Characterization of connective tissue growth factor expression in primary cultures of human tubular epithelial cells: modulation by hypoxia

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Am J Physiol Renal Physiol 298: F796–F806, 2010. First published December 23, 2009; doi:10.1152/ajprenal.00528.2009.—Tubular epithelial cells secrete connective tissue growth factor (CTGF, CCN2), which contributes to tubulointerstitial fibrosis. However, the molecular regulation of CTGF in human primary tubular epithelial cells (hPTECs) is not well defined. Therefore, CTGF expression was characterized in hPTECs isolated from healthy parts of tumor neoprectomies, with special emphasis on the regulation by transforming growth factor-β (TGF-β) and hypoxia, essential factors in the development of fibrosis. CTGF synthesis was strongly dependent on cell density. High CTGF levels were detected in sparse cells, whereas CTGF expression was reduced in confluent cells. Concomitantly, stimulation of CTGF by TGF-β or the histone deacetylase inhibitor trichostatin was prevented in dense cells. Exposure of hPTECs to low oxygen tension (1% O2) or the hypoxia mimetic dimethyl-oxalylglycine for 24 h reduced CTGF gene expression in most of the 17 preparations analyzed. Preincubation of the cells under hypoxic conditions significantly reduced TGF-β-mediated upregulation of CTGF. In line with these data, CTGF mRNA was only induced in interstitial cells, but not in tubular cells in kidneys of mice exposed to hypoxia. Longer exposure to hypoxia or TGF-β (up to 72 h) did not induce hPTECs to adopt a mesenchymal phenotype characterized by upregulation of α-smooth muscle actin, downregulation of E-cadherin, or increased sensitivity of the cells in terms of CTGF expression. Sensitivity was restored by inhibition of DNA methylation. Taken together, our data provide evidence that exposure to hypoxia decreased CTGF gene expression. Furthermore, hypoxia per se was not sufficient to induce a mesenchymal phenotype in primary tubular epithelial cells.

hypoxia inducible factor; human primary tubular epithelial cells; cell density; transforming growth factor-β; connective tissue growth factor

CHRONIC HYPOXIA IS INCREASINGLY recognized as a pathogenic factor contributing to the development of interstitial fibrosis (33, 34, 37, 38). The cellular effects of hypoxia are mainly mediated by hypoxia inducible transcription factors (HIF). HIFs consist of a stable and constitutively expressed β-subunit and an inducible α-subunit. Under normoxic conditions, the α-subunit is hydroxylated by HIF prolyl-hydroxylases, which marks it as target for the E3 ubiquitin ligase von Hippel-Lindau protein, leading to proteosomal degradation. In hypoxic conditions, HIF prolyl-hydroxylases are inactive, as they utilize oxygen as co-substrate. Thus HIFs are stabilized and activate gene expression (31). A number of HIF-1α target genes (e.g., vascular endothelial growth factor, glucose transporters, and nitric oxide synthases) may contribute to the survival of cells in ischemia. Upregulation of these adaptive genes may relate to the beneficial effects, which are observed in animal models on short-term exposure to hypoxia (15, 16). Chronic hypoxia, however, has been associated with an increased expression of profibrotic proteins, among them lysyl oxidases, plasminogen activator-1, or connective tissue growth factor (CTGF, CCN2) (18).

CTGF is upregulated in kidney diseases of different etiology and associated with increased synthesis of extracellular matrix and fibrosis (37, 39). Regulation of CTGF has been studied in various renal cells, most intensively in mesangial cells and fibroblasts. However, a strong correlation between the number of CTGF mRNA-positive cells and the degree of chronic tubulointerstitial damage also suggested tubular epithelial cells as potential source and target of CTGF (20). CTGF mRNA and protein were not detected in normal human tubuli, but were present in severely damaged cells (20, 36). In the remnant kidney model of renal fibrosis, Frazier et al. (13) observed CTGF expression in close proximity to epithelial cells transdifferentiating to a myofibroblast phenotype. This suggested that CTGF might play a role in mesenchymal transition of tubular epithelial cells, which is considered to play a role in progressive kidney disease (5). However, markers of mesenchymal transition did not correlate with interstitial fibrosis, as analyzed in protocol transplant biopsies (40). While the concept of mesenchymal transition is well established in tumor cells or mouse models, the extent to which it contributes to human renal tubulointerstitial fibrosis is under debate (e.g., Ref. 1).

Transforming growth factor-β (TGF-β) is considered a major factor driving epithelial cells to adopt a mesenchymal phenotype, including expression of CTGF (3). Recently, it was suggested that hypoxia might also induce tubular epithelial cells to undergo phenotypic alterations contributing to chronic renal disease (1, 19). Hypoxia-mediated phenotypic alterations of tubular cells were first described in immortalized rat proximal epithelial cells (28) and recently confirmed in human cell lines (39). In mice, stable expression of HIF-1α by deletion of von Hippel-Lindau protein in tubular epithelial cells promoted progression of interstitial fibrosis (22). Based on studies with mice lacking HIF-1α, CTGF was implicated in hypoxia-mediated alterations of tubular epithelial cells (18).

