VASCULAR ENDOTHELIAL GROWTH FACTOR EXPRESSION IN PERITONEAL MESOTHELIAL CELLS UNDERGOING TRANSDIFFERENTIATION

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Objective: To analyze gene expression of localized peritoneal tissue structures in a rodent model of peritoneal fibrosis.

Methods: Female Sprague Dawley rats were treated with an intraperitoneal injection of an adenovirus expressing active transforming growth factor-beta or control adenovirus. Four and 7 days after infection, animals were sacrificed and frozen sections of parietal peritoneum were subjected to immunofluorescence-aided laser capture microdissection in order to isolate vascular, mesothelial, and submesothelial structures. RNA was extracted from microdissected tissue and gene expression was analyzed by quantitative reverse-transcript polymerase chain reaction. We analyzed genes involved in angiogenesis, epithelial-to-mesenchymal transdifferentiation, and fibrosis. Vascular endothelial growth factor and alpha-smooth muscle actin expression was analyzed with immunohistochemistry of formalin-fixed tissue.

Results: Transforming growth factor-beta (TGF-β) induced expression of Snail and alpha-smooth muscle actin genes in the peritoneal mesothelium. This same cell population also demonstrated increased gene expression of vascular endothelial growth factor. The distribution of this growth factor was confirmed by immunohistochemistry. The fibrogenic growth factor, connective tissue growth factor, was also strongly induced in the peritoneal mesothelium.

Conclusions: Using immunofluorescence-aided laser capture microdissection, we were able to study gene expression in subcompartments of the peritoneal tissue. We demonstrated that mesothelial cells exhibiting mesenchymal transdifferentiation are associated with increased expression of genes associated with fibrosis and angiogenesis.

Peritoneal dialysis (PD) patients develop increased peritoneal membrane thickness, fibrosis, and angiogenesis that progress with the duration of PD therapy (1). Peritoneal membrane dysfunction, manifested by ultrafiltration failure, appears to be the result of increased peritoneal vascularization leading to increased solute transport (2) combined with progressive submesothelial thickness and fibrosis (3). The processes of fibrosis and angiogenesis are linked in many respects, with common growth factors implicated in both (4). Recently, the mesothelial cell has taken on new importance in peritoneal membrane injury through the observed effect of epithelial-to-mesenchymal transdifferentiation (EMT). EMT is an early event in peritoneal membrane fibrogenesis and has been described as a consequence of transforming growth factor-beta (TGF-β) exposure, in mesothelial cell culture (5) and in vivo (6). EMT has also been observed in the peritoneum of PD patients (7). EMT involves a loss of cell–cell adhesion through downregulation of the intracellular adhesion molecule E-cadherin (8), basement membrane degradation, and cytoskeletal rearrangement with expression of alpha-smooth muscle actin (αSMA) (9). Snail is a zinc finger regulator of gene transcription that has been shown to be essential in the initiation of EMT (10).

The mesothelial cell may play a role in the fibrosis and angiogenesis associated with peritoneal membrane injury. Aroeira and colleagues demonstrated that injured mesothelial cells demonstrating with a transdifferentiated phenotype also appear to be a source of vascular endothelial growth factor (VEGF) (11).

The peritoneal membrane is a relatively simple structure consisting of a mesothelial cell layer with underlying submesothelial connective tissue and vasculature. Despite this simplicity, studies evaluating gene and protein expression by injured peritoneal tissues will tend to
group these substructures together. Gene expression studies using conventional RNA isolation from anatomically complex tissues are difficult to interpret because the cells of interest are contaminated by many different cell types. It is difficult to detect an early subtle change in gene expression within a specific type of cell from the RNA pools isolated from the bulk tissue. However, laser capture microdissection (LCM) of frozen tissue sections has enabled the isolation of specific cells from tissues based on direct morphologic visualization, and thus allows circumvention of the problem of tissue heterogeneity (12). This technique can be refined using immunofluorescence (IF-LCM) to identify cell populations for selection (13).

