCR. The MT-3 coding sequence was blunt end ligated into the *Eco*R V site of pcDNA3.1/Hygro(+) (Invitrogen, Carlsbad, CA, USA). This vector has a cytomegalovirus immediate-early promoter upstream of the multiple cloning site and a hygromycin B resistance gene driven by an SV40 early promoter. The DNA construct was linearized by *Fsp* I before transfection. The HK-2 cells were transfected with the MT-3 plasmid construct in the sense direction or the vector alone by using Effectene™ transfection reagent (Qiagen, Valenecia, CA, USA). Briefly, lipid DNA complexes were prepared according the manufacturer's protocol at a ratio of 1:10 plasmid to Effectene. Lipid complexes were added to cells at 2 μg DNA per 9.6 cm^2 well for 24 hours. The cells were then fed fresh media for 48 hours, trypsinized and seeded at 8% confluency for selection in growth medium containing 30 μg/mL hygromycin B. Clones were selected using cloning rings and propagated in media containing 30 μg/mL hygromycin B. The stable transfectants were identified, recloned and preserved in liquid nitrogen storage.

MT-3 protein determination

The immunoblot protocol used for the determination of the level of MT-3 protein in cell lysates has been described previously by this laboratory [23]. The MT-3 protein was detected using an antibody against human MT-3 that was generated using the dodecapeptide GGEAAEAEAEKC (corresponding to MT-3 amino acids 53 to 64, which contains the MT-3 unique amino acid insert) conjugated through the C-terminal cysteine SH group to keyhole limpet hemocyanine using maleimidobenzoyl-N-hydroxysuccinimide ester. This was used to immunize New Zealand White rabbits. The MT-3 antibody was affinity purified using the dodecapeptide linked to SulfoLink gel (Pierce, Rockford, IL, USA) through the C-terminal cysteine residue. MT-3 protein was quantified by comparing the optical density of the sample dots to the standard MT-3 curve using image analysis software (KS 400). For standard curves, known amounts of the conjugated synthetic peptide were applied to each blot. The assay has detection limits in the range of 0.5 to 2 pg MT-3 protein.

RESULTS

Basal expression of MT-3 mRNA in the human proximal tubule and confluent cultures of HPT and HK-2 cells

The basal expression of MT-3 mRNA in the proximal tubule of the in situ kidney was determined by RT-PCR on total RNA isolated from proximal tubules microdis-

