Epithelial-to-mesenchymal transition of the mesothelial cell—its role in the response of the peritoneum to dialysis

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Abstract
Peritoneal membrane fibrosis, ranging from mild inflammation to severe sclerosing peritonitis, is one of the complications of peritoneal dialysis (PD). In parallel with fibrosis, the peritoneum shows a progressive increase of capillaries and vasculopathy, involved in increased small solute transport across the membrane and ultrafiltration failure. Glucose and glucose degradation products from PD solutions are responsible of stimulating transforming growth factor-β (TGF-β) and vascular endothelial growth factor (VEGF) production by mesothelial cells (MCs). TGF-β is a potent pro-fibrotic factor and inducer of epithelial-to-mesenchymal transition (EMT) of the MC. Local production of VEGF by transitional MC appears to play a central role in the processes leading to peritoneal angiogenesis.

This review addresses the mechanism involved in peritoneal structural alteration by dialysis and points to the EMT of MC as the initiating mechanism of peritoneal injury. Information from multiple origins about TGF-β and VEGF is integrated into EMT process in a comprehensive manner. Regulation and new targets for inhibition of EMT or its deleterious effects are discussed.

Keywords: angiogenesis; epithelial-to-mesenchymal transition; mesothelial cells; peritoneal dialysis; transforming growth factor; vascular endothelial growth factor

Introduction
After reaching a short- and medium-term success as treatment of ESRD, the most important challenge of peritoneal dialysis (PD) is the long-term preservation of the peritoneal integrity [1]. Prolonged exposure to hyperosmotic, hyperglycaemic, high content of glucose degradation products (GDP) and low pH dialysate, and repeated episodes of peritonitis or haemoperitoneum, cause peritoneal injury with progressive mesothelial cell (MC) denudation, neovascularization and fibrosis [2]. Such structural alterations are considered the major cause of ultrafiltration failure and, potentially of encapsulating–sclerosing peritonitis [2–4]. The progressive increase of capillary number (neoangiogenesis) has been related to the local production of vascular endothelial growth factor (VEGF), a potent pro-angiogenic cytokine [5].

However, the pathophysiology of peritoneal changes during each stage of PD remains elusive. Two questions are still unanswered: what is the first step in the process, and what is the contribution of the different peritoneal cells to it?

We have demonstrated that soon after the PD is initiated, peritoneal MCs from dialysis effluents show a progressive loss of epithelial phenotype and acquire fibroblast-like characteristics [6]. In addition, by immunohistochemical studies of peritoneal biopsies from PD patients, we demonstrated the expression of the mesothelial markers in stromal α-smooth muscle actin (α-SMA) -positive myofibroblasts [7], suggesting that these cells stemmed from the local conversion of MC. All these changes of the MC are reminiscent of those that take place during the epithelial-to-mesenchymal transition (EMT), also called transdifferentiation [8]. For a long time MCs have been considered as mere victims of the peritoneal injury during PD, whereas peritoneal stromal fibroblasts have been classically observed as the main cells responsible of the structural and functional peritoneal alterations. Our aim is to demonstrate that the first peritoneal change in response to PD is the acquisition by MCs of fibroblast capabilities by suffering EMT, and that
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Table 1. The sequential pattern of peritoneal changes in response to peritoneal dialysis

- TGF-β present in the ECM, activated by exogenous dialysate and inflammatory mediators activates quiescent fibroblasts and MCs.
- TGF-β, cooperating with FGF, induces the EMT of MC.
- MC loses microvilli and intercellular junctions, and detaches.
- MC migrates into submesothelial tissue, after disrupting the basement membrane.
- MC transforms into myofibroblast.
- Myofibroblasts and fibroblasts synthesize ECM components (collagen I, fibronectin).
- Fibroblastoid MCs and myofibroblasts synthesize large amounts of VEGF.
- High local levels of VEGF promote immediate vasodilation and medium-term neoangiogenesis.
- The peritoneum is denuded of MC line and the submesothelium is plenty of MCs transformed into very productive fibroblasts.
- The replacement of the normal peritoneum by a large acellular and variably vascularized fibrotic tissue is the subjacent phenomenon.

with this view, the whole process of peritoneal fibrosis is satisfactorily explained.