In this study, we used IF-LCM to isolate mRNA from different substructures within the peritoneal membrane of rats after exposure to an adenovirus expressing TGF-β1. We previously demonstrated that transient overexpression of TGF-β1 induces EMT (6). We were specifically interested in evaluating the in vivo expression of vascular growth factors in epithelium undergoing EMT. Using IF-LCM, we were able to isolate and evaluate gene expression in epithelium, vessels, and submesothelium separately. We confirmed the activity of the mesothelium in this model using increased expression of EMT-related genes. This same cell population also demonstrated significantly increased expression of VEGF, supporting previous ex vivo observations (11).

METHODS

ADENOVIRAL CONSTRUCT

A replication-deficient recombinant adenovirus expressing the biologically active form of porcine TGF-β1 (AdTGF-β1) was used as previously described (14). In this construct, the TGF-β1 gene is mutated at residues 223 and 225, and the resulting protein does not bind to its latency-associated protein, allowing production of active TGF-β1. An empty (null) virus (AdDL) was used as a virus control. Adenovirus preparations were purified by CsCl gradient centrifugation and PD-10 Sephadex chromatography (Amersham Pharmacia, Baie d’Urfé, Quebec, Canada), and plaques were titrated on 293 cells as described previously (15).

ANIMAL EXPERIMENT

All animal studies were performed according to the Canadian Council on Animal Care guidelines. Food and water were provided ad libitum. Sprague Dawley rats (225 – 275 g; Harlan, Indianapolis, Indiana, USA) were anesthetized with isoflurane (MTC Pharmaceuticals, Cambridge, Ontario, Canada) and were given intraperitoneal injections of AdTGF-β1 or AdDL. A total of 16 rats were used in this experiment. They received 1 × 10⁹ plaque-forming units of AdTGF-β1 or AdDL diluted in 100 μL phosphate-buffered saline injected into the peritoneal cavity on day 0. The animals were euthanized on the fourth or seventh day after infection (4 animals/group). The anterior abdominal wall was taken and divided in two. The lower (caudal) section was frozen for LCM and the upper (rostral) half was placed in 10% formalin for histological analysis.

HISTOLOGIC ASSESSMENT

Fresh tissue samples were fixed in 10% formaldehyde for 24 hours and then paraffin-processed and embedded; 3-μm sections were cut. Sections were stained with Masson’s trichrome. For VEGF staining, we used proteinase K (Dako, Glostrup, Denmark) antigen retrieval followed by anti-VEGF antibody (Santa Cruz Biotechnology, Santa Cruz, California, USA) and EnVision Plus (Dako) secondary reagent. Negative controls using a blocking peptide (Santa Cruz) were run concurrently. For αSMA staining, sections were incubated overnight in an anti-αSMA antibody (Dako) with EnVision Plus secondary reagent.

LASER CAPTURE MICRODISSECTION (LCM)

Tissues from the anterior abdominal wall were placed in a mold containing Tissue-Tek OCT compound (Sakura Finetek Torrance, California, USA), frozen on dry ice, and then stored at −70°C. Using RNase-free technique, frozen sections were sectioned at 8 μm and stored at −80°C until microdissection. Immediately before LCM, the frozen sections were thawed for 30 seconds, fixed in cold acetone, and then stained with purified mouse anti-rat CD31 (BD Biosciences Pharmingen, Oakville, Ontario, Canada), followed by TRITC-labeled anti-mouse IgG (Jackson ImmunoResearch, West Grove, Pennsylvania, USA). RNase inhibitor (Invitrogen, Burlington, Ontario, Canada) was added to antibodies to prevent tissue RNA from degradation. Laser capture microdissection of peritoneal tissues was performed using a PixCell Laser Capture Microscope (Arcturus, Mountain View, California, USA), as described previously (12). In brief, immunostained sections were overlaid with a thermoplastic membrane mounted on transparent caps. Laser spots of 7.5 μm size and pulse power of 40 – 100 mW were used. A brief (750 – 1000 ms) laser pulse shot through the cap directly above the target tissues caused the thermoplas-
tic film to adhere to the target tissue. Lifting the cap removed the target cells or tissues attached to the cap. Using specific immunofluorescence, blood vessels were identified and selected by LCM. Mesothelium was identified by autofluorescence and anatomic location, and was selected separately by LCM. Remaining interstitium (without vasculature or mesothelial cells) was captured on a third cap. Over 1000 laser spots were used for each sample. Staining and LCM was limited to 2 hours to maintain RNA integrity.