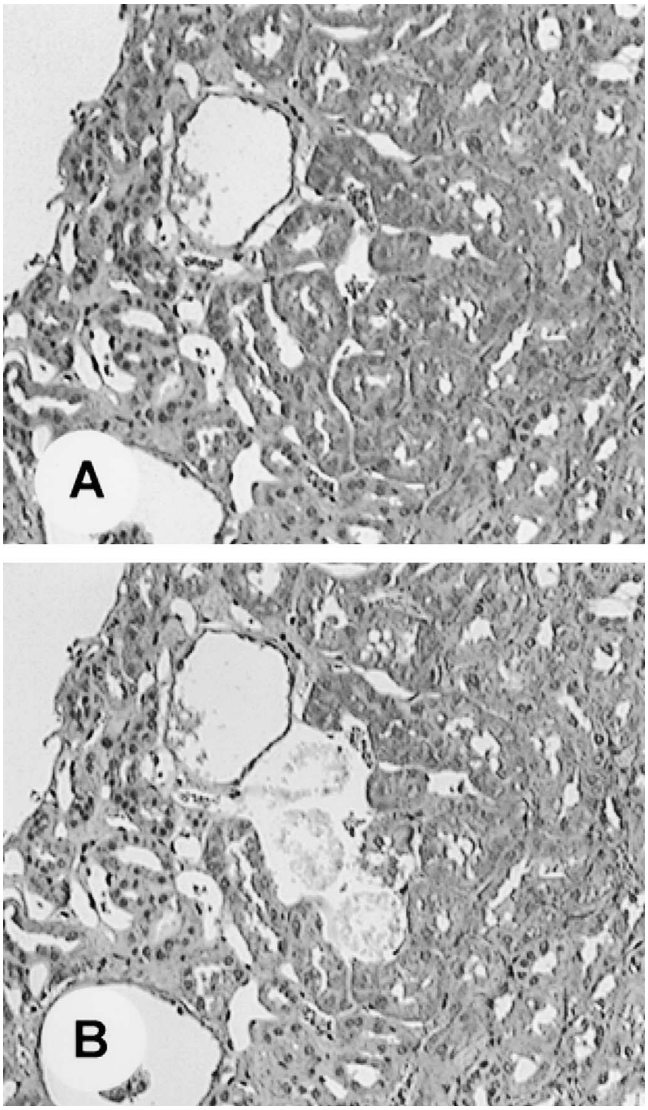

Fig. 1. Microdissection of human proximal tubules from adult kidney. Paraffin blocks of formalin fixed tissue from pathology archives were used to obtain sections from disease-free areas of kidneys removed for neoplastic disease. (*A*) Five-micron thick section of kidney stained lightly with hematoxylin and eosin before laser capture. (*B*) The residual tissue section shown after laser capture.

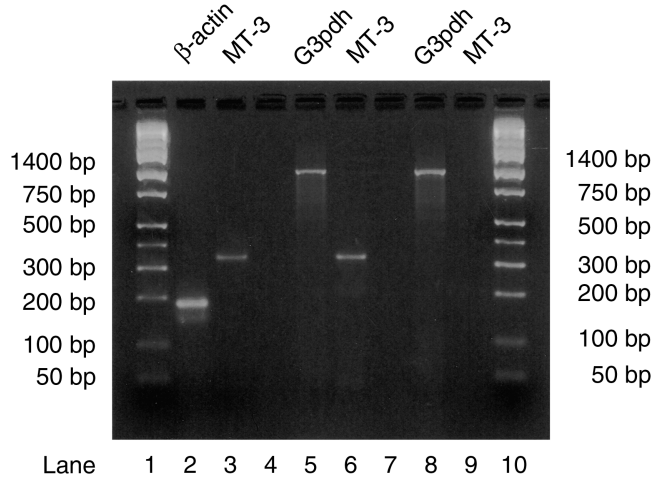

Fig. 2. Basal expression of metallothionein isoform 3 (MT-3) in human proximal tubule. Lanes 1 and 10, DNA ladder. Lanes 2 and 3, RT-PCR was performed on total RNA isolated from microdissected proximal tubules. Bands representing PCR products for β-actin (nested) (194 bp) and MT-3 (325 bp) are shown. Lanes 5 and 6, RT-PCR was performed on total RNA (500 ng) isolated from cultured human proximal tubule cells. Bands representing PCR products for GAPDH (G3pdh at 983 bp) at 25 PCR cycles and MT-3 at 35 PCR cycles are shown. Lanes 8 and 9, RT-PCR was performed on total RNA isolated from the cell line HK-2. Band representing PCR product for GAPDH at 25 cycles is shown. No PCR product was detected for MT-3 in HK-2 cells at an RNA input of 500 ng and 40 PCR cycles.

