Oncostatin M-induced effects on EMT in human proximal tubular cells: differential role of ERK signaling

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Oncostatin M (OSM) is a multifunctional cytokine that belongs to the interleukin-6 (IL-6) family, which includes IL-6, IL-11, leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-1), and novel neurotrophin-1/B cell-stimulating factor-3 (NNT-1/BSF-3) (54, 63). Among the family members, OSM is most closely related to LIF and is able to utilize the LIF receptor in addition to its specific human OSM receptor. Human OSM is secreted as a glycoprotein monomer of approximately 28 kDa, mainly from activated T cells, monocytes, and neutrophils (54). In adult rodent species, it is constitutively expressed in tissues such as pancreas, kidney, testes, spleen, stomach, and brain but not in liver or lung (63). While OSM was originally recognized by its activity to inhibit the proliferation of tumor cells, accumulating evidence now indicates that OSM exhibits many unique biological activities in inflammation, remodeling of extracellular matrix, hematopoiesis, organ development, and regeneration (13, 54). In addition, it has recently been proposed that OSM might be involved in renal proximal tubular epithelial-mesenchymal transition (EMT), a phenotypic conversion that is fundamentally linked to the pathogenesis of renal interstitial fibrosis (35).

Tubular EMT represents a biological process, which is induced and/or promoted by pro-EMT cytokines such as transforming growth factor-β1 (TGF-β1), epidermal growth factor (EGF), and fibroblast growth factor-2 (FGF-2) after tubular injury and/or inflammation (20, 25, 32). Injured tubules undergo apoptosis, leading to tubular atrophy, and, under the influence of pro-EMT factors, some tubular epithelial cells acquire mesenchymal properties similar to those of fibroblasts and myofibroblasts (9, 10, 25, 32). Evidence is accumulating that these mechanisms contribute to the pool of cells, which are directly involved in tubulointerstitial fibrogenesis in vivo. The existence of EMT was first demonstrated in a mouse model of antitubular basement membrane disease utilizing fibroblast-specific protein (FSP1; S100A4) as a marker (52). Subsequent studies provided morphological and phenotypic evidence for the occurrence of EMT in remnant kidneys after 5/6 nephrectomy (33) as well as in obstructive nephropathy induced by unilateral urethral obstruction (59, 60). In accordance with the results of these animal studies, EMT was also observed in human renal biopsy tissues (19, 43). By genetically tagging renal proximal tubules, Iwano et al. (18) found that, after obstructive injury, LacZ-tagged epithelia exhibited abnormal morphology, became disorganized, and moved into the interstitium. To model the effects of an EMT microenvironment consisting of multiple cytokines in vitro, Healy et al. (16) stimulated human tubular epithelial cells with activated peripheral blood mononuclear cell (PBMC)-conditioned medium and described alterations in renal epithelial cell differentiation indicative of EMT. With the utilization of a similar approach, it has been demonstrated recently that OSM-specific receptor β-subunit (OSMRβ) expression is upregulated by activated EMT in human proximal tubular cells.

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ONCOSTATIN M (OSM) IS A MULTIFUNCTIONAL CYTOKINE THAT BELONGS TO THE INTERLEUKIN-6 (IL-6) FAMILY, WHICH INCLUDES IL-6, IL-11, LEUKEMIA INHIBITORY FACTOR (LIF), CILIARY NEUROTROPHIC FACTOR (CNTF), CARDIOTROPHIN-1 (CT-1), AND NOVEL NEUROTROPHIN-1/B CELL-STIMULATING FACTOR-3 (NNT-1/BSF-3) (54, 63). AMONG THE FAMILY MEMBERS, OSM IS MOST CLOSELY RELATED TO LIF AND IS ABLE TO UTILIZE THE LIF RECEPTOR IN ADDITION TO ITS SPECIFIC HUMAN OSM RECEPTOR. HUMAN OSM IS SECRETED AS A GLYCOPROTEIN MONOMER OF APPROXIMATELY 28 KD, MAINLY FROM ACTIVATED T CELLS, MONOCYTES, AND NEUTROPHILS (54). IN ADULT RODENT SPECIES, IT IS CONSTITUTIVELY EXPRESSED IN TISSUES SUCH AS PANCREAS, KIDNEY, TESTES, SPLEEN, STOMACH, AND BRAIN BUT NOT IN LIVER OR LUNG (63). WHILE OSM WAS ORIGINALLY RECOGNIZED BY ITS ACTIVITY TO INHIBIT THE PROLIFERATION OF TUMOR CELLS, ACCUMULATING EVIDENCE NOW INDICATES THAT OSM EXHIBITS MANY UNIQUE BIOLOGICAL ACTIVITIES IN INFLAMMATION, REMODELING OF EXTRACELLULAR MATRIX, HAMATOPOIESIS, ORGAN DEVELOPMENT, AND REGENERATION (13, 54). IN ADDITION, IT HAS RECENTLY BEEN PROPOSED THAT OSM MIGHT BE INVOLVED IN RENAL PROXIMAL TUBULAR EPITHELIAL-MESENCHYMAL TRANSITION (EMT), A PHENOTYPIC CONVERSION THAT IS FUNDAMENTALLY LINKED TO THE PATHOGENESIS OF RENAL INTERSTITIAL FIBROSIS (35).

TUBULAR EMT REPRESENTS A BIOLOGICAL PROCESS, WHICH IS INDUCED AND/OR PROMOTED BY PRO-EMT CYTOKINES SUCH AS TRANSFORMING GROWTH FACTOR-β1 (TGF-β1), EPIDERMAL GROWTH FACTOR (EGF), AND FIBROBLAST GROWTH FACTOR-2 (FGF-2) AFTER TUBULAR INJURY AND/OR INFLAMMATION (20, 25, 32). INJURED TUBULES UNDERGO APOPTOSIS, LEADING TO TUBULAR ATROPHY, AND, UNDER THE INFLUENCE OF PRO-EMT FACTORS, SOME TUBULAR EPITHELIAL CELLS ACQUIRE MESENCHYMAL PROPERTIES SIMILAR TO THOSE OF FIBROBLASTS AND MYOFIBROBLASTS (9, 10, 25, 32). EVIDENCE IS ACCUMULATING THAT THESE MECHANISMS CONTRIBUTE TO THE POOL OF CELLS, WHICH ARE DIRECTLY INVOLVED IN TUBULOINTERSTITIAL FIBROGENESIS IN VIVO.

