Activation of nuclear factor of activated T cells 5 in the peritoneal membrane of uremic patients

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1Division of Nephrology, Department of Internal Medicine, Robert-Bosch-Hospital, Stuttgart, Germany; 2Department of General, Visceral, and Trauma Surgery, Robert-Bosch-Hospital, Stuttgart, Germany; 3Department of Diagnostic Medicine, Division of Pathology, Robert-Bosch-Hospital, Stuttgart, Germany; 4Dr. Margarete Fischer-Bosch Institute of Clinical Pharmacology, University of Tuebingen, Stuttgart, Germany; 5Department of Pathophysiology, University of Medical Sciences, Poznan, Poland; 6Department of Nephrology and Medical Intensive Care, Charité-Universitätsmedizin Berlin, Campus Virchow-Klinikum, Berlin, Germany; 7Division of Nephrology, University Hospital Zurich, Zurich, Switzerland; and 8Institute of Physiology and Zurich Center for Integrative Human Physiology, University of Zurich, Zurich, Switzerland

Submitted 11 November 2014; accepted in final form 25 March 2015

Kitterer D, Latus J, Ulmer C, Fritz P, Biegger D, Ott G, Alscher MD, Witowski J, Kawka E, Jörres A, Seeger H, Segerer S, Braun N. Activation of nuclear factor of activated T cells 5 in the peritoneal membrane of uremic patients. Am J Physiol Renal Physiol 308: F1247–F1258, 2015. First published April 1, 2015; doi:10.1152/ajprenal.00617.2014.—Peritoneal inflammation and fibrosis are responses to the uremic milieu and exposure to hyperosmolar dialysis fluids in patients on peritoneal dialysis. Cells respond to high osmolarity via the transcription factor nuclear factor of activated T cells (NFAT5). In the present study, the response of human peritoneal fibroblasts to glucose was analyzed in vitro. Expression levels of NFAT5 and chemokine (C-C motif) ligand (CCL2) mRNA were quantified in peritoneal biopsies of five nonuremic control patients, five uremic patients before PD (pPD), and eight patients on PD (oPD) using real-time PCR. Biopsies from 5 control patients, 25 pPD patients, and 25 oPD patients were investigated using immunohistochemistry to detect the expression of NFAT5, CCL2, NF-κB p50, NF-κB p65, and CD68. High glucose concentrations led to an early, dose-dependent induction of NFAT5 mRNA in human peritoneal fibroblasts. CCL2 mRNA expression was upregulated by high concentrations of glucose after 6 h, but, most notably, a concentration-dependent induction of CCL2 was present after 96 h. In human peritoneal biopsies, NFAT5 mRNA levels were increased in uremic patients compared with nonuremic control patients. No significant difference was found between the pPD group and oPD group. CCL2 mRNA expression was higher in the oPD group. Immunohistochemistry analysis was consistent with the results of mRNA analysis. CD68-positive cells were significantly increased in the oPD group. In conclusion, uremia results in NFAT5 induction, which might promote early changes of the peritoneum. Upregulation of NFAT5 in PD patients is associated with NFκB induction, potentially resulting in the recruitment of macrophages.

nuclear factor of activated T cells 5; chemokine (C-C motif) ligand 2; monocyte chemoattractant protein-1; mesothelial cell; human peritoneal fibroblasts

LOSS OF PERITONEAL MEMBRANE FUNCTION is a major contributor to treatment failure in patients on peritoneal dialysis (PD) (14, 16, 17). Nonspecific morphological findings of patients with ultrafiltration failure are thickening of the submesothelial layer by expansion of the extracellular matrix (peritoneal fibrosis), neangiogenesis, vasculopathy, and mesothelial cell alterations (with denudation). Low biocompatibility of PD fluids, such as low pH, lactate buffer, glycation degradation products, and high osmolality, may all promote these changes (12, 13, 56, 58, 59). Importantly, the thickening of the submesothelial layer was already found to be present in uremic patients not yet on PD (31).

