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

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1Division of Nephrology, Department of Internal Medicine, and 2Division of Hygiene and Social Medicine and 3Division of Physiology, Innsbruck Medical University, Innsbruck, Austria; 4Department of Cell Physiology and Metabolism, School of Medicine, University of Geneva, Geneva, Switzerland; and 5Department of Nephrology and Rheumatology, Georg-August-University Medical Center, Göttingen, Germany

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Pollack V, Sarközi R, Banki Z, Feifel E, Wehn S, Gstraunthaler G, Stoiber H, Mayer G, Montesano R, Strutz F, Schramek H. Oncostatin M-induced effects on EMT in human proximal tubular cells: differential role of ERK signaling. Am J Physiol Renal Physiol 293: F1714–F1726, 2007. First published September 19, 2007; doi:10.1152/ajprenal.00130.2007.—Growing evidence suggests that a proportion of interstitial myofibroblasts detected during renal tubulointerstitial fibrosis originate from tubular epithelial cells by a process called epithelial-mesenchymal transition (EMT). The IL-6-type cytokine oncostatin M (OSM) has been recently implicated in the induction of EMT. We investigated OSM effects on the expression of both cell-cell contact proteins and mesenchymal markers and studied OSM-induced intracellular signaling mechanisms associated with these events in human proximal tubular cells. Human recombinant OSM attenuated the expression of N-cadherin, E-cadherin, and claudin-2 in human kidney-2 (HK-2) cells associated with the induction of HK-2 cell scattering in 3D collagen matrices. Conversely, expression of collagen type I, vimentin, and S100A4 was induced by OSM. OSM-stimulated cell scattering was inhibited by antibodies against gp130. Besides inducing phosphorylation of Stat1 and Stat3, OSM led to a strong concentration- and time-dependent phosphorylation of the mitogen-activated protein kinases ERK1, ERK2, and ERK5. MEK1/2 inhibitor U0126 (10 μM) blocked basal and OSM-induced ERK1/2 phosphorylation but not phosphorylation of either ERK5 or Stat1/3. Both synthetic MEK1/2 inhibitors U0126 and CI-1040, when used at concentrations which inhibit ERK1/2 phosphorylation but not ERK5 phosphorylation, restored N-cadherin expression in the presence of OSM, inhibited basal claudin-2 expression, but did not affect either basal or OSM-induced E-cadherin expression or OSM-induced expression of collagen type I and vimentin. These results suggest that in human proximal tubular cells ERK1/2 signaling represents an important component of OSM’s inhibitory effect on N-cadherin expression. Furthermore, functional ERK1/2 signaling is necessary for basal claudin-2 expression.

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), cardiotoxin-1 (CT-1), and novel neurotrophin-1/B cell-stimulating factor-3 (NTNT/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...
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.

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+ 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).

Scattering 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 as prepared (28). Aliquots of the cell suspension were then dispensed into the wells of multiwell 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 × 105 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 μg/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

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.
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 microbicinchoninic 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) polyacrylamide gels, and electrophoresed on a 10% polyacrylamide gel (for N-cadherin protein expression). 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 dually 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 dually phosphorylated ERK5 (Thr218/Tyr220) (all Cell Signaling Technology); rabbit anti-anti-MEK2, goat anti-ERK5, and goat anti-E2K (all Santa Cruz Biotechnology, Santa Cruz, CA); rabbit anti-phospho Stat1 (Cell Signaling Technology), which detects phosphorylated Stat1-α and -β (Tyr 701); rabbit anti-anti-Stat3 (Cell Signaling Technology); rabbit anti-anti-Stat1 (Cell Signaling Technology); and rabbit anti-anti-Stat1 (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 (Cell Signaling Technology), goat anti-mouse IgG (for analysis of E-cadherin and -N-cadherin), and secondary horseradish peroxidase-conjugated goat anti-rabbit IgG (Cell Signaling Technology) with the addition of DNase- and RNase-free H2O. Amplification was started at 95°C for 30 s and incubated up to 1 h. The final product was extended and finalized at 95°C for 1 h and 30 s.