sected from formalin-fixed kidney sections. The proximal tubules for microdissection were identified based on periodic-acid-Schiff (PAS) staining of serial sections and the corresponding H&E stained proximal tubules removed for analysis (Fig. 1). The results of this analysis demonstrated that the in situ proximal tubule expressed a high basal level of MT-3 mRNA as judged by a relative comparison to the β-actin housekeeping gene (Fig. 2). Using the same sample of total RNA from the microdissected proximal tubules, MT-3 mRNA expression could be determined at 40 PCR cycles and a 500 ng total RNA input, whereas a second nested PCR had to be performed to demonstrate the expression of β-actin. This would indicate that the relative expression of MT-3 mRNA was much higher than that of the housekeeping gene. The basal expression of MT-3 mRNA also was determined on total RNA obtained from confluent cultures of the mortal HPT cells and the immortal HK-2 cell line. It was demonstrated that the HPT cells had moderate expression of MT-3 mRNA when compared relative to that of the GAPDH housekeeping gene. MT-3 mRNA could be detected following 35 PCR cycles and a 500 ng total RNA input, while mRNA for the GAPDH housekeeping gene was detected at 25 PCR cycles at an identical total RNA input (Fig. 2). It was demonstrated that HK-2 cells had no expression of MT-3 mRNA at a 500 ng total RNA input and 40 cycles of PCR, but mRNA for the GAPDH housekeeping gene was detected at 25 PCR cycles using the same total RNA sample (Fig. 2). The average basal expression of MT-3 protein in the three isolates of HPT cells was 0.51 ± 0.04 ng MT-3 per μg total protein. The MT-3 protein was below the limit of detection in the HK-2 cells.

Expression of MT-3 mRNA and protein as a function of HPT cell growth

The expression of MT-3 mRNA and protein was determined during HPT cell growth and when the cells were maintained at confluence. This was accomplished by determining the MT-3 mRNA (relative to GAPDH) and protein levels on HPT cells that had been maintained at

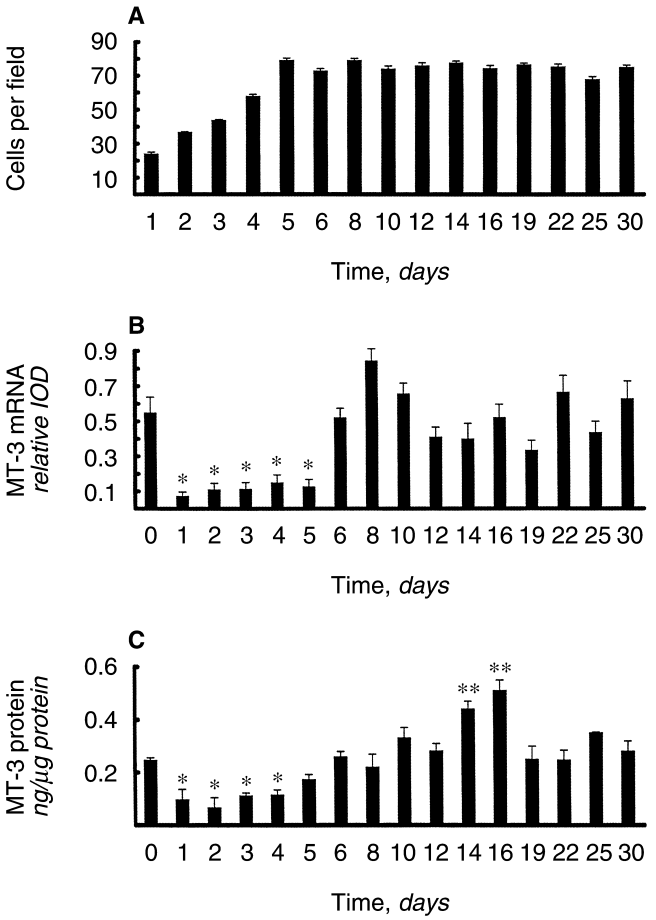

Fig. 3. Expression of MT-3 as a function of cell growth. HPT cells were seeded at a ratio of 1:3 in six-well plates and harvested at the indicated number of days after seeding. Day 0 represents cells taken before seeding. (*A*) Cell number reported as DAPI-stained nuclei per field. (*B*) MT-3 mRNA expression assessed by RT-PCR with gene specific primers. The integrated optical density (IOD) of the 40-cycle PCR DNA product band on ethidium bromide stained agarose gels was normalized to that obtained for GAPDH at 30 cycles. (*C*) MT-3 protein expression. Protein extracts were prepared from cells harvested on the indicated days and assessed for levels of MT-3 protein by immuno-dot blot using an MT-3 specific antibody. Shown are the means and SE of triplicate cell samples. Statistically significant decrease, $*P < 0.01$, and statistically significant increase, $**P < 0.01$.