THE EXISTENCE OF EMT WAS FIRST DEMONSTRATED IN A MOUSE MODEL OF ANTITUBULAR BASEMENT MEMBRANE DISEASE UTILIZING FIBROBLAST-SPECIFIC PROTEIN (FSP1; S100A4) AS A MARKER (52). SUBSEQUENT STUDIES PROVIDED MORPHOLOGICAL AND PHENOTYPIC EVIDENCE FOR THE OCCURRENCE OF EMT IN REMNANT KIDNEYS AFTER 5/6 NEXPHRECTOMY (33) AS WELL AS IN OBSTRUCTIVE NEPHROPATHY INDUCED BY UNILATERAL URETHRAL OBSTRUCTION (59, 60). IN ACCORDANCE WITH THE RESULTS OF THESE ANIMAL STUDIES, EMT WAS ALSO OBSERVED IN HUMAN RENAL BIOPSY TISSUES (19, 43). BY GENETICALLY TAGGING RENAL PROXIMAL TUBULES, IWANO ET AL. (18) FOUND THAT, AFTER OBSTRUCTIVE INJURY, LACZ-TAGGED EPITHELIA EXHIBITED ABNORMAL MORPHOLOGY, BECAME DISORGANIZED, AND MOVED INTO THE INTERSTITIUM. TO MODEL THE EFFECTS OF AN EMT MICROENVIRONMENT CONSISTING OF MULTIPLE CYTOKINES IN VITRO, HEALY ET AL. (16) STIMULATED HUMAN TUBULAR EPITHELIAL CELLS WITH ACTIVATED PERIPHERAL BLOOD MONONUCLEAR CELL (PBMC)-CONDITIONED MEDIUM AND DESCRIBED ALTERATIONS IN RENAL EPITHELIAL CELL DIFFERENTIATION INDICATIVE OF EMT. WITH THE UTILIZATION OF A SIMILAR APPROACH, IT HAS BEEN DEMONSTRATED RECENTLY THAT OSM-SPECIFIC RECEPTOR β-SUBUNIT (OSMRβ) EXPRESSION IS UPREGULATED BY ACTIVATED ERK SIGNALING.

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PBMC-conditioned medium (35). When applied in a medium containing EGF, insulin, and fetal calf serum, OSM-induced Jak/Stat signaling was associated with the induction of morphological alterations of human proximal tubular cells, with the reduction of E-cadherin expression as well as with the induction of α-smooth muscle actin (α-SMA) expression and fibronectin synthesis (35), suggesting that, in a mitogen-stimulated background, OSM is able to induce cellular events indicative of tubular EMT.

Structural and functional alterations in epithelial tight junctions (TJ) and adherens junctions (AJ), loss of epithelial cytokeratins, rearrangement of actin stress fibers as well as expression of FSP1 (S100A4), vimentin, interstitial collagens, and occasionally α-SMA mark the morphological transition of epithelial cells into fibroblasts (23, 32). The early loss of E-cadherin expression, for example, has been described as one hallmark of tubular EMT both in vitro and in vivo, which is later followed by enhanced migration and invasion of myofibroblasts into the peritubular interstitium (1, 3, 4, 5, 25, 40). Moreover, TGF-β1-induced EMT was associated with reduced expression of ZO-1 (39, 58) and E-cadherin (58, 59). However, in vivo, N-cadherin seems to be the predominant classic cadherin in the proximal tubule of human and rat (42, 55), and the TJ protein claudin-2 is abundantly expressed in the proximal tubule but is essentially absent in most other nephron segments (11, 44). Therefore, we decided to investigate the effects of OSM on the two AJ proteins E-cadherin and N-cadherin, the TJ protein claudin-2, and the EMT marker proteins vimentin, collagen type I, and FSP1 in human proximal tubular cells. In the course of this analysis, we assessed OSM-induced intracellular signaling pathways with a specific emphasis on the three ERK isoforms ERK1, ERK2, and ERK5. The results show that, when administered as the only ligand in the absence of any additional growth supplements, OSM attenuates N-cadherin, E-cadherin, and claudin-2 expression while increasing vimentin, collagen type I, and FSP1 expression. Associated with these effects, OSM induces HK-2 cell scattering in three-dimensional (3D) collagen matrices but inhibits basal and mitogen-stimulated HK-2 cell proliferation without affecting cell viability. Moreover, human recombinant OSM is a strong activator of the mitogen-activated protein kinases ERK1, ERK2, and ERK5 in these cells. The synthetic MEK1/2 inhibitors U0126 and CI-1040 blocked basal and OSM-induced ERK1/2 phosphorylation but neither ERK5 nor Stat1/3 when used at concentrations of 10 and 1 μM, respectively. Long-term experiments utilizing these pharmacological inhibitors provided evidence for a differential role of ERK1/2 signaling on N-cadherin and claudin-2 expression in HK-2 cells. While OSM-induced ERK1/2 activity is involved in the inhibition of N-cadherin expression, functional MEK1/2-ERK1/2 signaling seems to be necessary for basal claudin-2 expression.

MATERIALS AND METHODS

Reagents and antibodies. Cell culture reagents were obtained from Gibco (Life Technologies, Lofer, Austria). U0126 was purchased from Calbiochem (Nottingham, UK). OSM and EGF were purchased from Sigma (St. Louis, MO) or Peprotech, bovine pituitary extract (BPE) from Gibco or Clonetics, and anti-human gp-130 monoclonal antibody (MAB628) from R&D Systems. All other reagents were obtained from Sigma.

