Ex vivo reversal of in vivo transdifferentiation in mesothelial cells grown from peritoneal dialysate effluents

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Abstract

Background. During peritoneal dialysis (PD), epithelial–mesenchymal transition (EMT) is likely involved in aberrant healing and progressive peritoneal fibrosis. Recently, EMT of the kidney was actively reversed into the opposite direction, into mesenchymal–epithelial transition (MET), by treatment with bone morphogenic protein-7 (BMP-7). In this study, the potential for ex vivo interconversion of in vivo transdifferentiation processes was investigated in mesothelial cells.

Methods. In vivo EMT was assessed in mesothelial cell cultures randomly grown from peritoneal effluents of seven patients on chronic PD. Then, ex vivo treatment with modulating factors was performed by incubating cobblestone-like cell cultures with transforming growth factor (TGF-β1) and fibroblast-like cultures with BMP-7. Effects were assessed by morphological characterization, western analysis and reverse transcription–polymerase chain reaction of marker proteins ezrin and α-smooth muscle actin (α-SMA).

Results. PD caused progressive in vivo EMT with loss of the epithelial phenotype in the majority of mesothelial cell cultures over a 12-month period. EMT was reproducible by ex vivo treatment of cultured cells with TGF-β1, converting the epithelial to the fibroblast-like phenotype. Ex vivo treatment with BMP-7 reversed in vivo and ex vivo EMT. During rhBMP-7 incubation the fibroblast-like growth pattern reversed into a more epithelial morphology, the expression of ezrin increased and α-SMA decreased.

Conclusion. Our study shows that modulating factors of transdifferentiation, such as BMP-7, may be attractive tools in the balance between normal healing and aberrant profibrotic processes in mesothelial cells during peritoneal dialysis. Peritoneal-effluent-derived mesothelial cells are not mere biomarkers for in vivo EMT in the peritoneal cavity, but also represent an assay to test ex vivo interventions to reverse the profibrotic phenotype.

Keywords: BMP-7; epithelial–mesenchymal transition; mesenchymal–epithelial transition; mesothelial cells; peritoneal dialysis; peritoneal effluent; TGF-β1

Introduction

Peritoneal dialysis (PD) is a frequently used mode of renal replacement therapy in end-stage renal disease. However, glucose-related toxicity of PD fluids and peritoneal infections may converge in epithelial–mesenchymal transition (EMT) that results in aberrant healing processes, peritoneal fibrosis and, ultimately, technical therapy failure [1]. EMT, however, is a dynamic cellular process and might be actively reversed into the opposite direction, into mesenchymal–epithelial transition (MET) [2]. MET has long been known to be paramount for normal embryonic development, but has only recently been shown to represent an attractive concept in counteracting fibrotic damage in acute and chronic renal disease processes [2–4].

In this study, EMT was prospectively assessed in mesothelial cells grown from peritoneal effluents of children on chronic PD. We hypothesized that ex vivo reversal of in vivo transdifferentiation in these cells may be performed by incubating cultures with epithelial phenotype with transforming growth factor (TGF-β1) and cultures with fibroblast-like phenotype with bone morphogenic protein-7 (BMP-7).

Patients and methods

From June 2004 to May 2005, mesothelial cells were cultured from randomly collected peritoneal effluents in seven
clinically stable children (four boys and three girls, aged 5.9 ± 6.1 years) undergoing PD for 0–34 months with commercial PD fluid.

Peritoneal cells were concentrated by centrifugation of dialysis fluid effluent and then cultured in Earle’s M199 medium, 10% fetal calf serum, 50 U/ml penicillin and 50 μg/ml streptomycin. Non-adherent cells were removed 2 days later by two brief washes with medium. When the primary mesothelial cultures reached confluence (after 9–55 days), they were split (in a ratio of 1:2) 2–3 times and their morphological features were assessed (see subsequently, these features remained stable during 2–3 cell passages).