confluence for seven days, and then subcultured at a 1:3 ratio, and determining the cell growth, MT-3 mRNA and MT-3 protein each day for a 30 day period (Fig. 3). Following subculture, the HPT cells attained confluence within five days and maintained a constant cell number for 25 additional days (Fig. 3A). The level of MT-3 mRNA was reduced tenfold following subculture and remained reduced for the next five days following subculture (Fig. 3B). On day 6 following subculture, the level of MT-3 mRNA increased significantly to a level similar to that which preceded subculture of the cells, and remained elevated and close to this original level for the remaining 24 days of the time course (Fig. 3B). In general, the level of MT-3 protein followed that of MT-3 mRNA, with a significant reduction in MT-3 protein level following subculture and a return to pre-subculture levels by day 6 of the time course (Fig. 3C). The magnitude of the changes in the level of MT-3 protein were not as pronounced at that for the corresponding MT-3 mRNA.

Stable transfection of MT-3 in HK-2 cells and restoration of vectorial active transport

Since the HK-2 cells were shown to have no expression of MT-3 mRNA or protein, this cell line was used as a recipient for stable transfection with a vector designed to overexpress the *MT-3* gene. The coding sequence of the *MT-3* gene was obtained from HPT cell RNA by RT-PCR, blunt end ligated into the *Eco*R V site of pcDNA3.1/Hygro(+), and linearized by *Fsp* I prior to transfection of the HK-2 cells. The HK-2 cells were transfected with the MT-3 plasmid construct in the sense direction or with the vector without insert using the Effectene protocol. Following selection in hygromycin B-containing growth medium, five clones containing the MT-3 sequence, two clones containing the vector only sequence, and one clone of the wild-type HK-2 cells were selected for further characterization. The first line of characterization was a simple examination of the routine light level morphology of the cells as they proliferated and attained confluence. The light level morphology of the HK-2 cells possessing the MT-3 construct were similar during growth, but markedly altered at confluence as noted by the formation of domes (arrows), structures not present in either the vector-only control cells or in the HK-2 parent cells (Fig. 4). The presence of domes are a routine feature of the HPT cells (Fig. 4D) [24]; the formation of domes are a hallmark of cultured renal epithelial cells that retain the in situ property of vectorial active transport [24]. These out-of-focus areas of the cell monolayer seen upon light microscopic examination represent raised areas where fluid has become trapped underneath the monolayer owing to active transport of ions and water across the cell monolayer in an apical to basolateral direction. This in turn traps a bubble of fluid between the cell layer and the culture dish, forcing local detachment of the monolayer from the plastic surface forming a raised area with an underneath reservoir of accumulated fluid. Each of the five MT-3 transfected clones formed domes, while neither the vector-only control cells or parental cells formed any domes. The growth rates of the MT-3 expressing HK-2 clones, the vector-only controls and the parental cells were all similar and the cells attained confluence within four days following a 1:2 subculture (data not shown). Upon reaching confluence, the MT-3 expressing HK-2 cells did appear to become more tightly packed compared to control cells and this was quantified below along with MT-3 gene expression and dome formation.