Cell culture. Human kidney 2 (HK-2) cells were cultured in keratinocyte-serum-free medium (KSFM) containing 10% FBS, 5 ng/ml recombinant epidermal growth factor (rEGF), 0.05 mg/ml BPE, 100 U/ml penicillin, and 100 μg/ml streptomycin (46, 47). The cells were grown at 37°C in a humidified 5% CO2 atmosphere and split at a 1:5 ratio once a week. After growth to a subconfluent state, cells were washed once, made quiescent by incubation in serum- and supplement-free medium for 48 h, and then used for experiments. Stimulations with ligands such as OSM, LIF, epidermal growth factor (EGF), transforming growth factor-β1 (TGF-β1), or combinations thereof were performed in the absence of serum and any other growth supplements.

LLC-PK1/D+/D- and LLC-PK1/D- cell clones were a kind gift of Dr. A. Wohlwend (Univ. of Geneva Medical School, Geneva, Switzerland). They were grown in Eagle’s MEM supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 1.2 mg/ml NaHCO3, and 5% FBS at 37°C in a humidified 5% CO2 atmosphere, and split at a 1:10 ratio, once a week (56).

Scanning assay in 3D collagen gels. HK-2 cells were harvested by trypsinization from confluent cultures, resuspended in KSFM, and mixed with a type I collagen solution prepared as described (28). Aliquots of the cell suspension were then dispensed into the wells of multwell plates (Nunc, Kamstrup, Roskilde, Denmark). After a 10-min incubation at 37°C to allow collagen gelation, KSFM was added above the gels with or without 10% FCS and the indicated supplements (50 μg/ml BPE and/or 5 ng/ml rEGF), and the cultures were either left untreated or treated with 10 ng/ml OSM. For experiments designed to study the effect of OSM on preformed colonies, HK-2 cells were grown in a 1:1 mixture of KSFM and advanced DMEM/F12 (cat. No. 12634, Invitrogen Gibco) supplemented with 10% FCS (in the absence of BPE and EGF). Media and treatments were renewed every 2–3 days. After 7–10 days, the cultures were monitored using a Nikon Diaphot TMD inverted photomicroscope. For quantitative analysis of 3D scattering, 10 randomly selected colonies per well in each of 3 independent experiments (i.e., a total of 30 colonies/experimental condition) were inspected and scored as “scattered” when comprising at least 5 isolated cells. Data are expressed as mean percentage of scattered colonies ± SE, and statistical significance was determined using the Student’s unpaired t-test.

Flow cytometric DNA analysis. The effect of OSM treatment on the viability of HK-2 cells was investigated by the dye exclusion method, using propidium iodide (PI; Sigma) staining of unfixed cells (8). For this purpose, cells were serum and supplement starved for 48 h, then stimulated with 10 ng/ml OSM for 4 days and compared with unstimulated controls. As a positive control, apoptosis was induced by the addition of 20% DMSO for 24 h. After the experiment, culture medium was collected, the cells were harvested in 0.02% EDTA solution containing 0.1% trypsin and then resuspended in KSFM containing 10% FCS. The cell suspensions (1 × 106 cells) were then added to their respective culture medium, pelleted, resuspended in 500 μl cold PBS containing 1% BSA, and stained with PI at a concentration of 0.2 mg/ml. Samples were measured by FACScan flow cytometer in FL2 and analyzed by CellQuest software (Beckton Dickinson).

Cell proliferation assay. The number of cells per square centimeter of culture dish was evaluated by light microscopy utilizing an inverted microscope equipped with a CCD camera (Siemens C725) connected to a videographic printer (Sony UP-890 CE) and a superimposed test frame as described previously (27, 49). For determination of cell number, HK-2 cells were made quiescent by 48 h of incubation in serum- and supplement-free KSFM. Thereafter, the medium was changed and cells were stimulated with 10 ng/ml human recombinant OSM and/or 50 μg/ml BPE on days 0 and 2 compared with unstimulated controls. One measurement (n = 1) represents the mean of cells per square centimeter determined from 10 videoprints randomly taken from one petri dish at days 0, 2, and 4.

For the measurement of cell proliferation in 3D collagen gels, HK-2 cells were suspended in collagen gels in KSFM supplemented
with FCS, BPE, and EGF. Twenty-four hours later, triplicate wells were either left untreated or treated with OSM (10 ng/ml). After 6 days, collagen gels were digested with 4 mg/ml Clostridium histolyticum collagenase (Worthington Biochemical, Freehold, NJ). The released cell clumps were dissociated with 3 mM EDTA, and the released cells were counted with a hemocytometer.

**Western blot analysis.** Cells were washed with ice-cold PBS and lysed in ice-cold RIPA lysis buffer [50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 50 mM NaF, 5 mM EDTA, 0.5% deoxycholic acid, 40 mM β-glycerophosphate, 1 mM sodium orthovanadate, 10% protease inhibitor cocktail for mammalian tissues (Sigma), 0.1% SDS, 1% Triton X-100] for 20 min at 4°C. Insoluble material was removed by centrifugation at 12,000 g for 15 min at 4°C. For the examination of E-cadherin, N-cadherin, and claudin-2 protein expression, cells were harvested into 1× Laemmli buffer, and total cell homogenates were analyzed. The protein content was determined using a microbicinichinic acid assay (Pierce) or Coomassie protein assay (Pierce) with BSA as a standard. Cell lysates were matched for protein, separated on 10% (7.5% for N-cadherin and E-cadherin) SDS-PAGE, and transferred to a polyvinylidene difluoride membrane. Subsequently, membranes were stained with one of the following specific antibodies: mouse anti-E-cadherin and mouse anti-N-cadherin (Transduction Laboratories, San Diego, CA); rabbit anti-claudin-2 (Zymed Laboratories, San Francisco, CA); mouse anti-β-actin (Sigma); rabbit anti-phospho MEK1/2, which detects usually phosphorylated MEK1/2 (Ser217/Ser221), rabbit anti-phospho-ERK1/2, which detects phosphorylated Thr202/Tyr204 of both ERK1 and ERK2, and rabbit anti-phospho-ERK5, which recognizes usually phosphorylated ERK5 (Thr218/Tyr220) (all Cell Signaling Technology); rabbit anti-MEK1, rabbit anti-MEK2, goat anti-ERK5, and goat anti-Erk2 (all Santa Cruz Biotechnology, Santa Cruz, CA); rabbit anti-phospho Stat1 (Cell Signaling Technology), which detects phosphorylated Stat1α and -Stat1β (Tyr 705); rabbit anti-phospho Stat3 (Cell Signaling Technology), detecting phosphorylated Stat3α and -Stat3β (Tyr 705); rabbit anti-Stat1 (Cell Signaling Technology); and rabbit anti-Stat3 (Cell Signaling Technology). After extensive washing of the sheets in TBS, 0.1% Tween 20, the primary antibodies were detected using horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology), goat anti-mouse IgG (for analysis of E-cadherin protein expression, Sigma) or goat anti-goat IgG (for analysis of ERK2 and ERK5 protein expression, Santa Cruz Biotechnology), visualized by Lumiglo Western Blot Detection system (Cell Signaling Technology).