For incubation experiments, the medium was replaced in selected cultures by a medium with lower (2%) serum. Mesothelial cells with cobblestone-like phenotype were then exposed for 1 week with 3 ng/ml recombinant human TGF-β1 (R&D Systems, Minneapolis, MN, USA) and then transferred to the original medium containing 10% fetal calf serum (FCS) for a further 24 hours. In contrast, mesothelial cell cultures with fibroblast-like phenotype were exposed for 1 week with 100 ng/ml recombinant human BMP-7, dissolved in 24 mM sodium acetate containing 1% mannitol (R&D Systems, Minneapolis, MN, USA) and then transferred to the original medium containing 10% FCS for a further 24 hours. In both protocols, parallel cultures underwent sham treatment with original mediums as controls, and were simultaneously harvested for protein analysis immediately after morphological assessment.

In an additional series of three cultures of mesothelial cells with cobblestone-like phenotype, the incubation protocols were performed sequentially, i.e. the same culture was first incubated with TGF-β1 for 1 week and subsequently incubated with BMP-7 for another week. Aliquots for mRNA analysis were harvested before the treatments, and at the end of the TGF-β1 and of the BMP-7 incubation periods, respectively.

The EMT phenotypes of mesothelial cell cultures were microscopically characterized into cobblestone-like epithelial, fibroblast-like or an intermediate phenotype (Figure 1).

For western blotting, protein content was determined by Bradford assay (BioRad) and equal amounts of protein samples (3 μg/lane) were separated by standard sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using a Pharmacia Multiphore II unit. Size-fractionated proteins were then transferred to polyvinylidene fluoride membranes by semi-dry transfer in a Pharmacia Multiphore II Novablott unit. The membranes were blocked in 5% dry milk in Tris-buffered saline-Tween (TBS-Tween) (10 mM Tris, 150 mM NaCl, 0.05% Tween-20, pH 7.4), and were incubated with the respective primary antibody for 16 h [α-SMA, NeoMarkers, USA; ezrin (clone 3212, Sigma, USA)]. The detection was accomplished by incubation with secondary, peroxidase-coupled antibodies (anti-mouse IgG, Dako Cytomation, USA) and enhanced chemiluminescence (ECL) using ECL western blotting analysis system and protocols (Renaissance, NEN-Life Science Products, Boston, MA, USA).

For reverse transcription-polymerase chain reaction (RT-PCR) analysis, total RNA was extracted with the RNeasy Mini Kit followed by on-column digestion of contaminating genomic DNA by DNase 1 (Qiagen, Hilden, Germany). First-strand cDNA was synthesized from total RNA with Superscript II RNase H Reverse Transcriptase (Gibco BRL, Life Technologies) using random hexamer primers. Real-time PCR was performed on the ABI Prism 7000 detection system (Applied Biosystems Inc., Foster City, CA, USA) using TaqMan® Universal PCR Master Mix (Applied Biosystems Inc., Foster City, CA, USA) and the measurements were carried out in a two-step protocol using optimal temperatures for annealing and elongation. The relative transcription was calculated using the ΔΔCt method [5].

**Results**

PD-effluent-derived mesothelial cell cultures had markedly varied morphological features, ranging from a cobblestone-like appearance similar to that of mesothelium derived from omentum in 24 cultures, to non-epithelial fibroblast-like or intermediate cell populations in 17 cultures (Figure 1). The differences in phenotypes were also confirmed by differential expression of the marker proteins ezrin and α-SMA. There was no correlation between the period required to reach confluence and the cell phenotype ($P = 0.41$).
The loss of the epithelial phenotype was related to long-term PD treatment. Whereas 21 of 27 (78%) cultures exhibited a cobblestone-like phenotype during the first year of PD, only 3 of 14 (21%) retained the epithelial phenotype during the second year ($P = 0.0007$).

The effects of ex vivo reproduction of EMT are demonstrated in Figure 2. Upon continuous treatment with TGF-β1 for 7 days, the epithelial morphology with its characteristic cobblestone-like growth pattern converted to a fibroblast-like morphology. In parallel to morphological changes, TGF-β1 also decreased the expression of the epithelial marker ezrin and increased the expression of the fibromyoblast marker α-SMA in parallel cultures. Western blot data are representative for five different cell cultures from three donors.

Then, we tested the effects of ex vivo treatment of mesothelial cell cultures with severe in vivo EMT. As shown in Figure 3, rhBMP-7 reversed the EMT phenotype in cultures with fibroblastoid aspect into an epithelial morphology and also reversed the typical alterations of the marker proteins ezrin and α-SMA.