To understand the molecular mechanisms of phenotypic alterations of tubular cells observed in vivo, multiple analyses have been performed in vitro using different types of tubular

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epithelial cells, most often immortalized cell lines of different origin. Regulation of CTGF expression in tubular epithelial cells was primarily investigated in rodent cells or the human proximal tubular cell lines, HK-2 and HKC-8, whereas little data are available in human primary tubular epithelial cells (hPTECs). Stimulation of hPTECs with TGF-β for 24 h leads to an upregulation of CTGF, which is comparable to results obtained with human cell lines (27). By contrast, regulation of CTGF by hypoxia was less concordant. Upregulation was observed in mouse tubular cells (17), whereas downregulation was reported in human proximal tubule cells in microarray analyses (7). In a recent study, our laboratory analyzed hypoxia-mediated regulation of CTGF in the human cell lines HK-2 and HKC-8 and observed a HIF-1α-dependent reduction of CTGF gene expression (26).

Primary cultures of tubular epithelial cells seem to differ from cell lines in terms of phenotypic changes: TGF-β-induced expression of α-smooth muscle actin (α-SMA) has been reported in HKC-8 (42) and HK-2 cell lines (14), whereas it was not induced in primary cultures (12). Regulation of CTGF has not been analyzed in detail in primary tubular epithelial cells. Primary cultures of tubular epithelial cells are expected to vary, depending on the donor. Therefore, it was the aim of the present study to analyze CTGF expression in a large number of well-characterized primary cultures of tubular epithelial cells obtained from different donors. CTGF gene expression proved to be strongly dependent on cell density and was downregulated when the cells were exposed to hypoxia.

**MATERIALS AND METHODS**

**Materials.** DMEM/Ham’s F12 medium was purchased from Biocrom (Berlin, Germany), DMEM medium and Hank’s balanced salt solution from PAA Laboratories (Coelbe, Germany), insulin-transferrin-selenium supplement from Gibco (Karlsruhe, Germany), fetal calf serum (FCS) from PAN Biotech (Aidenbach, Germany), triiodothyronine from Fluka (Buchs, Switzerland), hydrocortisone from Sigma (Munich, Germany), epidermal growth factor from PeproTech (Hamburg, Germany), TGF-β, from tebu-bio (Offenbach, Germany), dimethyl-oxalylglycine (DMOG) from Axxora (Frontier Scientific, Carnforth, Lancashire, UK), trichostatin A (TSA) from Serva (Heidelberg, Germany), and 5-aza-2’-deoxyuridine from Sigma-Aldrich (Taufkirchen, Germany).

**Cell culture.** hPTECs were isolated from renal cortical tissues collected from tumor nephrectomies by two different methods. On the one hand, hPTECs were obtained according to a protocol of Detrisac and colleagues (9), which is based on cell outgrowth in epithelial cell-selective medium (DMEM/Ham’s F12 medium containing 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, insulin-transferrin-selenium supplement, 10 ng/ml epidermal growth factor, 36 ng/ml hydrocortisone, and 4 μg/ml triiodothyronine). Experiments were conducted at passages 1–3 (days 15–25), as described before (27). Second, after transport in Hank’s balanced salt solution, cortex tissue was cut into 1-mm3 pieces and digested with collagenase type II (Gibco, Karlsruhe, Germany) and DNase I grade II (Roche Diagnostics, Mannheim, Germany). Incubation with 10% FCS for 30 min, the cell suspension was collected from tumor nephrectomies by two different methods. On the other hand, hPTECs were isolated from renal cortical tissues collected from tumor nephrectomies by two different methods. On the one hand, hPTECs were obtained according to a protocol of Detrisac and colleagues (9), which is based on cell outgrowth in epithelial cell-selective medium (DMEM/Ham’s F12 medium containing 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, insulin-transferrin-selenium supplement, 10 ng/ml epidermal growth factor, 36 ng/ml hydrocortisone, and 4 μg/ml triiodothyronine). Experiments were conducted at passages 1–3 (days 15–25), as described before (27). Second, after transport in Hank’s balanced salt solution, cortex tissue was cut into 1-mm3 pieces and digested with collagenase type II (Gibco, Karlsruhe, Germany) and DNase I grade II (Roche Diagnostics, Mannheim, Germany) for 30 min, the cell suspension was centrifuged and cultured in epithelial cell-selective medium. Cells were subcultured by application of trypsin. This protocol enabled the isolation of higher cell numbers, and experiments could be conducted at passages 1–4 (days 5–20). For experiments, hPTECs (25 × 10³ cells/cm²) were seeded in medium containing 2.5% FCS to facilitate cell attachment, and medium was replaced after 4 h to FCS-free epithelial cell-selective medium. Experiments were started with confluent cells, unless indicated otherwise in the figure legends. Hypoxic exposure was performed in a Hera cell 150 incubator (Thermo Scientific Heraeus, Hamburg, Germany) with 1% O2, 5% CO2, and balance nitrogen. Bright-field pictures were recorded by Olympus CK40 microscope (Olympus, Hamburg, Germany) using Leica DC Viewer software (Leica, Herbrugg, Switzerland). Isolation of human cell lines was approved by the local ethics committee.