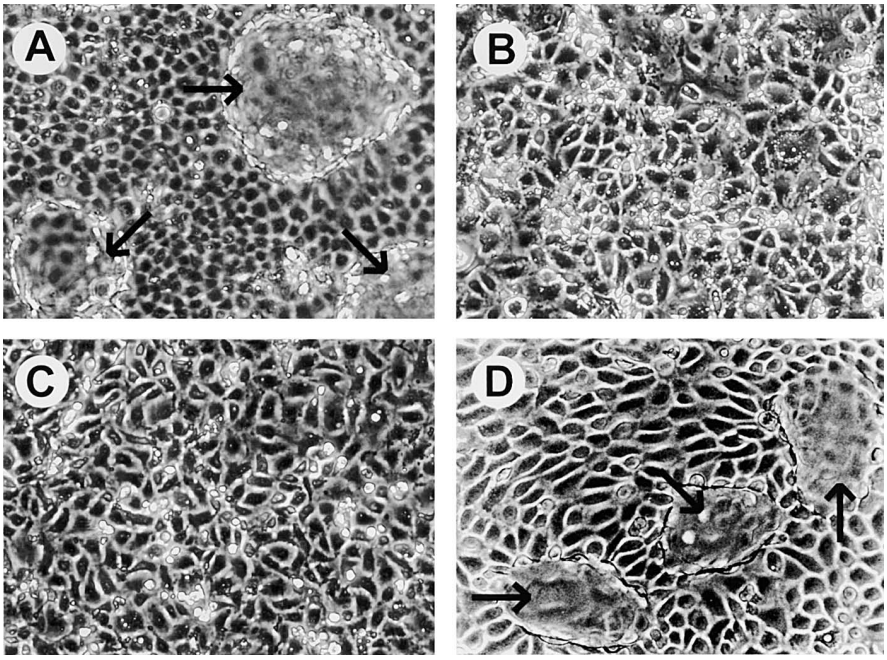

Fig. 4. Light level morphology of HK-2 cells with and without MT-3 expression. (*A*) HK-2 stably transfected with MT-3 (Clone #2). Arrows designate the presence of domes. (*B*) HK-2 stably transfected with the vector without MT-3. (*C*) HK-2 parental cells. (*D*) Normal HPT cells exhibiting domes (arrows). Cells were passaged at a 1:2 ratio and allowed to attain confluency for two weeks (original magnification ×40).

The extent of dome formation, MT-3 mRNA and MT-3 protein expression were determined 7, 14 and 21 days after subculture at a 1:2 split ratio for each of the five clones of HK-2 cells stably transfected with the MT-3 coding sequence and the two clones of vector-only control cells. Each of the seven cultures attained confluence seven days following cell culture and to remain contact inhibited for 14 additional days (Fig. 5A). The five clones of HK-2 cells transfected with the MT-3 coding sequence were all shown to reach a higher saturation density at confluent (cells per unit area) than the HK-2 cells transfected with the vector-only control (Fig. 5A). This difference in confluence was quite marked between the two sets of cultures, being increased by approximately 50% for the MT-3 transfected cells. There was no dome formation in any of the seven cultures one week following subculture (Fig. 5B). At 14 and 21 days, all five clones of HK-2 cells containing the MT-3 coding sequence had formed domes, while neither of the two vector-only controls had domes (Fig. 5B). The number of domes per unit area was similar among four of the five HK-2 clones containing the MT-3 sequence, while one clone (#4) had a reduced level of dome formation. Visual observation of the respective cultures suggested the higher cell density of the MT-3 transfected cells to be due to a tighter packing of the cells within the monolayer compared to control cells, rather than a multilayering of the MT-3 transfected cells. The expression of MT-3 mRNA was elevated approximately three- to fourfold when compared relative to that of the GAPDH housekeeping gene and expression was not influenced by the time the cells were maintained at confluency (Fig. 5C). One of the clones (#4) had a reduced expression of MT-3 mRNA (approximately equal to GAPDH expression), and this was the same clone that demonstrated a reduced amount of dome formation compared to the other MT-3 transfected clones. The two vector-only control clones demonstrated no expression of MT-3 mRNA at any of the three time points when assessed at total RNA inputs of 500 ng and 40 cycles of PCR (data not shown). In general, the expression of MT-3 protein followed that of MT-3 mRNA in the five clones of HK-2 cells stably transfected with MT-3 (Fig. 5D). Clone #4, which had comparatively reduced dome formation and MT-3 mRNA expression, also produced less MT-3 protein than the other MT-3 expression clones. The MT-3 protein expression in the five HK-2 clones was increased five- to 15-fold over that present in the HPT cells under basal conditions of growth. The two vector-only control clones demonstrated no expression of MT-3 protein at any of the three time points (data not shown).