Epithelial-to-mesenchymal transition of epithelial cells

EMT is a complex and generally reversible process that starts with the disruption of intercellular junctions and loss of apical–basolateral polarity, typical of epithelial cells, which are then transformed into fibroblast-like cells with increased migratory, invasive and fibrogenic features. The objective of this process is to repair tissue wounds by promoting the recovery of ancestor capabilities of epithelial cells. Cell migration, production of extracellular matrix and induction of neoangiogenesis are the main activities [8]. The process is conducted by the transforming growth factor-β (TGF-β) and the representative cell form is the myofibroblast. TGF-β synthesis may be stimulated by glucose, and infections, via peritoneal leucocyte-derived factors. TGF-β has been found to be up-regulated in peritoneal inflammatory processes and its over-expression has been correlated to worse PD outcomes [9]. TGF-β is a growth factor that has been implicated as the causal agent in fibrosis of different tissues and organs [10]. TGF-β exists in tissues, generally in a latent and inactive form, bound to the latency-associated peptide (LAP), and it is activated through proteolytic cleavage by thrombospondin, plasmin, cathepsin D, furin and glycosidases when exposed to acid pH as it occurs with PD solutions [11].

Mechanisms of TGF-β-induced epithelial-to-mesenchymal transition—the role of nuclear transcription factor snail

TGF-β is considered the master molecule in the genesis of peritoneal fibrosis, because it plays a central role in the triggering and perpetuation of many wound healing processes [12–16]. The importance of TGF-β in peritoneal fibrosis has been demonstrated in an in vivo mouse model, in which the TGF-β gene was transfected into the peritoneal cavity with an adenovirus vector, demonstrating in 28 days the whole process of peritoneal change, from normalcy to sclerosis throughout neoangiogenesis [5]. The process in mice seemed to sequentially reproduce the structural and functional alterations observed in PD patients.

The TGF-β family includes TGF-β1, TGF-β2, TGF-β3, activins and bone morphogenetic proteins (BMP). These cytokines interact with the TGF-β receptors type II, and then recruit and activate the subfamily of TGF-β receptors type I, which triggers the cellular signalling pathways through its serine/threonine kinase activity [17]. TGF-β is considered one of the most important inducers of EMT of different epithelial cells, including the MC, both in vitro and in vivo [18]. Four different intracellular signal pathways are triggered by TGF-β, the Smad cascade being the most important (Figure 1). TGF-β receptor I phosphorylates Smad 2 and 3 inducing their association with the common partner Smad 4, and then they translocate into the nucleus, where they control the expression of TGF-β-responsive genes, such as that encoding integrin-linked kinase (ILK) [17]. The activation of up-regulated ILK by β1 integrins results in strong phosphorylation of Akt and glycogen synthase kinase-3 (GSK-3) [19]. Phosphorylated Akt triggers NF-κB activation [20], which in turn induces the expression of Smad 7 [21], an inhibitory Smad molecule that interferes with the phosphorylation of Smad 2 and 3, and of snail, a key regulator of EMT. The transcription factor snail regulates EMT by inhibiting the expression of E-cadherin [22,23], and by inducing growth arrest and survival, which confer selective advantage to migrating trans-differentiated cells [24]. The phosphorylation of GSK-3 by ILK results in its inhibition and subsequent stabilization of β-catenin, released from the adherens junction, and of AP-1 [25]. Stabilized β-catenin, in conjunction with Lef-1/Tcf, may per se induce EMT [26], and AP-1 activates MMP-9 expression inducing the invasion of the extracellular matrix (ECM) [27]. One of the main Smad-independent signalling cascades triggered by TGF-β receptor I ligation, includes the RhoA- p160ROCK pathway that regulates cytoskeleton remodelling and cellular migration/invasion. In addition, RhoA induces the expression of α-SMA in a ROCK-independent manner [28]. Another signal transduction stimulated by TGF-β is the H-Ras/Raf/ERK pathway, which is also necessary for the induction of snail expression and EMT [29,30]. In this context, it has been described that TGF-β and fibroblast growth factor (FGF), a potent inducer of the
H-Ras/Raf/ERK signalling, cooperate in the triggering of EMT [31] (Figure 1).

Epithelial-to-mesenchymal transition of mesothelial cell in PD

The presence of a great number of fibroblasts is usually representative of fibrosis, but their origin is not necessarily unique. The classic concept suggests that fibroblasts are simply residual embryonic mesenchymal cells that remain in the tissues after organogenesis. However, the current view argues that fibroblasts may arise from local conversion of epithelial cells by EMT or from CD34⁺ cells (fibrocytes) of the bloodstream, after being recruited from bone marrow [32,33].