**Western blot analysis.** Cells were lysed in buffer containing 50 mM HEPES, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10% glycerol, 2 mM sodium vanadate, and protease inhibitors complete EDTA-free (Roche Diagnostics, Mannheim, Germany). For detection of HIF-1α, a buffer containing 9 M urea, 2% 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate, 1 mM DTT, 2 mM sodium vanadate, and protease inhibitors complete EDTA-free was used. For detection of secreted CTGF, cell culture supernatants were precipitated overnight with four volumes of ethanol at −20°C. After centrifugation (13,000 × g, 30 min, 4°C), pellets were dried and resuspended in gel loading buffer. Proteins were separated on 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Macherey-Nagel, Düren, Germany, and Bio-Rad Laboratories, Munich, Germany). Immunoreactive proteins were visualized by the enhanced chemiluminescence detection system (ECL-Plus, Amersham Biosciences, Freiburg, Germany). The immunoreactive bands were quantified using the luminescent image analyzer (LAS-1000 Image Analyzer, Fujiﬁlm, Berlin, Germany) and AIDA 4.15 image analyzer software (Raytest, Berlin, Germany). To ensure equal loading and blotting, all blots were redetected with an antibody directed against tubulin. The following antibodies were used: goat polyclonal anti-CTGF and rabbit anti-N-cadherin (SC-14939 and SC-7939, Santa Cruz, Heidelberg, Germany), mouse monoclonal anti-β-tubulin and mouse monoclonal anti-BNP3 (T0198 and B7931, Sigma, St. Louis, MO), rabbit polyclonal anti-HIF-1α (no. 10006421, Cayman Chemicals, Ann Arbor, MI), rabbit monoclonal anti-E-cadherin and p-Smad2 Ser465/467 (no. 3195 and no. 3101, 24E10, Cell Signaling, Danvers, MA), horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (SC-2020, Santa Cruz, Heidelberg, Germany), HRP-conjugated sheep anti-mouse IgG and donkey anti-rabbit IgG (no. NA931-1ML, no. NA934-1ML; GE Healthcare Bio-Sciences, Uppsala, Sweden).

**RNA isolation and real-time RT-PCR.** Total RNA was prepared from cultured epithelial cells using TriFast reagent from Peqlab (Erlangen, Germany). RNA (500 ng) were reverse transcribed with TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA), according to the manufacturer’s instructions. cDNA was amplified using Power SYBR MM reaction buffer (Applied Biosystems). The PCR reactions were carried out using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). The following primers were used: CTGF forward 5′-GTC CAC CGA CCA AGA TGG T-3′, reverse 5′-AAG TAC TCC CCG CTG CGA T-3′; TGF-β1 forward 5′-TGG AAG TGG TAC CTC GCC CCC AAG G-3′, reverse 5′-GCA GGA CGC CAT GAT CCT GGA C-3′, and 18 S forward 5′-ttg att aag tcc ctc ctc ttt gt-3′, reverse 5′-cga tgg gcc ctg ctc act a-3′.

**Immunocytochemistry.** Cells were fixed with paraformaldehyde (3.5% in PBS) for 10 min and afterwards permeabilized by 0.5% Triton X-100 in PBS for 10 min. After they were washed three times with PBS, cells were blocked in 1% BSA in PBS for 1 h at room temperature and washed once. Cells were incubated with rabbit anti-N-cadherin (1:250, SC-7939, Santa Cruz, Heidelberg, Germany) overnight at 4°C, and secondary Alexa Fluor 488-conjugated donkey anti-rabbit antibody (A21206, 1:500 in PBS, Invitrogen, Karlsruhe, Germany) for 1 h at room temperature. Subsequently, mouse anti-E-cadherin antibody (no. ab1416, 1:200 in PBS; Abcam, Cambridge, UK) was applied for 3 h at room temperature, and secondary Alexa
Fluor 555-conjugated donkey anti-mouse antibody (A21424, 1:500 in PBS, Invitrogen, Karlsruhe, Germany) for 1 h at room temperature. Cytokeratin was stained with mouse anti-cytokeratin antibody (no. 1918, KL1, 1:200, Immunotech, Marseille, France), and secondary Alexa Fluor 488-conjugated donkey anti-mouse antibody (A21202, 1:500 in PBS, Invitrogen, Karlsruhe, Germany) for 1 h at room temperature. After mounting, slides were viewed using a Nikon Eclipse 80i fluorescent microscope; digital images were recorded by Visitron Systems 7.4 Slider camera (Diagnostic Instruments, Sterling Heights, MI) using Spot Advanced software (Diagnostic Instruments).

**In situ hybridization.** C57BL/6 mice (Jackson Laboratories, Sulzfeld, Germany) were exposed to low oxygen tension (8% O₂) for 8 h and compared with their littermates (n = 2 each). Thereafter, the kidneys were removed and processed for in situ hybridization. Animal experiments were approved by the institutional review board for the care of animal subjects.

In situ hybridization was performed on 4-μm paraffin sections of kidneys fixed in 4% buffered formaldehyde for 24 h. A 542-bp cDNA fragment of rat CTGF (Genbank gi5070343 496-1037) was amplified by PCR and cloned in the PCR2.1-TOPO vector (Invitrogen, Breda, The Netherlands) (20). In vitro transcription of the purified insert was performed using SP6 or T7 RNA polymerases and digoxigenin (DIG)-conjugated UTP (Roche, Almere, The Netherlands) to produce DIG-labeled sense or antisense riboprobes, respectively. Hybridization of the probe was detected by an alkaline phosphatase-labeled sheep anti-DIG antibody and visualized with nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate. Sections were briefly counterstained with periodic acid Schiff.