The morphological changes and resulting disturbance of membrane physiology are caused and accompanied by an increase in various cytokines, including different chemokines, and growth factors [e.g., transforming growth factor (TGF)-β and VEGF] (8, 42, 63, 64). These factors induce inflammation and angiogenesis, which result in fibrosis (41, 57).

The osmosensitive transcription factor nuclear factor of activated T cells (NFAT)5, also known as toxicity-responsive enhancer-binding protein, plays a key role in the protection of cells against osmotic stress (51). NFAT5 regulates gene expression induced by high osmolarity in mammalian cells, accumulates in the nucleus, and stimulates the transcription of genes involved in the production and uptake of organic osmolytes under hyperosmolarity (11, 35). Recently, it has been shown that osmotic stress increases NF-κB activity through an interaction between NFAT5 and the transcription factor NF-κB (51). Chemokine (C-C motif) ligand (CCL2), also known as monocyte chemoattractant protein (MCP-1), is a key chemokine that regulates the migration and infiltration of monocytes/macrophages. It is produced by a variety of cell types after induction by oxidative stress, cytokines, or growth factors (18). An alternative pathway is the induction of CCL2 by osmotic stress. Remarkably, this mechanism can be suppressed by prednisolone via NF-κB inhibition (29, 40, 43). Osmotic stress induces both NFAT5- and NF-κB expression, leading to an upregulation of CCL2 in mesothelial cell cultures (36). It has been shown that the mesothelial cell layer is lost in a large proportion of peritoneal biopsies from PD patients (19). Furthermore, submesothelial fibroblast cells produce matrix proteins, which lead to further thickening and fibrotic remodeling of the submesothelial zone. These cells seem to play a key role in this process and are specific progenitors of α-smooth muscle actin-positive myofibroblasts in an animal model of peritoneal fibrosis induced by hyperosmolality (10). Additionally, CCL2

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increases formation of the extracellular matrix in cell cultures (39, 49).

Therefore, in the present study, we aimed to describe the activation of NFAT5 in vitro and in vivo in human peritoneal biopsies. The hypothesis was that hyperosmotic conditions, such as exposure to glucose-containing peritoneal dialysate, result in activation of NFAT5, which might promote inflammation of the peritoneal membrane.

**METHODS**

**Patients**

Demographic information, underlying renal disease, comorbidities (diabetes, hypertension, and smoking status), details on PD prescriptions, peritonitis rate, and medications were documented through chart reviews. Baseline characteristics of all patients are shown in Tables 1 and 2.

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**Table 1. Clinical data of control patients, pPD patients, and oPD patients (immunohistochemistry)**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control Patients</th>
<th>pPD Patients</th>
<th>oPD Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>5</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>Age, yr</td>
<td>54 ± 13.0</td>
<td>54.5 ± 15</td>
<td>54.2 ± 15.3</td>
</tr>
<tr>
<td>PD duration, mo</td>
<td></td>
<td>30.0 ± 26.6</td>
<td></td>
</tr>
<tr>
<td>Peritonitis</td>
<td></td>
<td>25 in 749 mo (1:30)</td>
<td></td>
</tr>
<tr>
<td>PD fluids</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutral</td>
<td>3/14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acidic</td>
<td>11/14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Both or ND</td>
<td>6/20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Icodextrin</td>
<td>1/14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transporter status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High/high average</td>
<td>6/11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low/low average</td>
<td>5/11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ND</td>
<td>14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes</td>
<td>0/5</td>
<td>12/25</td>
<td>8/24 (1 ND)</td>
</tr>
<tr>
<td>Smoker</td>
<td>0/5</td>
<td>6/13 (12 ND)</td>
<td>20/25</td>
</tr>
<tr>
<td>Hypertension</td>
<td>0/5</td>
<td></td>
<td></td>
</tr>
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</table>

Laboratory findings:

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control Patients</th>
<th>pPD Patients</th>
<th>oPD Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin, g/dl (range: 13–18 g/dl)</td>
<td>12.8 ± 2.7</td>
<td>10.4 ± 1.5</td>
<td>11.5 ± 1.9</td>
</tr>
<tr>
<td>Leukocytes, g/l (4.0–11.3 g/l)</td>
<td>5.0 ± 1.9</td>
<td>8.1 ± 2.2</td>
<td>7.6 ± 2.1</td>
</tr>
<tr>
<td>C-reactive protein, mg/dl (&lt;0.1 mg/dl)</td>
<td>0.2 ± 0.2</td>
<td>0.9 ± 1.1</td>
<td>1.7 ± 1.7</td>
</tr>
<tr>
<td>Phosphate, mmol/l (range: 0.68–1.68 mmol/l)</td>
<td>ND</td>
<td>1.8 ± 0.5</td>
<td>1.7 ± 0.6</td>
</tr>
<tr>
<td>Calcium, mmol/l (range: 1.90–2.70 mmol/l)</td>
<td>2.2 ± 0.1</td>
<td>2.3 ± 0.3</td>
<td>2.3 ± 0.2</td>
</tr>
<tr>
<td>Parathyroid hormone, pmol/l (range: 1.1–7.3 pmol/l)</td>
<td>ND</td>
<td>23.0 ± 11.7</td>
<td>22.3 ± 20.7</td>
</tr>
<tr>
<td>Urea N, mg/dl (range: 10–25 mg/dl)</td>
<td>ND</td>
<td>149.4 ± 67.6</td>
<td>122.7 ± 71.4</td>
</tr>
<tr>
<td>Creatinine, mg/dl (range: 0.5–1.4 mg/dl)</td>
<td>0.8 ± 0.1</td>
<td>6.8 ± 2.5</td>
<td>7.9 ± 3.4</td>
</tr>
</tbody>
</table>

Values are means ± SD; n, number of patients. PD, peritoneal dialysis; pPD patients, patients before the start of PD; oPD, patients on PD; ND, not determined.

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**Table 2. Clinical data of control patients, pPD patients, and oPD patients (using RT-PCR)**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control Patients</th>
<th>pPD Patients</th>
<th>oPD Patients</th>
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<tbody>
<tr>
<td>n</td>
<td>5</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>Age, yr</td>
<td>56.2 ± 12.3</td>
<td>54.4 ± 18.1</td>
<td>59.1 ± 14.3</td>
</tr>
<tr>
<td>PD duration, mo</td>
<td></td>
<td>49.8 ± 39.4</td>
<td></td>
</tr>
<tr>
<td>Peritonitis</td>
<td>16 in 398 mo (1:25)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PD fluids</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutral</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acidic</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ND</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Icodextrin</td>
<td>4/7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transporter status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High/high average</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low/low average</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ND</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes</td>
<td>0/5</td>
<td>4/5</td>
<td>2/8</td>
</tr>
<tr>
<td>Smoker</td>
<td>0/5</td>
<td>2/5</td>
<td>2/8</td>
</tr>
<tr>
<td>Hypertension</td>
<td>0/5</td>
<td>3/5</td>
<td>7/8</td>
</tr>
</tbody>
</table>

Laboratory findings:

<table>
<thead>
<tr>
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<th>Control Patients</th>
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<td>Hemoglobin, g/dl (range: 13–18 g/dl)</td>
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<td>11.5 ± 2.0</td>
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<tr>
<td>Leukocytes, g/l (4.0–11.3 g/l)</td>
<td>5.4 ± 1.3</td>
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<td>6.1 ± 1.7</td>
</tr>
<tr>
<td>C-reactive protein, mg/dl (&lt;0.1 mg/dl)</td>
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<td>1.1 ± 1.6</td>
<td>1.6 ± 2.3</td>
</tr>
<tr>
<td>Phosphate, mmol/l (range: 0.68–1.68 mmol/l)</td>
<td>N.D.</td>
<td>1.7 ± 0.3</td>
<td>1.4 ± 0.4</td>
</tr>
<tr>
<td>Calcium, mmol/l (range: 1.90–2.70 mmol/l)</td>
<td>2.2 ± 0.1</td>
<td>2.0 ± 0.1</td>
<td>2.2 ± 0.1</td>
</tr>
<tr>
<td>Parathyroid hormone, pmol/l (range: 1.1–7.3 pmol/l)</td>
<td>N.D.</td>
<td>34.3 ± 8.3</td>
<td>32.8 ± 23.0</td>
</tr>
<tr>
<td>Urea N, mg/dl (range: 10–25 mg/dl)</td>
<td>N.D.</td>
<td>133.2 ± 54.7</td>
<td>88.0 ± 44.5</td>
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<tr>
<td>Creatinine, mg/dl (range: 0.5–1.4)</td>
<td>0.8 ± 0.1</td>
<td>5.1 ± 1.0</td>
<td>6.3 ± 3.8</td>
</tr>
</tbody>
</table>

Values are means ± SD; n, number of patients.
Peritoneal Biopsies

All peritoneal biopsies were obtained from the peritoneal biopsy registry at the Robert-Bosch-Hospital (Stuttgart, Germany). The collection of human peritoneal tissue, blood, and peritoneal dialysate for research purposes was approved by the local ethics committee (no. 322/2009BO1, Ethic Committee of Eberhard-Karls University, Tuebingen, Germany). All patients gave written informed consent concerning the scientific workup of tissues taken during surgery.

Biopsies from the parietal peritoneum were taken from 25 patients on PD (oPD) and 25 uremic patients before the start of PD (pPD). Clinical information regarding exposure times to PD fluids of the peritoneal membrane was available from 21 of 25 biopsies from PD patients. In 11 of 21 patients, exposure to PD fluids was paused within <8 h before surgery. In 10 of 21 patients, PD fluids were paused for >8 h before surgical biopsy. In the group of patients in whom biopsies were available from 21 of 25 biopsies from PD patients, PD was paused 8 h before surgical biopsy. In 4 of 8 patients, PD was paused <8 h before surgery, and in 4 of 8 patients, PD was paused >8 h before biopsy.

Five biopsies from healthy control patients without kidney disease and systemic inflammation were collected during elective cholecystectomy. In the other patients, samples were taken at the time of catheter removal, correction of a catheter malposition, or during abdominal surgery (e.g., hernia repair or cholecystectomy). Peritoneal biopsies of uremic patients before the start of PD were obtained during catheter insertion. All biopsies were formalin fixed and paraffin embedded following routine protocols (7, 32). Patients with an episode of peritonitis within the last 6 mo were excluded. Peritoneal biopsies were washed in 0.9% saline solution, placed in RNAlater (Ambion, Woodlands, TX) shortly after tissue excision, and stored at −80°C for subsequent RNA extraction. On these samples, real-time RT-PCR was performed and mRNA expression was analyzed.

RNA Isolation

RNA was isolated from tissue immersed and frozen in RNAlater (Ambion). Frozen tissue samples were incubated in 700 µl QIAzol reagent and homogenized using a rotor-stator homogenizer (T8, IKA-Werke, Staufen, Germany) for 1 min. The homogenate was extracted with 140 µl chloroform, and phase separation of the solution was achieved by centrifugation. The clear, aqueous supernatant containing total RNA was removed, placed in a new microcentrifuge tube, mixed with 100% ethanol, applied to a spin column, and eluted in nuclease-free water. DNA was digested on column.

Quality Control

RNA was measured using a NanoDrop 2000c UV spectrophotometer (NanoDrop Technologies, Wilmington, DE). RNA integrity was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA).

Quantitative Real-Time RT-PCR

First-strand cDNA was synthesized with TaqMan RT reagents (Applied Biosystems, Darmstadt, Germany). Predeveloped TaqMan reagents were used for human CCL2 and NFAT5 and the housekeeping genes human β-actin and 18S rRNA (Applied Biosystems). mRNA expression was analyzed by standard curve quantification as previously described (15). For in vitro experiments, reverse transcription was performed, and the expression of candidate genes in samples was analyzed by the ΔΔCt method, where Ct is threshold cycle (15).