DISCUSSION

The goal of the present study was to expand on our earlier observations that MT-3 mRNA and protein are expressed in the human kidney and cultured human proximal tubule cells [20, 21]. The first goal was to confirm the presence and level of expression of MT-3 mRNA in the intact human proximal tubule. While the previous studies had shown that total RNA isolated from fresh human kidney tissue contained MT-3 mRNA and that MT-3 protein could be immunolocalized in the proximal

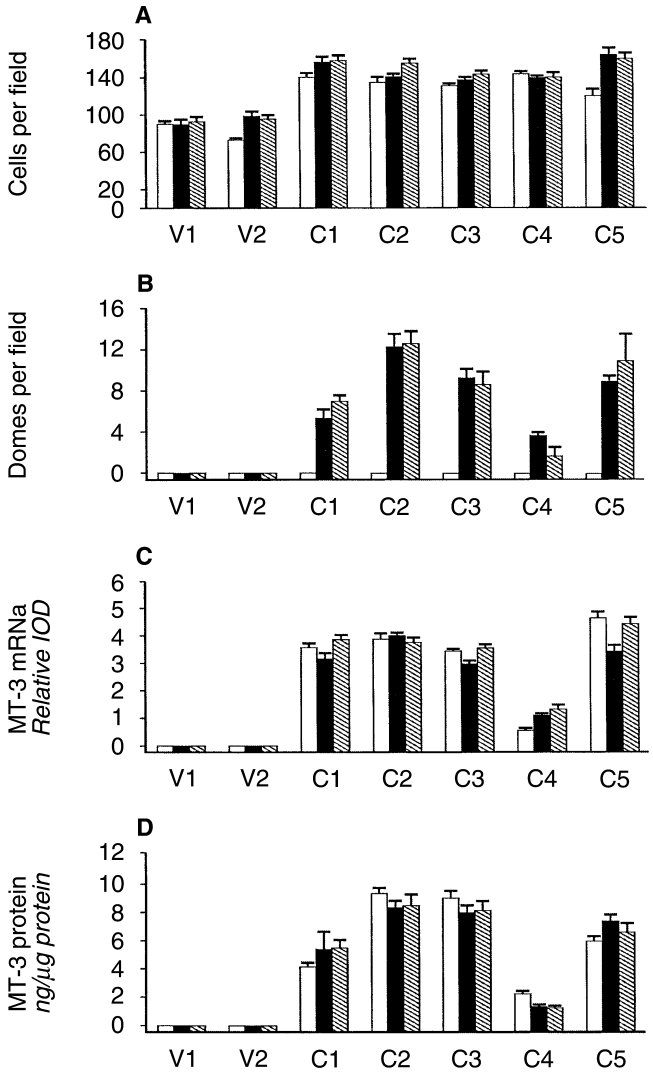

Fig. 5. MT-3 expression and dome formation in MT-3 transfected clones. Each MT-3 expressing clone (designated C1 through C5) and two vector only transfected clones (V1 and V2) were passaged 1:2 in 25 cm^2-T flasks. Pictures were taken and the cells were harvested for protein and RNA at (□) 1, (■) 2, and (▨) 3 weeks. (*A*) Average cell count per field. (*B*) Number of domes per ×4 objective field (mean ± SE of three ×4 objective fields). (*C*) MT-3 mRNA expression assessed by RT-PCR. Shown are the IOD of the MT-3 PCR product band at 30 cycles normalized to that of GAPDH at 30 cycles of PCR. (*D*) MT-3 protein expression. Protein extracts were prepared from cells at 1, 2, and 3 weeks, and assessed for levels of MT-3 protein by immunodot blot.