**Immunohistochemistry.** Immunohistochemistry was essentially performed as described by Willam et al. (41). In brief, paraffin sections (4 μm) were dewaxed in xylene and rehydrated in graded ethanol washes. Antigen retrieval was performed with a commercial kit (Biocare Medical, Concord, CA). After blocking, sections were incubated with the primary antibodies and then with the appropriate secondary antibodies. Slides were counterstained with hematoxylin. Digital images were recorded using a Nikon Eclipse 80i fluorescence microscope (Nikon, Melville, NY) equipped with a DXM1200F digital camera (Nikon, Andover, MA).

Fig. 1. Characterization of human primary tubular epithelial cells (hPTECs). A: human renal cortical tissues collected from tumor nephrectomies were fixed in 4% buffered formaldehyde for 24 h. A 542-bp cDNA fragment of rat CTGF (Genbank gi5070343 496-1037) was amplified by PCR and cloned in the PCR2.1-TOPO vector (Invitrogen, Breda, The Netherlands) (20). In vitro transcription of the purified insert was performed using SP6 or T7 RNA polymerases and digoxigenin (DIG)-conjugated UTP (Roche, Almere, The Netherlands) to produce DIG-labeled sense or antisense riboprobes, respectively. Hybridization of the probe was detected by an alkaline phosphatase-labeled sheep anti-DIG antibody and visualized with nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate. Sections were briefly counterstained with periodic acid Schiff.

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**Fig. 1. Characterization of human primary tubular epithelial cells (hPTECs). A:** human renal cortical tissues collected from tumor nephrectomies were fixed in methyl Carnoy’s solution. Paraffin sections were stained for the cell-cell adhesion molecules E-cadherin in red and N-cadherin in green. A bright-field picture of confluent hPTECs was recorded (left). hPTECs isolated by sieving were stained for cytokeratin as a marker for epithelial cells (middle), and N-cadherin (green) and E-cadherin (red) as markers for proximal and distal tubular epithelial cells, respectively (right). C: hPTECs at passages P1–P5 were cultivated until reaching confluence. Expression of E-cadherin and N-cadherin was analyzed by Western blotting. To confirm equal loading and blotting, the blot was reprobed with an anti-tubulin antibody. A representative blot of 4 preparations of hPTECs is shown. D: hPTECs were plated at 2.5 × 10^4 cells/cm² and harvested at the indicated time points. A representative Western blot for cellular and secreted connective tissue growth factor (CTGF) is shown. E: hPTECs were plated at 25 × 10^3 cells/cm². On day 2, the DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine (Aza, 2.5 μM) was added, and cells were harvested at the indicated time points. Expression values of cells treated with Aza and harvested after 72 h were set to 100%. Values are means ± SD of 5 independent experiments with 3 different cell preparations. **P < 0.01, ***P < 0.001 for treated vs. untreated cells. F: hPTECs were plated at low-(10 × 10^3 cells/cm²), medium-(25 × 10^3 cells/cm²), and high-cell density (50 × 10^3 cells/cm²) and cultivated for 2 days before harvesting. The graph displays the statistical analysis of cellular CTGF expression of 13 independent experiments with 7 different preparations of hPTECs. For normalization, CTGF expression of cells plated at low density was set to 100%. Values are means ± SD.
cial antigen retrieval solution (DAKO, Glostrup, Denmark) using a pressure cooker and boiling the slides for 6 min. HIF-1α (no. 10006421; Cayman Chemicals) was used as primary antibody. Detection of HIF-1α and chromogenic visualization was performed, applying a tyramine amplification technique, using a commercial kit, according to the manufacturer’s recommendations (CSA, DAKO). N-cadherin and E-cadherin were detected with the antibodies described above using secondary Alexa Fluor-labeled antibodies (Invitrogen, Eugene, OR).

Statistical analysis. To compare multiple conditions, statistical significance was calculated by one-way ANOVA with Tukey-Kramer multiple-comparison post hoc tests, unless otherwise indicated. Paired Student t-test was used to compare two conditions using the original data. A value of $P < 0.05$ was considered to indicate significance.

RESULTS

Characterization of primary human tubular epithelial cells. Epithelial cells are characterized by the expression of cadherins as cell-cell adhesion molecules. Renal proximal tubular cells express primarily N-cadherin, whereas distal tubular cells express predominantly E-cadherin (35). This distribution was also observed in the healthy renal cortical tissue specimens collected from tumor nephrectomies, which were used for the isolation of hPTECs (Fig. 1A). hPTECs were isolated by two methods, which were based on outgrowth and sieving, as described in detail in MATERIALS AND METHODS. Cells isolated by both protocols displayed positive staining for the epithelial cell marker cytokeratin (>99%, 6 cell preparations; Fig. 1B). Furthermore, hPTECs expressed either E-cadherin or N-cadherin as markers for distal and proximal tubular cells, respectively (Fig. 1B). Cells isolated by outgrowth consisted mainly of distal cells (>95%, 3 preparations of hPTECs), while the sieving protocol yielded a mixture of 60–80% distal and 20–40% proximal cells (3 preparations). When the cells were propagated, the expression of N-cadherin and E-cadherin decreased after passages 3 and 4 (Fig. 1C). Concomitantly, the proliferation rate of hPTECs declined (data not shown). Therefore, cells were used in passages 1–4. A total of 17 different preparations of hPTECs was analyzed in this study; $n$ indicates the number of independent experiments.