**Northern blot analysis.** Total RNA was isolated from cultured cells with TRI-Reagent (Molecular Research Center, Cincinnati, OH), according to the manufacturer’s instructions. RNA samples (25 μg/ lane) were electrophoresed on a 0.95% agarose/1.6% formaldehyde gel (51), transferred to GeneScreen Plus membranes (New England Nuclear), and hybridized with a 32P-labeled human E-cadherin cDNA probe (generous gift of Dr. Frans Van Roy, University of Ghent, Ghent, Belgium) (31). Signals were detected by autoradiography at −80°C. Sample integrity and equal loading were monitored by staining with ethidium bromide after electrophoresis.

**RNA isolation and real-time PCR.** HK-2 cells were made quiescent by incubation for 48 h in serum- and supplement-free medium. Cells were then incubated for 6, 12, and 24 h with OSM at a concentration of 10 ng/ml. Moreover, in dose-response experiments, the 10 ng/ml dose was compared with 1 and 50 ng/ml doses after stimulation for 6 h. In selected experiments, cells were coincubated with U0126 (10 μM), Cells stimulated with either medium, U0126 (10 μM), or TGF-B1 (10 ng/ml) served as controls. All experiments were performed in triplicate and repeated at least three times. Total RNA from cell culture experiments was extracted using the phenol-guanidine isothiocyanate reagent RNA-Be (Tel-Test, Friendswood, TX). Oligo dT-primed reverse transcription was performed at 42°C for 50 min after denaturation of the RNA at 70°C for 10 min. E-cadherin, collagen type I, vimentin, and GAPDH PCR of reverse-transcribed RNA (3 μg) was performed using the following primer sets:

GAPDH forward: 5’-CCC TTC ATT GAC CTC AAC TAC 3’
GAPDH reverse: 5’-TGA GTC CTT CCA CGA TAC C-3’
Vimentin forward: 5’-GAT TCT TCC GCT TCT TCT TCC AAA CTVT-3’
Vimentin reverse: 5’-GGG TAT CAA CCA GAG GGA GTG A-3’
E-cadherin forward: 5’-CCA ACG GGA ATG CAG TTG A-3’
E-cadherin reverse: 5’-TGA ATT CGG GTT GTG TGT CA-3’
Collagen I forward: 5’-CAA TGC TGC CCT TTG TGC TCC TTT-3’
Collagen I reverse: 5’-CAC TTG GGT GTG TGA GCA TTA TTT-3’

Real-time PCR was performed using Stratagene Mx3000P with the following reagents (all Stratagene, La Jolla, CA); the reaction mixture consisted of 12.5 μl of real-time PCR Master Mix and 0.25 μl of each primer (100 nM), 0.375 μl Rox dye solution, and 1 μl probe solution, and the final volume of the mixture was adjusted to 25 μl with the addition of DNase- and RNase-free H2O. Amplification was started at 95°C for 30 s as the first step, followed by 40 cycles of PCR at 95°C for 60 s, at 62°C for 60 s, and at 72°C for 60 s, respectively. The final product was extended and finalized at 95°C for 60 s, 62°C for 30 s, and 95°C for 30 s. Results are given relative to unstimulated controls (calibrator).

**Indirect immunofluorescence.** To confirm RNA and Western blot findings, indirect immunofluorescence stainings were performed for claudin-2, E-cadherin, N-cadherin, vimentin, and S100A4 as described earlier (53). HK-2 cells were cultured as described above. Cells were stimulated with 10 ng/ml OSM or were left untreated for 48 h. Incubation in the presence of TGF-β1 (10 ng/ml) served as the positive control (data not shown). All primary antibodies were used in a concentration of 1:50 and an incubation time of 60 min. The secondary rhodamine-labeled antibodies were added sequentially at a concentration of 1:20. After several washings, nuclear counterstaining was performed by DAPI (1:50) for 2 min, before slides were mounted. Pictures were taken either by double or by sequential exposure using a Zeiss camera and software (MC 200 Chip). Negative controls consisted of substitution of the primary antibody with an irrelevant rabbit polyclonal antibody.

**Statistical analyses.** All values are expressed as means ± SE. One-way ANOVA was used to determine statistical differences between growth factor-treated groups and controls using Sigma-Stat software 3.11 (Jandel Scientific, San Rafael, CA) for quantitative RT-PCR or SPSS for Windows software 15.0 (SPSS, Chicago, IL) for the cell proliferation assay. Bonferroni’s method was used to control for multiple testing. P values <0.05 were considered significant. Data of the scattering assay (Table 1) were expressed as mean percentage

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Values are means percentage of scattered colonies ± SE. BPE, bovine pituitary extract. HK-2 cells were suspended in collagen gels in keratinocyte-seum-free medium (KSFM) with or without 10% FCS and the indicated supplements and either left untreated or treated with oncostatin M (OSM; 10 ng/ml) every 2–3 days. After 10 days, the cultures were monitored for induction of 3-dimensional scattering. Ten randomly selected colonies per well in each of 3 independent experiments (i.e., a total of 30 colonies/experimental condition) were inspected using a Nikon Diaphot TMD inverted photomicroscope. A colony was scored as “scattered” when comprising at least 5 isolated cells. *P < 0.0025 vs. control (KSFM + FCS + BPE without OSM) by Student’s unpaired t-test. †P < 0.0125 vs. control (KSFM + FCS + BPE + EGF without OSM) by Student’s unpaired t-test.
of scattered colonies ± SE, and statistical significance was determined using Student’s unpaired t-test. Data of the cell proliferation assay in 3D collagen gels represent the mean number of cells per well ± SE from four independent experiments (n = 12) and were compared using Student’s unpaired t-test.