**GAPDH forward:** 5′-CCC TTC ATT GAC CTC AAC TAC-3′

**GAPDH reverse:** 5′-TGA TTC CTT CCA CGA TAC C-3′

**Vimentin forward:** 5′-GAT TGC TCT GCC TCT TCT TCC AAA CTT-3′

**Vimentin reverse:** 5′-GGG TAT CAA CCA GAG GGA GTG A-3′

**E-cadherin forward:** 5′-CCA ACG GGA ATG CAT TGG A-3′

**E-cadherin reverse:** 5′-TGA ATT CGG GTG TGT TGT CA-3′

**Collagen I forward:** 5′-CAA TGC TGC CCT TGC TGC TCC TTT-3′

**Collagen I reverse:** 5′ CAC TGT GGT GTT TGA GGA TTA-3′

**Refined data and statistical analyses.** 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 stained with 10 ng/ml OSM or were left untreated for 48 h. Incubation in the presence of TGFB-1 (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, nuclei were counterstained with Alexa Fluor 488 (for Alexa Fluor 488-labeled secondary antibodies) and Alexa Fluor 555 (for Alexa Fluor 555-labeled secondary antibodies) from Molecular Probes. Images were obtained using a Zeiss microscope (Zeiss, Oberkochen, Germany) equipped with a cooled charge-coupled device camera ( cooled 10.220.33.1 on June 21, 2017 http://ajprenal.physiology.org/ Downloaded from).
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-PK1D− 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-PK1D+ 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-PK1D−, suggesting that E-cadherin expression alone does neither 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 coinubcation 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
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 multicellular tissue-like structures (38). When grown for 7–10 days in a collagen gel in complete medium (KSFM supplemented with FCS, BPE, and EGF) without OSM, HK-2 cells formed compact colonies (Fig. 4A). In contrast, when cultured for the same time period in complete medium containing 10 ng/ml OSM, HK-2 cells disassociated from each other and invaded the surrounding matrix, mostly as individual cells (Fig. 4B). 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 (Fig. 4C). When grown in collagen gels in serum-free medium without BPE and EGF, HK-2 cells proliferated slowly and formed smaller cell clusters (Fig. 5A). Remarkably, however, even under these conditions of growth factor deprivation, OSM was able to induce 3D scattering (Fig. 5B). 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 × 10^4 ± 0.36 cells; n = 12) compared with untreated controls (4.5 × 10^4 ± 0.33 cells; n = 12), with values of OSM-treated cultures being not significantly different from those of control cultures (P < 0.35).

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 KSFM and advanced DMEM/F12), allowing the formation of larger and more compact colonies than KSFM 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 18.25 × 10^3 ± 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 for 4 days with 50 μg/ml BPE, cell number per square centimeter increased 5.12-fold from a value of 19.58 ± 1.07 (d0; n = 12) to 100.3 × 10^3 ± 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 3.38-fold from 17.83 × 10^3 ± 1.85 (n = 12) at d0 to 60.25 × 10^3 ± 4.17 at d4 (n = 12; P = 0.075 compared with untreated cells at d4). In addition, 10 ng/ml OSM reduced...
BPE-stimulated cell proliferation to a value of 3.93-fold from $21.92 \times 10^3 \pm 1.9$ (n = 12) at d0 to $86.08 \times 10^3 \pm 5.32$ at d4 (n = 12; P = 0.086 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 ± 0.30% of HK-2 cells (n = 9), while in unstimulated controls 3.73 ± 0.47% of HK-2 cells showed signs of reduced cell viability (n = 9). In contrast, administration of 20% DMSO for 24 h led to a strong induction of cell death in 71.34% of cells. 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
OSM-induced ERK signaling in HK-2 cells. After 10 min of incubation, OSM led to a concentration-dependent increase in phosphorylation of MEK1/2, ERK1/2 (Fig. 8A) and ERK5 (Fig. 8B) without affecting protein expression of MEK1, MEK2, and ERK2 (Fig. 8A) or ERK5 (Fig. 8B). While slight phosphorylation of these signaling molecules was already detectable at a concentration of 0.5 ng/ml OSM (MEK1/2, ERK1/2) and 1.0 ng/ml OSM (ERK5), respectively, maximal phosphorylation occurred at a concentration of 10 ng/ml. Moreover, stimulation of quiescent HK-2 cells with a concentration of 10 ng/ml OSM led to a strong time-dependent phosphorylation of MEK1/2 and ERK1/2, which was obtained as early as 5 min after incubation and lasted for 30 min (Fig. 8C).

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 μM 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-

Fig. 4. Effect of OSM on the behavior of HK-2 cells grown in 3-dimensional (3D) collagen gels. A: HK-2 cells grown for 8 days in a collagen gel in complete medium [keratinocyte-serum-free medium (KSFM) supplemented with FCS, bovine pituitary extract (BPE), and EGF] but without OSM form compact ball-like colonies. B: under the same culture conditions, addition of 10 ng/ml OSM results in the dispersal of HK-2 cells throughout the collagen matrix. C: addition of function-blocking antibody against the OSM receptor subunit gp130 (2 μg/ml) for 2 h before treatment with OSM prevents cell dissociation and 3D scattering. Magnification = ×165.
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 CI-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 CI-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 CI-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 presence of synthetic MEK1/2 inhibitors, both CI-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 CI-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. 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...