Expression of CTGF was largely dependent on cell culture conditions. When cells were seeded at medium density ($25 \times 10^3$ cells/cm$^2$), high levels of cellular CTGF were detectable by Western blot analysis 24 h after seeding (Fig. 1D, top). Over time, cellular CTGF expression decreased. Accordingly, the levels of secreted CTGF increased until days 2 and 3 and remained essentially unaltered thereafter (Fig. 1D). CTGF appeared as a double band in cellular homogenates, representing glycosylated and nonglycosylated CTGF (6). In the cell culture supernatant, a higher molecular weight band showed immunoreactivity, which was not further characterized. No low-molecular-weight bands were detectable, indicative of high stability of secreted CTGF in this cell culture system (data not shown). Time-dependent downregulation occurred at the level of gene expression, as CTGF mRNA was reduced after 48 h by $86 \pm 8\%$ (means ± SD, $n = 4$).

Expression of CTGF has been shown to be sensitive to DNA methylation of CpG islands in the promoter region (22). Therefore, we analyzed whether alterations of chromatin structure were involved in the time-dependent downregulation of CTGF. Incubation with an inhibitor of DNA methyltransferases, 5-aza-2’-deoxycytidine, increased CTGF expression and attenuated the decline (Fig. 1E).

Furthermore, cell density proved to be essential for the expression level of CTGF. When plated at medium density ($25 \times 10^3$ cells/cm$^2$), the cells formed a confluent monolayer after 2 days. Thereafter, cell density increased due to contin-
hypoxic proliferation of the cells. This suggested that CTGF downregulation was related to the increased cell density. To confirm this correlation, cells were seeded at different cell densities (10–50 × 10^3 cells/cm²), and CTGF was detected after 48 h. Cellular CTGF was highly expressed in hPTECs of low-cell density, whereas CTGF expression was weak at high-cell density (Fig. 1F).

Cell density-dependent regulation of CTGF by TGF-β. CTGF was consistently upregulated about threefold in primary epithelial cells on incubation with TGF-β for 6 and 24 h (Fig. 2A). Of interest, TGF-β-induced CTGF expression was significantly reduced in cells cultured at high-cell density (Fig. 2, B and C). Cell density might have affected TGF-β receptor expression. However, Smad2 was phosphorylated upon TGF-β treatment independently of cell density (Fig. 2B). This correlated with translocation of Smad3 to the nucleus, as characterized by immunocytochemistry (data not shown). Furthermore, to circumvent any potential changes in TGF-β signaling as possible cause for reduced CTGF expression at high-cell density, we also used TSA, an inhibitor of histone deacetylases, to directly enable stimulation of CTGF synthesis (23). Similar to the treatment with TGF-β, CTGF expression was strongly upregulated by TSA in subconfluent cells, whereas TSA was without effect when added to dense cells (Fig. 2D).

CTGF is downregulated by hypoxia in human tubular epithelial cells. To analyze the regulation of CTGF under hypoxic conditions, we first incubated primary epithelial cells with the hypoxia-mimicking compound DMOG, which leads to the accumulation of active HIF-1α by inhibition of 2-oxoglutarate-dependent dioxygenases, including HIF prolyl-hydroxylases and the asparaginyl-hydroxylase factor inhibiting HIF-1 (21). Upon treatment with 1 mM DMOG, HIF-1α protein accumulated as expected (Fig. 3A). Concomitantly, CTGF protein expression was significantly reduced (Fig. 3A). Downregulation was consistently observed after incubation for 24 h, whereas the response was variable after 6 h, depending on the cell preparation. The slow downregulation of CTGF gene expression was confirmed at the mRNA level. CTGF mRNA expression was barely affected after 3 h, but was significantly reduced after 6 and 24 h (Fig. 3B).

Cellular, as well as secreted, CTGF were reduced when hPTECs were exposed to hypoxia (1% O₂) or DMOG for 24 h (Fig. 4A). The comparison of multiple cell preparations obtained from different donors revealed some variability in the hypoxic response. A total of 53 experiments were performed with 17 preparations of hPTECs at different passages. Fifteen preparations showed inhibition of CTGF expression by hypoxia, whereas cells obtained from two donors showed slightly increased CTGF levels or no change in CTGF expression (Fig. 4B, “All prep.”). Comparing the methods of cell preparation, cells isolated by outgrowth, which consisted primarily of distal tubular cells, showed a tendency toward stronger inhibition compared with sieved cells [Fig. 4B, 55 ± 16%, eight preparations of hPTECs isolated by outgrowth (O); 71 ± 25%, nine preparations of sieved hPTECs (S); means ± SD]. This was also demonstrated by cells obtained from one donor, where both methods were used in parallel (Fig. 4B, “Prep. #7”).