Immunohistochemistry

Immunohistochemistry was performed as previously described (5, 47). Dewaxed and rehydrated tissue sections were incubated in Peroxidase Blocking Solution (S 2023, DAKO, Hamburg, Germany). Pretreatment and characteristics of applied antibodies are shown in Table 3. For immunostaining, we used an Autostainer system (Autostainer Plus, DAKO). The staining method used a dextran-coated peroxidase coupled polymer system (Dako REAL EnVision Detektion Kit, peroxidase/DAB+, rabbit/mouse, K 5007, DAKO), and hema-toxylin counterstaining was performed.

Analysis of Immunohistochemistry

Two experienced observers (one pathologist and one nephrologist) blinded to the specimen’s diagnosis evaluated each section in two independent rounds. Any discrepant scores were reevaluated jointly on a second occasion, and an agreement was reached.

Quantification of fibrosis. Hematoxylin and eosin staining was performed on sections from each tissue block for morphological analysis. Tissue sections were scanned and visualized, and the thickness of the fibrosis zone was measured using the software program Image Manager (version 4.0, Leica). Slides were investigated at ×50 magnification to identify regions of interest. The submesothelial compacta was identified. Additionally, fibrosis was graded semiquantitatively, and the thickness of the submesothelial compacta was measured as previously described (6, 31, 55, 56). Five different representative high-power fields (HPFs) on each slide were analyzed in a standardized manner.

Evaluation of NFAT5 immunoreactivity. NFAT5 was evaluated semiquantitatively in endothelial cells of vessels (where 0 = absent, 1 = area of 1–10% of 5 HPFs, 2 = area of 11–50% of 5 HPFs, and 3 = area of >50% of 5 HPFs) and mesothelial cells (MCs; where 0 = absent, 1 = <3 cells/HPF, 2 = 4–10 cells/HPF, and 3 = >10 cells/HPF).

Evaluation of CCL2 immunoreactivity. Morphological CCL2 immunoreactivity was described and semiquantitatively scored in the endothelia of vessels, fibrocytes (FCs), fibroblasts (FBs), mononuclear cells (MNCs), and MCs. MNCs were defined as round cells with CCL2 reactivity in the cytoplasm and nucleus. Vessel endothelial cells were analyzed in a low-power field (LPF) and scored as follows: 0 = absent, 1 = 1–10% of 5 LPFs, 2 = 11–50% of 5 LPFs, and 3 = >50% of 5 LPFs.

Evaluation of NF-κB p50/κB p65 immunoreactivity. NF-κB p50/NF-κB p65 were evaluated in 5 HPFs (the mesothelial layer, FBs, FCs, vessels, round cells, and acute inflammation). NF-κB p50/NF-κB p65 expression was mainly detected in FCs, FBs, and mesothelial cells. Cells were analyzed in HPFs as follows: 0 = absent, 1 = 1–10% of 5 HPFs, 2 = 11–50% of 5 HPFs, and 3 = >50% of 5 HPFs. Acute inflammatory reaction was defined by the presence of neutrophilic granulocytes. Chronic inflammatory reaction was defined by the presence of MNCs without taking into consideration further sub-
classes (lymphocytes, plasma cells, monocytes, and histiocytes). Inflammatory cells and MNCs were scored in HPFs as follows: 0 = absent, I = 1–10% of 5 HPFs, 2 = 11–50% of 5 HPFs, and 3 = >50% of 5 HPFs.

**Evaluation of CD68 immunoreactivity.** CD68-positive cells were analyzed in 5 midpower fields (MPFs) as follows: 0 = absent, I = 1–10% of 5 MPFs, 2 = 11–50% of 5 MPFs, and 3 = >50% of 5 MPFs (HPF: 0.26 mm², MPF: 0.91 mm², and LPF: 3.2 mm²).