Fig. 10. Effects of the synthetic MEK1/2 inhibitor U0126 on OSM-induced ERK and Stat signaling. HK-2 cells were serum and supplement starved for 48 h and were then stimulated for different periods of time (5 min to 3 h) with 10 ng/ml OSM either in the absence or in the presence of 10 μM U0126. A: 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 ERK1/2 phosphorylation (p-ERK1/2) as described in MATERIALS AND METHODS. The results from 1 representative Western blot of 3 separate experiments are depicted. B: protein-matched samples of stimulated cells and unstimulated controls were separated on SDS 10% PAGE and analyzed by Western immunoblotting for Stat1 phosphorylation (p-Stat1) and Stat3 phosphorylation (p-Stat3) as described in the MATERIALS AND METHODS. The results from 1 representative Western blot of 3 separate experiments are depicted.

Fig. 11. Long-term effects of the 2 synthetic MEK1/2 inhibitors U0126 and Cl-1040 on the expression of E-cadherin, N-cadherin, and claudin-2 in the presence or absence of OSM. HK-2 cells were serum and supplement starved for 48 h and were then either stimulated for 48 (d2) or 96 h (d4) with ng/ml OSM or for 48 h (d2) with 10 μM U0126 and 1 μM Cl-1040, respectively, in the absence or in the presence of 10 ng/ml OSM compared with unstimulated controls (d0). Protein-matched samples of stimulated cells and unstimulated controls were separated on SDS 7.5 and 12% PAGE and analyzed by Western immunoblotting for E-cadherin, N-cadherin, claudin-2, and β-actin expression as described in MATERIALS AND METHODS. The results from 1 representative Western blot of 3 separate experiments are depicted.

Fig. 12. OSM-induced signaling pathways in HK-2 cells. OSM is a strong activator of Stat1/3, ERK1/2, and ERK5 phosphorylation. OSM inhibits expression of N-cadherin, E-cadherin and claudin-2. Conversely, OSM stimulates expression of collagen type I and vimentin. Selective inhibition of MEK1/2 by either 1 of the 2 synthetic MEK1/2 inhibitors U0126 or Cl-1040 restored N-cadherin expression in the presence of OSM, suggesting that the MEK1/2-ERK1/2 signaling module mediates OSM-induced N-cadherin suppression. In contrast, blockade of MEK1/2 did not affect either basal or OSM-mediated inhibition of E-cadherin expression or OSM-induced expression of collagen type I and vimentin. However, both synthetic MEK1/2 inhibitors led to a strong inhibition of basal claudin-2 expression, which is consistent with the idea that in HK-2 cells functional ERK1/2 signaling is necessary for basal claudin-2 expression.
In two strains of Madin-Darby canine kidney (MDCK) cells, increased and those of claudins-3 and -5 remained unchanged; claudin-2 declined to undetectable levels compared with vector. N-cadherin expression remained unaltered (Fig. 1). Selective inhibition of MEK1/2 by either one of the two synthetic MEK1/2 inhibitors U0126 or CI-1040 restored N-cadherin expression in the presence of OSM, suggesting that the MEK1/2/ERK1/2 signaling module at least partially mediates the OSM-induced N-cadherin suppression. However, blockade of MEK1/2 using these inhibitors did not affect either basal or OSM-mediated inhibition of E-cadherin expression, inhibited basal claudin-2 expression, and did not affect the OSM-driven induction 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 stimulator of the MAPKs ERK1, ERK2, and ERK5, which are important regulators of cell differentiation and proliferation (36, 48). In a healthy renal tubular system several different pathways modulating EMT and MET might be active, finally leading to a balance, which allows the maintenance of an epithelial phenotype. Even in response to tubular injury, we propose that cellular mechanisms acting in both directions might be turned on. Within a certain time frame and depending on the specific injury (its duration, size, the cellular context, and the ligands produced), the balance of the cellular mechanisms involved is then either shifted toward epithelial differentiation (MET, partial/full tubular epithelial repair, stable disease) or driven toward de-/transdifferentiation (EMT, fibrogenesis, progressive disease). Together, it is tempting to speculate that OSM might represent a cytokine, which, depending on the cellular microenvironment and/or a specific injury, has the ability to act as both a pro-EMT molecule and a pro-MET mediator.

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