and other tubules of tissue sections, it had not been shown that MT-3 mRNA was present in the proximal tubule or the relative level of expression. To accomplish this analysis, proximal tubules were identified in tissue sections obtained from paraffin-embedded, formalin-fixed kidneys using PAS staining and the proximal tubules removed from surrounding tissue using laser-assisted microdissection. Total RNA was then isolated from the proximal tubules and MT-3 and β-actin mRNA determined using RT-PCR. This analysis demonstrated that not only was MT-3 mRNA present in the proximal tubule, but that it was a very abundant transcript. This conclusion was based on the finding that the relative expression of MT-3 was much greater than the β-actin housekeeping gene. This finding was somewhat surprising since the MT-3 immunostaining found in the proximal tubules of the human kidney was of moderate intensity, but well below that of many of the tubules present in the medullary rays and in the astrocytes of control brain, all of which showed strong immunostaining for MT-3 [21]. Thus, these results confirm the presence of MT-3 mRNA in the proximal tubules and suggest that MT-3 may have an even higher level of expression in other tubule cell types of the human nephron.

The finding that MT-3 is expressed in the human kidney impacts on the initial observation that MT-3 was reported to be a brain-specific metallothionein [7]. First is the important point that the initial observation was based on expression results in mouse tissues, and there is no reason—technical or otherwise—to suggest that these observations were in error. Rather, it is quite possible that a new pattern of MT-3 expression was gained in the human as a result of evolutionary change. In the mouse, the organization of the *MT* genes is not complex. The genes encoding the MT-1 and MT-2 isoforms are both single copy genes, are located approximately 6 kb apart on mouse chromosome 8, are coordinately regulated, and the proteins are thought to be functionally equivalent [5, 27]. The MT-3 and MT-4 isoforms also are single copy genes and are closely linked to, but not coordinately regulated with, the *MT-1* and *MT-2* genes on mouse chromosome 8 [7, 8, 28]. In the human, a gene duplication event took place, in the time scale between mouse and human, that greatly increased the complexity of the organization of the human *MT* genes [28, 29]. This duplication event was mediated at the level of DNA, and not that of RNA, as evidenced by the fact the duplicated genes have intact 5′ promoter, regulatory regions and 3′ untranslated regions (UTRs). As a result, the human MT locus consists of a family of genes located at 16q13 consisting of ten functional (MT-1A, MT-1B, MT-1E, MT-1F, MT-1G, MT-1H, MT-1X, MT-2A, MT-3, MT-4) and six non-functional MT isoforms (MT-1C, MT-1D, MT-1I, MT-1J, MT-1K, MT-1L). That the gene duplication event in the human was of practical significance is suggested by the fact that the human *MT-1* and *MT-2* genes are not coordinately regulated and have been shown to exhibit unique expression profiles and examples of inducer-specific and tissue-specific [22, 26, 30–35], and developmental-specific [36] regulation have been demonstrated. The *MT-3* and *MT-4* genes in the human are within 85 kb of the other *MT* genes on human chromosome 16 [7, 8]. Although there is only limited information currently available, it can hypothesized that the tissue distribution pattern of MT-3 gene expression is different

between mouse and humans due to regulatory differences arising from the gene duplication event that occurred in the human MT gene locus.

Several other studies using immunohistochemical staining with the MT-3 antibody suggest that the increased distribution of MT-3 in normal human tissues will not be extensive or even a common occurrence. Staining with the MT-3 antibody showed no reactivity in tissue sections from formalin-fixed paraffin-embedded samples of normal human bladder [37].