Hypoxia represses TGF-β-mediated upregulation of CTGF in hPTECs. Downregulation of CTGF expression by hypoxia also affected the responsiveness of the cells to TGF-β. When the cells were preexposed to hypoxia for 24 h and then stimulated with TGF-β for 6 h, CTGF synthesis was significantly reduced compared with cells cultured under normoxic conditions (Fig. 5A). Comparable results were obtained when the cells were incubated with TGF-β during hypoxia treatment (data not shown).

It was shown previously that hypoxia induced TGF-β₁, secretion in human tubular epithelial cells after 24 h (36). In
our preparations, we observed no alteration of TGF-β1 mRNA expression on treatment with DMOG for 3 h and a slight, but not significant, increase after 6 h (Fig. 5B). Exposure to DMOG or hypoxia for 24 h significantly induced TGF-β1 mRNA approximately twofold. Therefore, neither exogenous nor endogenous TGF-β were able to counteract the hypoxia-mediated downregulation of CTGF, which was observed during the first 24 h of exposure to DMOG or low oxygen.

Effects of hypoxia on CTGF expression in vivo. CTGF is barely detectable in tubular epithelial cells in vivo (Fig. 6A). Therefore, we wondered whether CTGF expression was upregulated in tubular epithelial cells by hypoxia in the microenvironment of a functional kidney. Mice were exposed to 8% hypoxia for 8 h and then analyzed for HIF-1α and CTGF expression. Nuclear HIF-1α protein was detectable in a subset of tubuli in the boundary region of cortex and medulla in animals exposed to hypoxia (Fig. 6D), whereas no HIF-1α was detectable in control mice (Fig. 6C). In the same area, more interstitial cells stained positive for CTGF mRNA in hypoxic kidneys (Fig. 6B) than in normoxic kidneys (Fig. 6A), whereas no tubular cells expressed CTGF upon exposure to hypoxia (Fig. 6B). In situ hybridization using the control sense riboprobe did not yield any staining (data not shown). The lack of

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Fig. 4. CTGF expression is reduced upon hypoxic treatment of hPTECs. A: hPTECs were cultured under normoxic conditions (control: C), hypoxia (1% O₂, H), or treated with 1 mM DMOG (D) for 24 h. A representative blot analyzing cellular and secreted CTGF is shown. The graph displays the statistical analysis for CTGF expression of 4 and 6 independent experiments (2 and 4 different preparations) performed under hypoxia and with DMOG, respectively. ***P < 0.001 for treated vs. untreated cells. B: the scatter blot represents the statistical analysis of CTGF expression in hPTECs cultured for 24 h under hypoxia. The scatter blot displays the mean values of CTGF expression of 17 preparations of hPTECs of which 8 were obtained by outgrowth (O) and 9 by sieving (S), as well as single values of 4 and 8 independent experiments performed with preparation 7 cells, which were prepared by both outgrowth and sieving.

Fig. 5. Hypoxia reduces TGF-β-mediated CTGF induction in hPTECs. A: hPTECs were incubated under either normoxic or hypoxic conditions for 24 h followed by 6-h incubation with 2 ng/ml TGF-β under corresponding conditions. A representative blot for the analysis of CTGF is shown. The graph summarizes the results of 8 independent experiments performed with 6 preparations. Values are means ± SD. *P < 0.05 and ***P < 0.001 for hypoxic vs. normoxic cells. B: hPTECs were exposed to 1 mM DMOG or hypoxia for the indicated time periods, and TGF-β mRNA expression was analyzed by real-time RT-PCR. The graph summarizes the results from at least 3 independent experiments for control (C), hypoxia (H), and DMOG (D) (5 preparations of hPTECs for 24 h); control cells were set to 100% for each time point. Values are means ± SD. ***P < 0.001 for 24-h DMOG vs. control cells. *P < 0.05 for 24-h hypoxia vs. normoxia, paired Student t-test.
CTGF induction in tubular cells by hypoxia in vivo was thus consistent with the findings in isolated tubular epithelial cells.

Long-term exposure of hPTECs to hypoxia. Confluent hPTECs were incubated under normoxic or hypoxic conditions for 24, 48, and 72 h. As described above, CTGF expression declined upon prolonged normoxic incubation (Fig. 7A). Continuous accumulation of HIF-1α was observed in cells cultured for up to 72 h under hypoxic conditions (Fig. 7A). Furthermore, BNIP3, a known target gene of HIF-1α (e.g., Ref. 2), was elevated at all time points. Hypoxia reduced CTGF expression during the first 24 h; however, the decrease slowed down subsequently, leading to slightly enhanced CTGF levels after 72 h compared with normoxia (Fig. 7A). Comparable results were obtained upon treatment with DMOG (data not shown).

When hPTECs were cultured in normoxic or hypoxic conditions for 72 h and then treated with TGF-β for 6 h, CTGF expression was slightly increased, but the total levels remained close to the detection limit (Fig. 7B). Prolonged exposure of hPTECs to hypoxia thus did not increase the sensitivity to TGF-β stimulation.

Inhibition of DNA methylation increased the reactivity of the cells in terms of CTGF expression. Cells were treated for 72 h in the presence or absence of 5-aza-2’-deoxuridine and then exposed to DMOG or TGF-β for another 24 h. In 5-aza-2’-deoxuridine-treated cells, CTGF was downregulated by DMOG and highly sensitive to stimulation by TGF-β (Fig. 7C). However, inhibition of DNA methylation was not sufficient to reinduce CTGF expression after it had been downregulated for 72 h under normoxic or hypoxic conditions, reminiscent of the results obtained with TGF-β (Fig. 7B and data not shown).