**Cell Culture of HPFBs**

HPFBs were isolated from specimens of apparently normal omentum obtained from consenting patients undergoing elective abdominal surgery. Tissue was first treated with two 20-min rounds of digestion with trypsin-EDTA (0.05-0.02%) solution to remove MCs. Subsequently, tissue was treated for 90 min with a solution of collagenase (1 mg/ml, Sigma-Aldrich, St. Louis, MO) and hyaluronidase (0.1 mg/ml, Sigma-Aldrich). HPFBs were identified by their typical spindle-shape appearance, formation of parallel arrays and whorls at confluence, and positive immunostaining for fibroblast-specific protein 1. Cells were propagated in Ham’s F-12 culture medium supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), hydrocortisone (0.4 μg/ml), and 10% (vol/vol) FCS. HPFB cultures were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO₂. All experiments were performed using cells from the first three passages and with cells derived from separate donors.

Cells were seeded into six-well plates, cultured until ~90% confluence, and rendered quiescent by reducing the FCS concentration to 0.1% for 48 h. Cells were then exposed for up to 96 h to media containing D-glucose at concentrations ranging from 10 mM (control) to 125 mM. At designated time points, cells were washed with cold PBS, and total RNA was extracted with RNA Bee (Tel-Test, Friendswood, TX). Cells were then stored at −80°C until assayed.

**Statistical Analyses**

Two observers, blinded to the diagnosis, performed the semiquantitative analysis. The ordinal variables were discriminated as absent, low grade, moderate grade, and high grade. Variables were classified for the analysis as binary (present or absent) to improve χ-values as previously described (6). All continuous variables were tested for normality using a Kolmogorov-Smirnov test. Comparisons between groups were made using a Mann-Whitney U-test or Fisher’s exact test as appropriate. Analyses were performed using the GraphPad statistical software package (San Diego, CA). Statistical results of P < 0.05 were considered to be significant, P < 0.01 as highly significant, and P < 0.005 as very highly significant. Continuous data are expressed as means ± SD. Medians with interquartile range were used where distribution was not normal. Error bars depict means with SD.

**RESULTS**

**In Vitro Experiments in HPFBs**

Peritoneal fibroblasts play a pivotal role in peritoneal fibrosis by secreting extracellular matrix and proinflammatory cytokines (60). A static cell culture system was used, in which HPFBs were exposed to increasing concentrations of glucose. NFAT5 mRNA revealed a significant induction upon increasing glucose concentrations after 6 h (Fig. 1A, left). After 24 or 96 h, no further induction above baseline was detectable. Therefore, the osmotic response can be detected within 6 h, but beyond 24 h, no regulation of NFAT-5 was detectable in this in vitro system.

After 6 h, only the highest concentration of glucose (125 mM) led to a significant induction of CCL2 mRNA in HPFBs (Fig. 1A, right). Importantly, after 96 h, a prominent, dose-dependent induction of CCL2 was detectable, without a significant induction of NFAT5 at that time. Therefore, CCL2 expression during chronic glucose exposure to peritoneal fibroblasts did demonstrate a late induction, which does not fit to the expression of NFAT5 in HPFBs.

**Patient Characteristics**

Clinical and laboratory data of 25 pPD patients, 25 oPD patients, and 5 nonuremic control patients are shown in Table 1. Mean age was 54.2 ± 15.3 yr in the oPD group, 54.5 ± 15.0 yr in the pPD group, and 54.0 ± 13.0 yr in the control group. Mean PD duration was 30.0 ± 25.6 mo in the oPD group. In the pPD group, 9 of 25 patients were women; in the oPD group, 10 of 25 patients were women. In the nonuremic control group, four of five patients were women. Baseline characteristics of the 18 patients from whom mRNA expression was analyzed are shown in Table 2.