**RNA ISOLATION AND mRNA AMPLIFICATION FROM LCM TISSUE**

Isolation of RNA from the laser captured cells was performed with PicoPure RNA Isolation Kit (Arcturus) according to the manufacturer’s instruction. Captured cells were immersed in 50 μL extraction buffer and incubated for 30 minutes at 42°C. The cell extract was collected by centrifugation, resuspended in 50 μL 70% ethanol, passed through an RNA purification column, then washed and eluted in buffer for a final volume of 13 μL.

Total RNA concentration and integrity were assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Mississauga, Ontario, Canada). Isolated RNA was amplified with MessageAmp aRNA Kit (Ambion, Foster City, California, USA) according to the manufacturer’s instruction. Briefly, we primed the RNA with the T7 oligo (dT) primer to synthesize cDNA with a T7 promoter sequence by reverse transcription. After cDNA purification, *in vitro* transcription was induced to generate multiple copies of amplified RNA (aRNA) from the double-stranded cDNA templates. Amplified products were finally subjected to purification steps to remove unincorporated NTPs, salts, enzyme, and other contaminants and concentrated by vacuum centrifugation for a final volume of 13 μL. The amount and purity of aRNA was determined using the Agilent 2100 Bioanalyzer.

**REVERSE TRANSCRIPT POLYMERASE CHAIN REACTION (RT-PCR)**

RNA was DNase treated and reverse transcribed using a standard protocol (Invitrogen). Quantitative PCR for VEGF, angioptietin-2 (ANG2), αSMA, Snail, connective tissue growth factor (CTGF), and GAPDH was carried out using an ABI Prism 7500 Sequence Detector (Applied Biosystems, Foster City, California, USA). Negative control samples (no template or no reverse transcriptase) were run concurrently. Primers (Mobix, Hamilton, Ontario, Canada) and probes (Applied Biosystems) are shown in Table 1.

**STATISTICAL ANALYSIS**

Data are shown as average ± standard error of the mean. Analysis between groups was performed by ANOVA at each time point with least significant difference post *hoc* analysis. A p value less than 0.05 was considered statistically significant.

**RESULTS**

After exposure to AdTGF-β1, the rat peritoneal tissues demonstrated histological changes similar to our previous observations [Figures 1(a) and 1(b)] (16). Specifically, there was submesothelial thickening, increased collagen deposition (by Masson’s trichrome), and angiogenesis. The control adenovirus had no overt effect on the peritoneal tissues (Figure 1).

We were able to successfully label and isolate vascular structures, mesothelium, and submesothelial tissues using LCM immunofluorescently labeled sections (Figure 2). Extracted mRNA was of sufficient quality for subsequent amplification and RT-PCR based on visual inspection of microfluidic gel plot (data not shown).

We used quantitative PCR to analyze the expression of genes involved in angiogenesis (VEGF, ANG2), EMT (Snail, αSMA), and fibrosis (CTGF) (Figure 3). In captured mesothelial cells 4 and 7 days after infection with AdTGF-β1, there was a significant increase in VEGF gene expression in AdTGF-β1-treated compared with control adenovirus-treated animals [Figure 3(a)]. VEGF expression was also evident in the vasculature and, to a lesser extent, in the submesothelial tissue of AdTGF-β1-treated animals. In control (AdDL-) treated animals, VEGF expression was not detected in the mesothelium and there was minimal expression in the vasculature. These changes correlate with protein expression seen with immunohistochemistry [Figures 1(c) to 1(e)]. These sections demonstrate intense VEGF staining in the mesothelium at day 4 after AdTGF-β1 injection. There is observed vascular expression in both AdTGF-β1- and AdDL-treated animals, with no mesothelial expression in control animals. We also analyzed the gene expression of another vascular-related molecule, ANG2 (17). As expected, ANG2 expression was increased in vascular tissue of both AdTGF-β1- and AdDL-treated animals [Figure 3(b)]. By day 7, ANG2 gene expression remained significantly elevated in the vasculature of AdTGF-β1- compared with AdDL-treated control animals. Angiopoeitin-2 was also expressed in the mesothelial tissue and, to a lesser extent, the submesothelial tissue of animals treated with AdTGF-β1. ANG2 gene expression was undetectable in the mesothelium and submesothelium of AdDL-treated animals.
TABLE 1