Finally, we tested whether EMT induced by TGF-β1 could be reversed (within the same culture) by subsequent BMP-7 treatment. As shown in Figure 4, TGF-β1 resulted in a marked down-regulation of ezrin and up-regulation of α-SMA. Subsequent treatment of these cultures with BMP-7 reversed the effects of TGF-β1 at protein and mRNA levels.

**Discussion**

This study confirms recent reports of progressive EMT of mesothelial cells grown from peritoneal effluents of patients on chronic PD [1,6]. As a novel finding, these in vivo transdifferentiation processes could be reversed ex vivo by incubating mesothelial cell cultures with epithelial phenotype with TGF-β1 and cultures with fibroblastoid phenotype with BMP-7.

Classically, fibrotic changes of the peritoneum are regarded as a ‘one way street’. In the international peritoneal biopsy registry, duration of PD treatment correlated with submesothelial fibrosis [7]. Thus, the majority of previous studies have focused on the loss of the epithelial phenotype by EMT. In vitro, EMT was induced in primary cultures of omental mesothelial cells from healthy donors by treatment with TGF-β1 and/or II-1 [6,8]. In the in vivo rat model of PD, Margetts et al. [9] recently performed an intraperitoneal transfection of TGF-β1, and also found clear evidence of mesothelial EMT. Our data extend the role of TGF-β1 in EMT to the ex vivo system of PD-effluent-derived mesothelial cells obtained from uraemic patients. To our knowledge, only Leavesley et al. [10] have used this system also to describe the
effects of ex vivo treatment (in vitro wounding and epidermal growth factor incubation) during reparative mesothelial proliferation. In our study, the epithelial phenotype of mesothelial cells, obtained from peritonitis-free patients in the early course of their PD treatment, underwent marked ex vivo EMT upon incubation with TGF-β1. These results corroborate with a role of TGF-β1 during clinical PD — as levels of TGF-β1 have been reported to be elevated in PD effluents—but, more importantly, also reflect the potential to reproduce transdifferentiation in that cell type ex vivo [11].

However, the more attractive aspect of these active cellular processes is certainly their potential reversibility. Interconversion between mesenchyme and epithelium is of paramount importance during organ formation in normal embryonal development [2]. These transdifferentiation processes are known to be tightly controlled by modulating factors, such as TGF-β1 and BMP-7, creating a balanced reciprocity. Whereas TGF-β1 signalling tends to promote EMT, BMP-7 signalling seems not only to counteract EMT, but also to promote MET, even in adult tissue [3,4]. Experiments based on that conceptual framework have recently provided promising in vitro and in vivo evidence for active conversion from EMT to MET [4].

In the second set of our experiments, we therefore hypothesized that in vivo EMT might be reversed ex vivo into MET in mesothelial cells with fibroblast-like aspect. Upon incubation with rhBMP-7, these cultures indeed lost their spindle aspect and gained a cobblestone-like growth pattern. In addition, we also found down-regulation of the expression of α-SMA and up-regulation of ezrin in the PD-effluent-derived mesothelial cell cultures during their ex vivo transition to the more epithelial phenotype. Finally, in order to test the reversibility of EMT within the same cell culture, we performed consecutive ex vivo treatments with both the counteracting modulating factors. Indeed, EMT induced by TGF-β1 could be reversed by subsequent BMP-7 treatment. The concordant effect on protein and mRNA levels suggests the regulation of α-SMA and ezrin by de novo synthesis. Only recently, Zeisberg et al. [2,4] reversed the profibrotic changes, using similar protocols in renal injury models upon administration of exogenous rhBMP-7 and, studying glucose related toxicity; Wang et al. [3] also demonstrated the protective effects of BMP-7 treatment in diabetic rats [2–4]. To our knowledge, our study is the first report of actively induced MET in mesothelial cells, transferring this potential therapeutic approach to experimental PD.

Taken together, our study shows that modulating factors of transdifferentiation, such as BMP-7, may be attractive tools in the balance between normal healing and aberrant profibrotic processes in mesothelial cells. Future in vivo studies in animal models will be essential to further evaluate the biological relevance of such interventions in PD.

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Conflict of interest statement. None declared.

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