The thickness of the submesothelial compacts is shown in Fig. 2A. The extent of fibrosis was significantly different between the groups (range: 195–3,365 μm in the oPD group and 34–380 μm in the pPD group, P < 0.005). Importantly, uremic patients demonstrated a thickening of the peritoneal membrane before the start of PD. Furthermore, a significant proportion of oPD patients showed signs of simple peritoneal fibrosis, as reflected by the prominent thickening of the submesothelial zone.

**NFAT5 and CCL2 mRNA Expression in Peritoneal Biopsies**

Peritoneal biopsies from the three groups of patients were evaluated for NFAT5 expression (Fig. 2B). The five nonuremic control biopsies demonstrated very little NFAT5 mRNA expression. Both oPD and pPD groups demonstrated a significant induction of NFAT5. CCL2 demonstrated the same trend but did not quite reach the level of significance in the pPD group (Fig. 2C). In contrast, CCL2 demonstrated a strong induction in the oPD group. Unexpectedly, NFAT5 mRNA expression was not significantly different between biopsies taken from the peritoneal membrane of pPD or oPD patients. Separation of patients into groups with <8 h of time after the last exposure to PD solution versus those of >8 h did not change the results (Fig. 2, D and E).

**Immunohistochemical Analyses of NFAT5, CCL2, NF-kB p50, NF-kB p65, and CD68**

**Immunoreactivity of NFAT5.** NFAT5 was present in endothelial cells of vessels (consistent with nuclei of endothelial cells) and MCs (Fig. 3, A and B). MCs were strongly positive for NFAT5 in peritoneal biopsies of both oPD and pPD groups (Fig. 3, A and B). Rare vessels in healthy control patients showed positive staining for NFAT5, but MCs showed no immunoreactivity in control patients.

NFAT5 protein scores were not significantly different between pPD and oPD groups for MCs and endothelial cells (P = 0.4 and P = 0.7, respectively; Fig. 4). In the control group, no staining of MCs occurred (data not shown). Therefore, the immunohistochemistry results were consistent with the mRNA expression data of NFAT5. Scores were not different in patient groups according to the last exposure to PD solutions (Fig. 4, H and I).
**Immunoreactivity of CCL2.** In the pPD group, there was little positive staining for MCs. No positive reaction was observed for FBs (Figs. 3C and 4D). In the oPD group, positive staining of the MC layer and of MNCs was present. Compared with the pPD group, FBs and, rarely, FCs showed positive staining for CCL2 (Fig. 3, C and D). The number of CCL2-positive cells was significantly increased in the oPD group compared with the pPD group (P < 0.05; Fig. 4C). CCL2-positive FBs were more frequent in the oPD group compared with the pPD group (P < 0.05; Fig. 4D). In control patients, no staining of FBs could be detected (data not shown). Again, scores were not different in patient groups according to the last exposure to PD solutions (Fig. 4, J and K).

**Immunoreactivity of NF-κB p50.** NF-κB p50-positive cells were identified mainly as FBs and FCs (Fig. 3, E and F). Expression of NF-κB p50 was significantly higher in FCs/FBs in the oPD group compared with the pPD group (P < 0.01; Fig. 4). In the control group, no positive staining of MCs and FBs could be detected (data not shown).

**Immunoreactivity of NF-κB p65.** The most intensive staining pattern could be detected in FBs and FCs according to the pattern of NF-κB p50-positive cells (Fig. 3, G and H). Com-
parable with the expression of NF-κB p50, NF-κB p65 was more prominent in FCs/FBs in the oPD group compared with the pPD group (P < 0.001; Fig. 4). In the control group, no positive staining of MCs and FBs could be detected (data not shown).

Immunoreactivity of CD68. The total number of CD68-positive cells was analyzed semiquantitatively in peritoneal biopsies of both groups (Fig. 3, I and J). The number of CD68-positive cells was significantly higher in the oPD group compared with the pPD group (P < 0.0001; Fig. 4). In the control group, CD68-positive cells were very rare (data not shown).