Recent studies in proximal tubular epithelial cell lines suggested that hypoxia might induce tubular epithelial cells to adopt a mesenchymal phenotype similar to the treatment with TGF-β (28, 39). This transition is believed to be associated with a decrease of the cell-cell adhesion protein E-cadherin and an upregulation of N-cadherin. Using subconfluent and confluent cells, we observed a variable induction of N-cadherin by TGF-β, most prominently in low-density cells (1.5- to 6-fold in 4 different preparations) (Fig. 8A). A tendency toward higher expression of N-cadherin was also detectable on exposure to hypoxia. E-cadherin, by contrast, was not significantly altered by either TGF-β or exposure to hypoxia for up to 72 h.

Interestingly, the cells retained their cadherin phenotype. When analyzed by immunocytochemistry after 24-, 48-, or 72-h exposure to hypoxia, the cells expressed either E-cadherin or N-cadherin, but not both cadherins (Fig. 8B, 72 h). Expression of α-SMA is another marker of activated mesenchymal cells. However, α-SMA was not detectable in hPTECs exposed to long-term hypoxia or upon treatment with TGF-β, indepen-
dently of cell density (Fig. 8A, representative of 4 preparations).

**DISCUSSION**

In this study, we analyzed primary cultures of human tubular epithelial cells in terms of CTGF expression. Our studies provide evidence for a strong dependency of CTGF expression on cell culture conditions: in high-density cells, CTGF expression itself, as well as the inducibility by external stimuli, such as TGF-β, is inhibited. Furthermore, we demonstrate that CTGF expression was repressed in cells exposed to hypoxia.

Using primary tubular epithelial cells obtained from different donors, we observed striking differences in CTGF gene expression in subconfluent and confluent cells. Not only was the basal expression of CTGF strongly dependent on cell density, but also the inducibility by external stimuli, such as TGF-β. With increasing cell density, cells became resistant against upregulation of CTGF. The lacking induction could not be attributed to a downregulation of TGF-β receptors, because Smad2/3 were still activated. Furthermore, it was also observed, when CTGF expression was stimulated by the histone deacetylase inhibitor TSA. These data are in line with the concept that the methylation status of the CTGF promoter plays a role in CTGF expression. Further studies are necessary to analyze the methylation status of the CTGF promoter, not only in cancer cells, but also in tubular epithelial cells under various culture conditions.

In the porcine renal tubular cell line LLC-PK1, loss of the integrity of cell-cell contacts was shown to be essential for TGF-β to induce markers of epithelial-to-mesenchymal transition (30). At the molecular level, removal of calcium as a means to interfere with cell-cell contacts led to an activation of the RhoA/Rho kinase pathway (29). Increased activity of RhoA/Rho kinase signaling may be involved in CTGF expression in subconfluent cells. We have shown earlier that activation of this pathway by alterations of the cytoskeleton activates CTGF expression via the transcription factor, serum response factor (32). Furthermore, tyrosine kinases seem to be involved in the repression of CTGF in dense cells, because preliminary data show that the tyrosine kinase inhibitor genistein prevents cell density-dependent downregulation of CTGF (unpublished observation). Further studies are under way to analyze the molecular mechanisms of the cell density-dependent regulation of CTGF expression.

If one assumes that cells cultured at high density more closely represent tubular cells in vivo than cells cultured at low density do, our in vitro data would implicate that CTGF expression may not be easily upregulated in tubular epithelial cells in vivo. In line with this prediction, CTGF expression is barely observed in healthy human tubuli, but detectable in severely damaged tubular cells (36). In a detailed study of posttransplant biopsies analyzed by microarrays, unexpectedly CTGF expression was reduced in biopsies with tubulointerstitial damage (43). Furthermore, CTGF mRNA was primarily expressed in α-SMA-positive cells in the interstitium in biop-
sies obtained from patients with interstitial injuries of different origin (20). This implicates that upregulation of CTGF may be more restricted in tubular epithelial cells than in fibroblasts. This was confirmed in our study in kidneys of mice exposed to low-oxygen tension (8%) for 8 h. While no CTGF mRNA was detected in tubular cells, some interstitial cells stained positive for CTGF. It will be interesting to define further genes, which are sensitive to cell density in tubular epithelial cells in vitro, and to study their reactivity in renal tissues.