RESULTS

OSM inhibits expression of E-cadherin, N-cadherin, and claudin-2 in human proximal tubular cells. As structural and functional alterations in epithelial AJs and TJs are important components in the genesis of EMT, we first studied the expression pattern of E-cadherin, N-cadherin, and claudin-2 in human kidney-2 (HK-2) cells both in the absence and in the presence of OSM. HK-2 cells show a low expression of E-cadherin, which increases in a time-dependent fashion after cell seeding, while both N-cadherin and claudin-2 are highly expressed in subconfluent and confluent HK-2 cells (Fig. 1A). In contrast to these proximal tubular cells of human origin, two subclones of LLC-PK1 cells, which represent porcine proximal tubular cells, express E-cadherin at much higher levels (Fig. 1A) compared with both N-cadherin (Fig. 1A) and claudin-2 (Fig. 1A). Interestingly, LLC-PK1 D− cells, which are unable to build domes (D−) and have a low transepithelial resistance (TER) of 37 ± 9 Ω-cm² (55, 56), show the highest expression of E-cadherin (Fig. 1A), LLC-PK1 D+ cells, which are able to build domes (D+) and show a high TER of 230 ± 16 Ω-cm² (55, 56), express E-cadherin at a much lower level when compared with subclone LLC-PK1 D−, suggesting that E-cadherin expression alone does not determine the capability of these cells to form domes, nor their potential to establish a high TER. Moreover, 10 ng/ml OSM inhibited the expression of E-cadherin, N-cadherin, and claudin-2 in HK-2 cells after 48 and 96 h of incubation while β-actin expression remained unaltered (Fig. 1B). These inhibitory effects of OSM on E-cadherin protein expression were corroborated at the mRNA level by both Northern blot analysis (data not shown) and quantitative PCR. PCR analysis revealed a concentration-dependent inhibition of E-cadherin mRNA expression with a maximum downregulation to 23.3 ± 17.4% at 10 ng/ml OSM after 6 h compared with basal expression levels. This down-regulation was not significantly affected by addition of the synthetic MEK1/2 inhibitor U0126 (data not shown).

OSM-induced expression of collagen type I and vimentin in HK-2 cells. EMT is not only characterized by the repression of epithelial proteins but by the emergence of the EMT proteome as well. The latter includes expression of collagen type I and vimentin. Quantitative real-time PCR analysis revealed an OSM-induced, time- and concentration-dependent increase in the expression of collagen type I and vimentin, which peaked after 6 h and returned to baseline values after 24 h (data not shown). OSM (50 ng/ml) had the most robust effect on collagen type I expression whereas a concentration of 10 ng/ml induced vimentin the most. Figure 2 displays the results obtained with OSM at a concentration of 10 ng/ml after 6 h of incubation. OSM led to a significant induction of both collagen type I mRNA (Fig. 2A) as well as vimentin mRNA (Fig. 2B) in HK-2 cells. For the induction of both mesenchymal marker proteins, 10 ng/ml TGF-β1 served as a positive control (Fig. 2). Neither the OSM-mediated stimulation of collagen type I nor that of vimentin was significantly altered by coincubation with 10 µM synthetic MEK1/2 inhibitor U0126 (Fig. 2).

To confirm the mRNA data and the results obtained by Western blot analysis, we performed indirect immunofluorescence stainings of HK-2 cells for claudin-2, E-cadherin, N-cadherin, vimentin, and S100A4 in the absence and in the presence of 10 ng/ml OSM. Representative results are illustrated in Fig. 3. After 48 h of incubation in the presence of OSM, HK-2 cell staining revealed a robust downregulation of claudin-2 (Fig. 3B), E-cadherin (Fig. 3D), and N-cadherin (Fig. 3F), whereas expression of vimentin (data not shown) and S100A4 (Fig. 3H) was stimulated.

OSM effects on HK-2 cell adhesion and proliferation. We next asked whether the OSM-mediated repression of cell-cell
in independent experiments, all performed in triplicate. OSM is a protein that promotes the organization of epithelial cell into 3D extracellular matrix environments, and can induce epithelial-mesenchymal transition (EMT) in a 3D extracellular matrix environment. When grown in collagen gels, HK-2 cells can form compact colonies (Fig. A). Cell scattering was specifically induced by OSM, since the related cytokines LIF, IL-6, and IL-11 had no ostensible effect (data not shown), and was inhibited by antibodies against the OSM receptor subunit gp130 (data not shown). When grown in collagen gels in serum-free medium without BPE and EGF, HK-2 cells proliferated slowly and formed smaller cell clusters (Fig. B). Remarkably, however, even under these conditions of growth factor deprivation, OSM was able to induce 3D scattering (Fig. B). The results of these 3D scattering assays are summarized in Table 1. In serum-free medium, 10 ng/ml OSM induced 3D scattering in the absence of any supplement, as well as in the presence of EGF or of a combination of EGF and BPE. In contrast, in the presence of BPE alone, OSM had only a marginal effect. In serum-containing medium, 10 ng/ml OSM led to 3D scattering under all conditions tested (Table 1). Quantification of cell numbers in 3D collagen gels revealed only minimal inhibition of cell growth in the presence of OSM (4.29 ± 0.36 cells; n = 12) compared with untreated controls (4.5 ± 0.33 cells; n = 12), with values of OSM-treated cultures being not significantly different from those of control cultures (P < 0.05).

To determine whether OSM is also able to promote the disaggregation of preformed multicellular colonies, HK-2 cells were grown in a collagen gel in a richer medium (i.e., a 1:1 mixture of KSF and advanced DMEM/F12), allowing the formation of larger and more compact colonies than KSF alone. After the cells were grown for 6 days to allow colony formation, they were either left untreated (Fig. 6A) or treated with 10 ng/ml OSM (Fig. 6B) for an additional 7 days. Under these conditions, OSM induced partial disintegration of the established colonies, with extension of invasive cords and migration of single cells throughout the collagen matrix (Fig. 6B).