Rat Primers and Probes Used for Reverse-Transcript Polymerase Chain Reaction

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF</td>
<td>GAGCAGAAAAGCCCATGAAGTG</td>
<td>GGTCTCAATTGGACGGCAAT</td>
<td>AAGTTGACGGACGTCTACCCAGGCACG</td>
</tr>
<tr>
<td>ANG2</td>
<td>CTGCCAGGATCCACCTACAGGACTCA</td>
<td>CTGCTGCGGTTGCTATGCT</td>
<td>AGGCACGGGGCGAATAAC</td>
</tr>
<tr>
<td>αSMA</td>
<td>CCAGAGCAAGAGAGGATCCT</td>
<td>TGTCGTCCCAGTTGGTGATG</td>
<td>ACGCTGAAGTACCGATAGAACACGCA</td>
</tr>
<tr>
<td>Snail</td>
<td>GGTTCCTGCGTCTGCTCCTCT</td>
<td>AGCTGCTTCCAGAGCTACAC</td>
<td>CAGACTGCTTCTGACCCACAGAG</td>
</tr>
<tr>
<td>CTGF</td>
<td>AATGCGCTTGGCAAGGTAACT</td>
<td>CCTCCAAACACCAGTACAGTC</td>
<td>CAAACACTTCTGCAAGATCAGCC</td>
</tr>
</tbody>
</table>

VEGF = vascular endothelial growth factor; ANG2 = angiopoietin-2; αSMA = alpha-smooth muscle actin; CTGF = connective tissue growth factor.

Figure 1 — Histology of the parietal peritoneal membrane. Seven days after infection with adenovirus expressing transforming growth factor-beta, (AdTGF-B1), the peritoneum demonstrates increased submesothelial thickness, angiogenesis, and fibrosis (A) compared with control adenovirus-treated animals (B). Vascular endothelial growth factor (VEGF) expression is increased in mesothelium (closed arrows) and vascular cells (open arrows) in AdTGF-B1-treated animals at 4 (C) and 7 (D) days after infection. In animals treated with empty (null) virus (AdDL), VEGF infection is limited to vascular structures (E). In alpha-smooth muscle actin (αSMA) staining at 4 (F) and 7 (G) days after infection with AdTGF-B1, αSMA is expressed in mesothelium (closed arrows), immediate submesothelium, and vascular structures (open arrows); αSMA is limited to vascular structures after AdDL infection (H). (A) and (B) are Masson’s trichrome stained, (C) to (E) are immunohistochemical sections for VEGF, and (F) to (H) are immunohistochemically stained for αSMA. (All are ×200 magnification.)
Alpha-SMA gene expression [Figure 3(c)] was upregulated by TGF-β1 in both vascular and mesothelial tissues compared with control animals. αSMA was not detected in mesothelium or submesothelial tissues in control AdDL-treated animals. Again, this correlated in general with immunohistochemical analysis [Figures 1(f) to 1(h)], although αSMA protein expression appeared to be more persistent in the submesothelial tissues than the corresponding gene expression. Snail gene expression was found to be variable, with significant increase in the mesothelial tissues demonstrated at day 4 only [Figure 3(d)]. Connective tissue growth factor gene expression was significantly elevated in mesothelial cells 4 days after AdTGF-β1 infection [Figure 3(e)].

**DISCUSSION**

Tissue fibrosis is a dynamic process consisting of a series of events that have both temporal and spatial patterns. In order to understand these processes, we developed an animal model of peritoneal fibrosis and used IF-LCM to examine gene expression in distinct subcompartments of the peritoneal tissues. We confirmed that the mesothelial cell layer demonstrates evidence of EMT...
after gene transfer of active TGF-β1. We observed increased gene expression of the zinc finger regulatory protein Snail with increased gene and protein expression of αSMA in mesothelial cells.