We have demonstrated that soon after PD is initiated, MCs obtained from the PD effluents show a progressive loss of epithelial morphology and acquire a fibroblast-like phenotype [6]. These transformed MCs progressively lose the expression of epithelial markers such as E-cadherin and cytokeratins, up-regulate the expression of snail, fibronectin and collagen I, and acquire migratory capacity [6]. In immunohistochemical studies of peritoneal biopsies from PD patients, we demonstrated the expression of mesothelial markers in stromal spindle-like cells, suggesting that they stemmed from the local conversion of MC [6,7]. The transformed MC expresses the myofibroblast molecule α-SMA [7]. We and others have shown that this myofibroblastic conversion of MC can be induced in vitro with various stimuli [6,34]. Our findings, suggest that new fibroblasts may arise mainly from local conversion of MC by EMT during the repair response of the peritoneal tissue induced by PD. In contrast, we did not observe a significant contribution of CD34⁺ cells from bone marrow to the submesothelial fibroblast population in the fibrotic peritoneal tissue. In renal fibrosis models, it has been shown that 36% of the new fibroblasts derive from EMT and 15% from bone marrow. The rest comes from local proliferation of resident fibroblasts [35].

In PD, factors such as peritonitis through the synthesis of interleukin-1 (IL-1) and other pro-inflammatory cytokines, haemoperitoneum activating the plasminogen-fibrin pathway, low pH through activation of latent TGF-β, elevated advanced glycation end products (AGEs) in peritoneal membrane [9,10,12,36–38] and glucose direct stimulation of TGF-β secretion [39], may induce EMT. The confirmation of all these events and their temporary sequence has been recently published by Margetts et al. [40]. In a rodent model, the peritoneal over expression of TGF-β1 provokes the EMT of MC as the first response to transfection. After 7 days, myofibroblasts and epithelioid-shaped cells are intermingled in submesothelium. MCs are found in this erroneous position creating a second basement membrane.
They also showed the in vitro vs in vivo differences of the TGF-β1 effect on E-cadherin expression, reflecting the influence that local environment causes in vivo.

Role of epithelial-to-mesenchymal transition of MC in neovascularization and peritoneal transport disorders

There is emerging evidence that expansion of the peritoneal vasculature and augmented vessel permeability are important determinants of increased small solute transport across the peritoneal membrane and ultrafiltration failure [41]. VEGF is a pro-angiogenic cytokine involved in endothelial cell proliferation and vascular permeability. It has been proposed that local production of VEGF during PD plays a central role in processes leading to peritoneal angiogenesis [42]. The main source of VEGF in PD patients, as well as the mechanisms implicated in VEGF up-regulation during PD, remains unclear. Previous studies have shown that MC from omentum have the capacity to produce VEGF in vitro in response to a variety of stimuli such as GDPs, AGEs and TGF-β [38,42,43]. Furthermore, it has been reported that effluent-derived MCs produce spontaneously different levels of VEGF ex vivo, but the reason for these marked differences in VEGF production capacity has not been established [44]. Recently, we have found that the mechanism underlying VEGF up-regulation in MCs is the EMT of these cells. Our most recent findings show a clear association between EMT of MCs, synthesis of VEGF, secretion of ECM components and type-I peritoneal membrane failure. The observation that EMT of MC is the first event and a key in the process of peritoneal angiogenesis and fibrosis, opens new insights for early diagnosis and therapeutic intervention.

Figure 2 summarizes the implications of EMT of MC in the peritoneal membrane failure. EMT induces a decrease in fibrinolytic capacity by an increase in the PAI–tPA ratio pathway [45], stimulates CTGF production [46,47], increases chemokines and pro-inflammatory molecules [48,49], and VEGF production [42,44,50] (unpublished data).

The correspondence between phenotypic changes in MC taken from the effluent, and cultured ex vivo, and that in the peritoneum, promotes this method for early diagnosis purposes. The first phenotypic changes of MC precede the intervention of VEGF, which is a secondary consequence of the epithelial-to-mesenchymal transition. Therapeutic interventions to modify the EMT of MCs are the next step to interrupt, in time, the undesirable response of the peritoneum to dialysis. The role of fibroblasts of different origin and their relationship to the trans-differentiated MCs also remains to be explored.

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