With respect to HK-2 cell proliferation, both EGF and BPE have been reported to act as essential mitogens for these cells (46, 47). However, in the present study we observed that HK-2 cells proliferate even in the absence of growth supplements such as FBS, recombinant EGF or BPE (Fig. 7). When HK-2 cells were cultured for 48 h in the absence of growth supplements (d0 in Fig. 7) such as EGF, BPE, or FBS and were then grown for additional 2 or 4 days under these conditions, their cell number per square centimeter increased 4.15-fold from 17.83 + 2.16 (d0; n = 12) to 75.73 ± 7.73 (d4; n = 12; P < 0.005), suggesting that, even in the absence of mitogens, HK-2 cells do have a basal proliferation rate (Fig. 7). When growth supplement-starved HK-2 cells were stimulated with 10 μg/ml FBS, cell number per square centimeter increased 5.12-fold from a value of 19.58 ± 1.07 (d0; n = 12) to 100.3 ± 5.29 (d4; n = 12; P < 0.005). Administration of 10 ng/ml OSM inhibited the basal increase in HK-2 cell number per square centimeter to a value of 60.25 ± 4.17 at d4 (n = 12; P = 0.075 compared with untreated cells at d4). In addition, 10 ng/ml OSM reduced

...contact proteins and the induction of mesenchymal marker proteins are associated with alterations in cell morphology, cell-cell adhesion, and/or cell proliferation. As we were not able to detect obvious alterations of the HK-2 cell phenotype in conventional two-dimensional cell cultures during the time points of investigation (data not shown), we assessed the potential effects of OSM on HK-2 cell-cell adhesion and migration in collagen gels, a 3D extracellular matrix environment that promotes the organization of epithelial cell into...
BPE-stimulated cell proliferation to a value of 3.93-fold from $21.92 \times 10^3 \pm 1.9 \, (n = 12) \text{ at } d0$ to $86.08 \times 10^3 \pm 5.32 \, (n = 12; \, P = 0.086 \text{ compared with cells treated with BPE in the absence of OSM after 4 days}).$

To verify whether or not this slight reduction of basal and mitogen-induced cell proliferation in the presence of OSM is due to alterations of cell viability we performed flow cytometric DNA analysis using PI staining. FACS analysis of $n = 9$ independent experiments revealed no significant difference ($P = 0.6958$) between untreated and OSM-treated HK-2 cells. Stimulation of HK-2 cells with 10 ng/ml OSM for 4 days induced apoptosis/necrosis in $3.53 \pm 0.30\% \, (n = 9)$, while in unstimulated controls $3.73 \pm 0.47\% \, (n = 9)$. In contrast, administration of 20% DMSO for 24 h led to a strong induction of cell death in $71.34\% \, (n = 9)$. Together, these results suggest that OSM slightly inhibits both basal and BPE-induced HK-2 cell proliferation and that this inhibitory effect is not due to an OSM-mediated reduction of HK-2 cell viability.

OSM is a strong stimulator of ERK1/2 and ERK5 in HK-2 cells. As a possible role of OSM in renal tubular EMT has been reported recently (35) and as strong evidence exists that the ERK family of mitogen-activated protein kinases does play an important role in renal EMT (20, 25, 27, 29, 41, 48–50), we...
In addition to activating these ERK isoforms, OSM represents a strong stimulus for both Stat1 and Stat3 phosphorylation in HK-2 cells (Fig. 9). OSM (10 ng/ml) time dependently increased phosphorylation of Stat1 and Stat3 as early as 5 min after administration of the cytokine (Fig. 9). For both Stat1 and Stat3, the highest phosphorylation levels were reached between 5 min and 30 min of stimulation. In contrast to OSM-mediated Stat1 phosphorylation, which returned to control levels after 3 h of stimulation, OSM-induced Stat3 phosphorylation was reduced after 1 h but lasted, albeit at lower levels, for at least 48 h (Fig. 9). Compared with incubation of HK-2 cells with 10 ng/ml OSM for 10 min, LIF was unable to stimulate phosphorylation of Stat1 or Stat3 after 10 min at any of the concentrations (0.5–100 ng/ml) tested (data not shown). The same result was obtained for LIF-induced phosphorylation of ERK1/2 and ERK5. While 10 ng/ml OSM rapidly stimulated ERK1/2 phosphorylation and ERK5 phosphorylation after 10 min of incubation, 0.5–100 ng/ml LIF did not alter the phosphorylation state of these MAPKs compared with unstimulated control cells (data not shown). Together, these results suggest that, besides Stat1/3 signaling, human recombinant OSM is able to stimulate the protein kinases ERK1/2 and ERK5 via the human OSM receptor subtype but not via the human LIF receptor subtype in HK-2 cells.

Selective inhibition of ERK1/2 phosphorylation compared with ERK5 and Stat1/3 by synthetic inhibitors of MEK1/2. To further elucidate the signaling mechanisms involved in the OSM-mediated effects on epithelial and mesenchymal marker proteins, we next investigated differential effects of synthetic inhibitors of MEK1/2 on OSM-induced phosphorylation of ERK1/2, ERK5, Stat1, and Stat3 after 10 min of stimulation. MEK1 and MEK2 are known to phosphorylate and activate both ERK1 and ERK2, while ERK5 is activated by another dual-specificity kinase, namely, MEK5 (21, 47, 48). The MEK1/2 inhibitor U0126 has recently been reported to inhibit both the ERK1/2 as well as the ERK5 signaling pathway (21). As depicted in Fig. 10A, however, preincubation for 1 h with 10 mM U0126 completely blocked OSM-induced ERK1/2 phosphorylation without affecting OSM-stimulated ERK5 phosphorylation in HK-2 cells. This is in line with recently published data from our laboratory showing that both synthetic MEK1/2 inhibitors U0126 and CI-1040 represent inhibitors of ERK1/2 but not ERK5 phosphorylation in HK-2 cells (47). In addition, the MEK1/2 inhibitor U0126 altered neither OSM-induced Stat1 phosphorylation nor OSM-induced Stat3 phosphorylation (Fig. 10B), suggesting that, during short-term stim-
ulation and when utilized at a concentration of 10 μM, U0126 is a specific blocker of ERK1/2 signaling compared with ERK5 and Stat1/3. As identical results were obtained using Cl-1040 (47, data not shown), both pharmacological inhibitors of ERK1/2 signaling appeared as appropriate tools to further investigate the relative contribution of the two ERK family members ERK1 and ERK2 compared with ERK5, Stat1, and Stat3 in OSM-mediated inhibition of N-cadherin, claudin-2, and E-cadherin expression.