This examination assessed urothelial cells, smooth muscle cells of the bladder wall, stroma of the bladder wall and associated smooth muscle and endothelial cells of the vessels. The absence of MT-3 mRNA was also confirmed by the RT-PCR analysis of total RNA prepared from fresh human bladder tissue. The fact that bladder cancers similarly treated were stained with the MT-3 antibody demonstrated that the absence of reactivity in normal tissues was not a fixation artifact. In the normal human prostate, MT-3 reactivity was limited to only a weak to moderate staining in basal cells and the secretory cells of the prostatic ducts; with other cell types showing no MT-3 immunoreactivity [23]. Within the series of prostate samples examined, nerves were shown to stain intensely for MT-3, as were some prostate cancers. The low level of MT-3 expression in normal prostate was confirmed by finding a corresponding low level of MT-3 mRNA in total RNA prepared from fresh prostate tissue. An examination of MT-3 expression in the normal human breast also demonstrated no immuoreactivity for MT-3 in any cellular component, nor was MT-3 RNA found in total RNA extracted from fresh surgical tissue [38]. To our knowledge, these are the only studies to date that address the tissue distribution of MT-3 in the human. These limited studies indicate that the expression of MT-3 is not highly restricted in the human, nor is expression ubiquitous like that of the highly studied MT-1 and MT-2 isoforms.

An opportunity to begin to determine the functional significance of MT-3 expression in the proximal tubule was provided by the finding that the immortalized human proximal tubule cell line, HK-2, did not express detectable MT-3 mRNA or protein. This was a fortuitous finding, since the HK-2 cell line is one of the few immortal human cell lines that have been isolated from primary proximal tubule cultures derived from adult kidneys [25]. It retains many of the features associated with the proximal tubule and mortal cultures of HPT cells. This includes positivity for alkaline phosphatase, γ-glutamyltranspeptidase, leucine aminopeptidase, acid phosphatase, cytokeratin, $\alpha_3\beta_1$ integrin, and fibronectin; and negativity for Factor VIII-related antigen, 6.19 antigen and CALLA endopeptidase. The HK-2 cells also display sodium-dependent glucose transport and adenylate cyclase responsiveness to parathyroid hormone but not to antidiuretic hormone. A feature not present in the parent HK-2 cell line was the ability to retain the in situ property of vectorial active transport as evidenced by dome formation by confluent cell monolayers. Dome formation in epithelial cell cultures is acceptable presumptive evidence of the following processes that are required for its expression: functional plasma membrane polarization, formation of occluding junctions (tight junctions), and vectorial transepithelial active ion transport [39]. It is presently unknown which of these processes are deficient in the cultures of the HK-2 cells. However, when the HK-2 cell line was stably transfected with a vector containing the MT-3 coding sequence under the control of the CMV promoter, dome formation was induced in the HK-2 cell line. This finding provides the first indication that MT-3 could be of functional significance in the proximal tubule as it relates to vectorial active transport.

The induction of dome formation in the HK-2 cell line by stable transfection with MT-3 was reproducible, with dome formation being present in the cultures derived from five independent clones, all of which were chosen for analysis before measurement of MT-3 expression. In four of five cultures, the extent of doming was very similar as were the corresponding levels of MT-3 mRNA and protein. In the culture from the one clone that exhibited a reduced incidence of dome formation, there was a corresponding reduced expression of MT-3 mRNA and protein. However, the fact that this culture, which expressed lower levels of MT-3, still formed domes is important because the level of both doming and expression of MT-3 protein are closer to that noted to occur in the HPT cell cultures. No dome formation was noted in HK-2 cells transfected with the vector containing no MT-3 coding sequence. The induction of dome formation appears to be specific for MT-3, since the HK-2 cells do express basal levels of mRNA for the *MT-2A*, *MT-1X*, *MT-1F* and *MT-1E* genes at levels similar to those found in the HPT cells and in identity with those found in total RNA from fresh kidney tissue (unpublished observations). The only other obvious effect that MT-3 expression had on the HK-2 cells was that they appeared to pack more tightly into the monolayer than the parent cell line. Further studies will be required to determine the mechanism underlying the induction of dome formation by expression of the *MT-3* gene in HK-2 cells. The present studies confirm the presence of MT-3 mRNA in the human proximal tubule and provide the first evidence that MT-3 expression is involved in the transport function of a human renal cell line retaining properties of the proximal tubule.

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