Hypoxic regulation of CTGF has been studied in several cell types, leading to apparently contradictory results. In most cell types investigated, CTGF was upregulated, e.g., endothelial (25) and chondrosarcoma cells (24). Inconsistently, hypoxic upregulation of CTGF was observed in human breast cancer cell line MDA-231 (26, 40), whereas a lack of change was reported in MCF-7 breast cancer cells (10). Upregulation was also described in murine tubular epithelial cells (17), which is in contrast to downregulation of CTGF observed in human tubular cell lines (26), or in primary cell cultures analyzed by gene expression microarray (7). In this study, we analyzed a total of 17 different preparations of tubular cells obtained from healthy parts of renal cortical tissue removed in the course of tumor nephrectomies. Exposure of the cells to low-oxygen tension or treatment with the prolyl-hydroxylase inhibitor DMOG consistently reduced CTGF expression. Not unexpectedly, the degree of CTGF reduction varied between preparations obtained from different donors. Decreased levels of CTGF were observed in 15 preparations, whereas an increase (maximally 25% in 24 h) was observed in two preparations. Cells obtained by outgrowth were slightly more responsive to the suppressive effect of hypoxia than cells obtained by sieving. Whether this was due to the higher content of distal tubular cells in preparations obtained by outgrowth, or rather related to differences in isolation procedures is not clear. Outgrowth of epithelial cells from tissue samples was a slow process, which required ~14 days until cells were ready for further analysis compared with availability of cells within 5 days after sieving. Reactivity of the cells in terms of CTGF regulation was not correlated with the passage of cells used (passages 1–4), and it could not be attributed to other experimental details, such as the culture time (2–3 days) before exposure to hypoxia or treatment with DMOG. Tissue samples were taken from the cortical part of the kidneys, but varied with respect to their exact localization, which may add to the different reactivity of the cells to hypoxia. It is well known that the sensitivity to hypoxia varies in different regions of the kidney, with the proximal tubulus and the outer medulla being most sensitive to hypoxic injury (4). Collectively, our data clearly indicate that, in line with results that our laboratory previously obtained in human proximal tubular cell lines HKC-8 and HK-2 (27), CTGF is not a positively regulated HIF target in human tubular epithelial cells.

Downregulation of CTGF expression by hypoxia and up-regulation by TGF-β were consistently detected within 24 h. To detect phenotypic alterations of tubular epithelial cells, which might be connected with a mesenchymal phenotype, the cells were also incubated for 72 h and compared with cells cultured for shorter periods of times. The expression of CTGF dramatically dropped over time, most likely due to alterations in cell density, as discussed above. While CTGF was clearly downregulated during the first 24 h of exposure to hypoxia,
prolonged exposure to hypoxia attenuated the downregulation. Selective analysis of the 72-h time point might have interpreted the attenuation as induction. Multiple factors may have contributed to the enhanced CTGF levels in long-term hypoxia compared with cells cultured under normoxic conditions. Most obviously, we observed attenuated cell growth upon exposure to hypoxia for 72 h, leading to reduced cell density in hypoxic cells (data not shown). Given the strong cell density dependence of CTGF expression, this may contribute to the delayed reduction of CTGF expression in hypoxic compared with normoxic cells, as shown in Fig. 7A. Furthermore, upregulation of TGF-β by hypoxia or DMOG in hPTECs may contribute to increased CTGF expression over time. Hypoxia may also affect CTGF expression via alterations of the cytoskeleton, as demonstrated in alveolar epithelial cells (3). Molecular mechanisms were not investigated further, because CTGF expression levels under either normoxic of hypoxic conditions were too low after 72 h.

Expression of α-SMA is often considered a hallmark of mesenchymal transition. Upregulation of α-SMA upon exposure to hypoxia was described in tubular epithelial cells obtained from young mice (18). In the porcine renal tubular cell line LLC-PK1, mechanical injury, i.e., loss of cell-cell contacts, was shown to be a prerequisite for the TGF-β-mediated induction of α-SMA (11, 30). Therefore, we tested both subconfluent and confluent cells, but never detected upregulation of α-SMA in human tubular epithelial cells upon hypoxic incubation or after treatment with TGF-β for up to 72 h. Compared with murine epithelial cells or certain human cell lines, primary cultures of human tubular epithelial cells seem to be more resistant toward phenotypic alterations (28).

This was also true in terms of E-cadherin expression, the loss of which has been associated with epithelial-to-mesenchymal transition, especially in tumor cells. E-cadherin protein expression was reduced in some preparations, with upregulation being observed in other preparations. Immunocytochemical examination gave no indication of a loss of E-cadherin in a certain cell population when treated with TGF-β or exposed to hypoxia. By contrast, N-cadherin synthesis was upregulated by TGF-β and to a lesser extent in cells exposed to hypoxia. It was interesting to note that no phenotypic alteration was observed, i.e., there was no evidence for a switch from E-cadherin to N-cadherin, which is often observed in tumor progression (17).

Taken together, our data indicate that hypoxia alone is not sufficient to transform human primary epithelial cells into mesenchymal cells within 72 h. Furthermore, exposure of the cells to hypoxia reduced the susceptibility of the cells to TGF-β in terms of CTGF induction. Our data neither exclude a role for hypoxia in chronic kidney disease, nor do they contradict CTGF being involved in chronic kidney injury or ischemia-reperfusion-related injuries. However, they argue against a direct link between increased levels of HIF-1α and increased levels of CTGF. As shown in mice, chronic overexpression or stabilization of HIF-1α under normoxic conditions may lead to CTGF induction (22). Our data suggest that more complex molecular processes, most likely involving interaction between different cell types within the kidney, may contribute to the phenotypic changes observed in these transgenic mice, as well as to the functional alterations typical of chronic tubulointerstitial injury associated with hypoxia.

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DISCLOSURES
I am not aware of financial conflict(s) with the subject matter or materials discussed in this manuscript with any of the authors, or any of the authors’ academic institutions or employers.

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