**MEK1/2-ERK1/2 signaling is necessary for basal claudin-2 expression but inhibits N-cadherin expression.** Thus we next investigated the effects of the two synthetic MEK1/2 inhibitors U0126 and Cl-1040 on E-cadherin, N-cadherin, and claudin-2 expression in long-term incubation studies. OSM (10 ng/ml) inhibited E-cadherin, N-cadherin, and claudin-2 expression in HK-2 cells after 48 and 96 h (Fig. 11). Long-term MEK1/2 inhibition, and, thus, selective inhibition of ERK1/2 signaling using either 10 μM U0126 or 1 μM Cl-1040 inhibited claudin-2 expression both in the absence and in the presence of OSM (Fig. 11), suggesting that a functional MEK1/2-ERK1/2 signal is necessary and sufficient for basal claudin-2 expression in HK-2 cells. Interestingly, while OSM led to an inhibition of N-cadherin expression in the absence of synthetic MEK1/2 inhibitors, both Cl-1040 and U0126 restored N-cadherin expression in the presence of OSM (Fig. 11), suggesting that OSM-induced ERK1/2 signaling, at least partially, mediates the inhibitory effects of OSM on N-cadherin expression. In contrast, the two synthetic MEK1/2 inhibitors did not have any effects on E-cadherin expression, neither in the absence nor in the presence of OSM (Fig. 11). No effects on β-actin expression were obtained under any of the conditions studied in these experiments (Fig. 11).

**DISCUSSION**

OSM is a cytokine which may be involved in renal tissue remodeling and regeneration as well as in pathophysiological processes associated with inflammatory kidney disease. With respect to its potential role in the induction of EMT, a biological process involved in the generation of matrix-producing effector cells during renal fibrogenesis, we evaluated OSM-induced effects on the expression of both epithelial cell-cell contact proteins (E-cadherin, N-cadherin, claudin-2) and mesenchymal marker proteins (collagen type I, vimentin, FSP1) in human proximal tubular cells. Here we report that OSM inhibits the expression of the TJ protein claudin-2 and the AJ proteins N-cadherin and E-cadherin (Fig. 12). Conversely, OSM stimulates expression of collagen type I, vimentin, and FSP1 (S100A4), which are markers characteristic of a mesenchymal cell. Furthermore, OSM is able to induce 3D scattering of human kidney-2 (HK-2) cells in the absence of any other growth supplement and inhibits rather than stimulates basal and mitogen-induced HK-2 cell proliferation. Besides activating Stat1 and Stat3 signaling, OSM is a strong activator of ERK1/2 and ERK5 in these cells. Of these signaling molecules, the MAPKs ERK1 and ERK2 are likely to be involved in OSM-induced inhibition of N-cadherin expression. The fact that both synthetic MEK1/2 inhibitors U0126 and Cl-1040 led to a strong inhibition of basal claudin-2 expression is consistent with the idea that functional ERK1/2 signaling is necessary for basal claudin-2 expression in human proximal tubular cells.

Tubulointerstitial fibrosis represents a hallmark of many types of progressive renal diseases. In vivo, pathogenetic factors as diverse as hypertension, proteinuria, hyperlipidemia, and inflammatory mediators initiate and contribute to cellular events leading to the histological presentations finally summarized as renal fibrosis. The underlying cellular mechanisms include endothelial cell injury, mesangial cell and resident fibroblast activation, epithelial cell injury associated with tubular atrophy and EMT, monocyte/macrophage, and T cell infiltration, as well as cell apoptosis (9, 10, 20, 25, 32). In the course of these events, initial renal injury stimulates various types of kidney cells to produce inflammatory mediators,
which activate renal tubular epithelial cells and peritubular capillary endothelial cells and facilitate infiltration of mononuclear cells into the interstitium (10, 32). Cytokines, chemokines, and growth factors produced by these infiltrating inflammatory cells as well as by renal parenchymal cells are then likely to play important roles in the following steps finally leading to tubulointerstitial fibrosis. Of these mediators, TGF-β, ET-1, IL-8, MCP-1, RANTES, and fractalkine have been reported to be upregulated in proximal tubular cells challenged with protein overload (62, 64). Other ligands, which might be involved in the progression of kidney disease toward renal fibrosis include TNF-α, angiotensin II, PDGF-B, EGF, FGF-2, and CTGF (6, 25). Furthermore, IL-1β, IL-4, IL-10, and INF-γ expression was observed to be significantly elevated in the PBMC of patients with IgA nephropathy or non-IgA mesangial proliferative glomerulonephritis (61). Activated PBMC were shown to produce a variety of growth factors and cytokines, including TGF-β1, EGF, IL-1β, IL-2, IL-6, OSM, GM-CSF, TNF-α, and INF-γ (16, 35). Of these, OSM has recently been reported as a novel cytokine, which is able to induce cellular events indicative of tubular EMT (35). Activated PBMC-conditioned medium, which contained high levels of OSM, led to the upregulation of OSMRβ subunit expression from 4 to 48 h after exposure of kidney epithelial cells (35). Furthermore, and when applied in a medium containing EGF, insulin, and FCS, OSM-induced Jak-Stat signaling was associated with the induction of morphological alterations of human proximal tubular cells, with the reduction of E-cadherin expression as well as with the induction of both α-smooth muscle actin (α-SMA) expression and fibronectin synthesis (35). In our present study, we confirmed the finding that OSM is able to inhibit E-cadherin even in the absence of any other growth supplement at both the mRNA and the protein level.