Mesothelial cells that demonstrate increased expression of EMT-related genes also have significantly increased expression of angiogenic molecules such as VEGF and ANG2. Increased VEGF gene expression was confirmed at the protein level by immunohistochemical analysis. These results suggest that EMT, angiogenesis, and fibrosis may be interconnected processes mediated by injury and response of the mesothelial cell layer. There is substantial evidence that VEGF is induced in mesothelial cells through a variety of agents, including TGF-β1 (16) and advanced glycation end products (AGE) (18), and our in vivo data would confirm these results. Aroeira and colleagues suggested the link between mesothelial EMT and angiogenesis in an ex vivo study of patients using...
PD (11). They demonstrated that ex vivo VEGF production of cultured mesothelial cells correlated with the phenotype of the cells (epithelial vs non-epithelial) and also correlated with serum VEGF measured in the patients. Using IF-LCM, our present research provides definitive evidence for the co-localization of VEGF induction in mesothelial cells demonstrating evidence of EMT. This observation suggests that isolation and evaluation of mesothelial cells from PD effluent for EMT and angiogenic factors could be another method to assess the health of the peritoneal membrane.

The two main angiopoietins, ANG1 and ANG2, both interact with the Tie2 receptor, with ANG2 antagonizing the signaling action of ANG1 (19). ANG2 activates endothelial cells and, in the presence of VEGF, induces vascular sprouting and initiates angiogenesis. We analyzed the localized expression of ANG2, as its expression is usually limited to endothelial cells, except in some tumor epithelium (19), and we felt this would support the specificity of the IF-LCM. We observed ANG2 expression limited to captured vascular structures of animals in both treatment groups, but AdTGF-β1-treated animals demonstrated significant ANG2 expression in the isolated mesothelium. ANG2 expression is known to be induced by VEGF and hypoxia (19); perhaps the interaction between these two agents allowed for induction of ANG2 in mesothelial cells in our model. The association between these angiogenic markers and peritoneal fibrosis has been studied in a model of chlorhexidine-induced peritoneal sclerosis (20). In agreement with our findings, these authors described increased VEGF and ANG2 localized to the superficial proliferative layer at later time points in their experiment.

Mesothelial cells also demonstrated an increased gene expression of the fibrosis-related molecule CTGF. CTGF has been demonstrated to be an important downstream mediator of TGF-β1 (21), although the role of CTGF as a direct fibrogenic molecule is not clear (22). The evidence for the role of CTGF in angiogenesis is discrepant. Earlier studies suggest that CTGF may inhibit VEGF action (23); however, more recent work indicates that CTGF has direct angiogenic activity (24,25).

There are several limitations in our study. This model has certain similarities to human peritoneal injury in patients on PD, but there are important differences, including a lack of progression over time. These issues have been addressed previously (16). The nature of the model involves gene transfer, using an adenovirus vector, to mesothelial cells. This is not a natural system; although, with usual peritoneal injury, TGF-β1 is induced in the mesothelium by agents such as high glucose, inflammatory molecules, or advanced glycation end products. In our model, this induction step is circumvented by direct expression of TGF-β1 in this cell population. The adenovirus itself may induce significant changes in gene regulation in the mesothelium. We previously showed that the inflammatory response to the adenovirus resolves by the fourth day after infection (6). In animals infected with control adenovirus, the captured mesothelium demonstrated no detectable gene expression of VEGF, ANG2, or αSMA [Figures 3(a) to 3(c)]. This argues against an overall activation through a direct adenoviral effect. It is impossible to capture a cell population with 100% purity. This is especially important at day 7, when we know the mesothelial cells have undergone EMT and these cells now intermingle with submesothelial myofibroblasts (6). This mixture of cell types likely explains why the captured submesothelium shows high expression of Snail and αSMA [Figures 3(c) and 3(d)].

To our knowledge, the use of IF-LCM has not previously been applied to peritoneal tissues. This technique is novel and allows for careful assessment of gene regulation in highly specific tissue structures (13). As opposed to other measures of gene expression, such as in situ hybridization, the power of this technique is its ability to simultaneously assess and quantify gene expression of multiple genes in a selected cell population. This can be advanced to the next level by taking RNA from laser captured tissue and applying genome wide analysis (26). This technique can also be applied to murine tissue to take advantage of the wide range of genetically manipulated animals available. In our present study, we confirmed the central role of mesothelial cells undergoing EMT in the progressive fibrosis and angiogenesis of peritoneal membrane.

REFERENCES