Fig. 8. Effects of OSM on the phosphorylation of MEK/ERK kinases (MEK) 1, MEK2, and of ERK1, ERK2, and ERK5. HK-2 cells were serum and supplement starved for 48 h and were then stimulated with OSM. A: serum- and supplement-starved HK-2 cells were stimulated for 10 min with different concentrations of OSM (from 0.5 to 100 ng/ml). Protein-matched samples of stimulated cells and unstimulated controls were separated on SDS 10% PAGE and analyzed by Western immunoblotting for MEK1/2 phosphorylation (p-MEK1/2), MEK1 and MEK2 protein expression (MEK1, MEK2), ERK1/2 phosphorylation (p-ERK1/2), and ERK2 protein expression (ERK2) as described in MATERIALS AND METHODS. The results from 1 representative Western blot of 4 separate experiments are depicted.

B: serum- and supplement-starved HK-2 cells were stimulated for 10 min with different concentrations of OSM (from 0.5 to 100 ng/ml). Protein-matched samples of stimulated cells and unstimulated controls were separated on SDS 10% PAGE and analyzed by Western immunoblotting for ERK5 phosphorylation (p-ERK5) and ERK5 protein expression (ERK5) as described in MATERIALS AND METHODS. The results from 1 representative Western blot of 3 separate experiments are depicted.

C: serum- and supplement-starved HK-2 cells were stimulated with 10 ng/ml OSM for different periods of time (from 5 min to 48 h). Protein-matched samples of stimulated cells and unstimulated controls were separated on SDS 10% PAGE and analyzed by Western immunoblotting for MEK1/2 phosphorylation (p-MEK1/2), MEK1 and MEK2 protein expression (MEK1, MEK2), ERK1/2 phosphorylation (p-ERK1/2), and ERK2 protein expression (ERK2) as described in MATERIALS AND METHODS. The results from 1 representative Western blot of 5 separate experiments are depicted.
protein level. We now report that OSM as a single ligand also leads to an inhibition of the expression of both N-cadherin and claudin-2. Furthermore, OSM is able to induce mesenchymal marker proteins such as collagen type I, vimentin, and FSP1 (S100A4). As we were unable to detect clear-cut effects of OSM on HK-2 cell phenotype in conventional 2D cell culture, we assessed the potential impact of OSM on HK-2 cell-cell adhesion and migration in 3D collagen gels. Albeit largely composed of collagen type I, a typical product of mesenchymal
In two strains of Madin-Darby canine kidney (MDCK) cells, increased and those of claudins-3 and -5 remained unchanged while claudin-2 declined to undetectable levels compared with vector controls. In ras-overexpressing LLC-PK1 cells, in which the abundance of vimentin and collagen type I expression. This is in contrast to ras-overexpressing LLC-PK1 cells, in which the abundance of claudin-2 declined to undetectable levels compared with vector controls, whereas levels of occludin and claudins-1, -4, and -7 increased and those of claudins-3 and -5 remained unchanged (30). In two strains of Madin-Darby canine kidney (MDCK) cells, it has been shown that the ERK1/2 signaling pathway negatively controls claudin-2 expression (24). In a mouse hepatic cell line, oncogenic Raf-1-induced EMT was associated with a marked downregulation of occludin and claudin-2 expression at both the transcriptional and translational level (22). In distal tubular MDCK cells, expression of a constitutively active mutant of the ERK1/2 activator MEK1 induced full EMT, including loss of E-cadherin expression and a marked reduction of β- and α-catenin expression (27, 29, 41, 48, 49). Moreover, inhibition or loss of active MEK1-ERK1/2 signaling restored the epithelial phenotype and the ability of these distal tubular cells to undergo epithelial morphogenesis (50). Together, these data suggest that, in different species and/or depending on the renal cell type as well as the cellular microenvironment, distinct molecular and cellular programs for reversible transitions between epithelial and mesenchyme exist.

Besides our present data obtained in human renal proximal tubular cells, evidence exists that OSM-stimulated signaling pathways might indeed be involved in the regulation of cell differentiation in various systems. While OSM-induced Jak-Stat signaling was associated with the induction of morphological alterations indicative of renal tubular EMT (35), Stat3 signaling has been implicated in HGF-induced formation of epithelial tubules (2, 60). OSM-induced responses consistent with a stimulatory role in cell differentiation have been also reported for glioma cells, mammary epithelial cells, hematopoietic cells, fibroblasts, and endothelial cells (13), while in totipotent embryonic stem cells OSM has been shown to inhibit cell differentiation (45). Moreover, we now report that OSM does not only activate Stat1 and Stat3 but is also a potent pro-EMT molecule and a pro-MET mediator. Besides our present data obtained in human renal proximal tubular cells, evidence exists that OSM-stimulated signaling pathways might indeed be involved in the regulation of cell differentiation in various systems. While OSM-induced Jak-Stat signaling was associated with the induction of morphological alterations indicative of renal tubular EMT (35), Stat3 signaling has been implicated in HGF-induced formation of epithelial tubules (2, 60). OSM-induced responses consistent with a stimulatory role in cell differentiation have been also reported for glioma cells, mammary epithelial cells, hematopoietic cells, fibroblasts, and endothelial cells (13), while in totipotent embryonic stem cells OSM has been shown to inhibit cell differentiation (45). Moreover, we now report that OSM does not only activate Stat1 and Stat3 but is also a potent pro-EMT molecule and a pro-MET mediator. Besides our present data obtained in human renal proximal tubular cells, evidence exists that OSM-stimulated signaling pathways might indeed be involved in the regulation of cell differentiation in various systems. While OSM-induced Jak-Stat signaling was associated with the induction of morphological alterations indicative of renal tubular EMT (35), Stat3 signaling has been implicated in HGF-induced formation of epithelial tubules (2, 60). OSM-induced responses consistent with a stimulatory role in cell differentiation have been also reported for glioma cells, mammary epithelial cells, hematopoietic cells, fibroblasts, and endothelial cells (13), while in totipotent embryonic stem cells OSM has been shown to inhibit cell differentiation (45). Moreover, we now report that OSM does not only activate Stat1 and Stat3 but is also a potent pro-EMT molecule and a pro-MET mediator.

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