DISCUSSION

In the present study, we describe three new aspects. In human peritoneal biopsies from uremic patients, both oPD and pPD, expression of NFAT5 mRNA and protein were signifi-
Fig. 3. Immunohistochemistry of human peritoneal biopsies. A and B: both pPD patients (A) and oPD patients (B) showed strong reaction in vessels and mesothelial cells against NFAT5. C and D: CCL2 expression occurred mainly in fibroblasts (FBs) and mononuclear cells of PD patients. In the pPD group, weak positive staining for mesothelial cells occurred. E–H: both NF-κB p50 and NF-κB p65 were upregulated in biopsies of PD patients compared with pPD patients. I and J: CD68-positive cells were mainly present in biopsies from oPD patients. Magnification: ×400 in A–J.
significantly increased compared with biopsies from patients with normal renal function. An early induction of NFAT5 in HPFBs was found in response to increasing glucose concentrations. Finally, the prominent induction of CCL2 in the peritoneum did not correlate well with the temporal expression of NFAT5, indicating additional regulatory mechanisms.

Fibrosis of the peritoneal membrane is driven by inflammation, which might be related in part to the exposure of the...
peritoneum to hyperosmolar PD solutions (8, 27, 56, 62). Several groups have demonstrated thickening of the peritoneum in uremic patients before the start of PD, indicating that the uremic milieu already promotes peritoneal fibrosis (31, 56). The thickening of the submesothelial compacta was confirmed in our cohort. Here, we present the first data showing that NFAT5 expression is increased in human peritoneal biopsies in predialysis patients. A significant induction of NFAT5 was found in the peritoneum of uremic patients not yet on PD. NFAT5 is a transcription factor associated with osmotic stress (26, 44); its activity correlates with extracellular osmolality, and its regulation is quite fast (23). Hyperosmotic conditions lead to an increase in intracellular electrolyte concentration (intracellular ionic strength), which is directly sensed by the NH2-terminal transactivation domain of NFAT5. Nuclear (intracellular ionic strength), which is directly sensed by the NH2-terminal transactivation domain of NFAT5. Nuclear translocation and homodimer formation could play roles in increasing NFAT5 activity (23, 38, 45, 46). Furthermore, an interaction of NFAT5 with several kinases and a potential osmoreceptor that increases NFAT5 activity have been previously discussed (2, 3, 24, 34, 37, 50). Patients with loss of renal function develop increased osmolality (54). Compared with patients with normal renal function (mean osmolality: 294 mosM/kg), the measured osmolality was found to rise significantly in patients through stages 3–5 of chronic kidney disease (mean osmolality: 308, 316, and 323 mosM/kg, respectively) (54). The osmolar gap, defined as the difference between the measured and calculated serum osmolality, is also increased in uremic patients (9, 22, 54). The cause of this increased osmolar gap, i.e., the accumulating solutes and relative role of urea, is currently unknown (54).

Nonosmotic factors have been demonstrated to induce NFAT5, including cytokines and growth factors like platelet-derived growth factor-BB (28). In oPD patients, mechanical stress, glucose toxicity, exposure to dialysate with low pH, the catheter, and peritonitis might all increase those factors. As NFAT5 was not significantly elevated, these factors are unlikely to be important for the NFAT5 expression in this situation.

To date, there are no published data combining morphological characteristics and clinical data with the expression of potentially involved molecules in the human peritoneal membrane. We suggest that the increased osmolality in patients with chronic renal failure might trigger NFAT5 expression, promoting an inflammatory milieu, resulting in a thickening of the peritoneal membrane. This mechanism might also be a cause of the systemic inflammatory response in uremia.

The primary hypothesis was that the high osmolality of the dialysis solutions would trigger NFAT5 expression and chemokine induction. We could not confirm the hypothesis in vivo, as patients on PD did not demonstrate higher NFAT5 expression than uremic patients not on PD. Due to these findings, we performed a chart review of the timing of the last PD exchange before surgical intervention. Unfortunately, there was a gap of hours to days between the peritoneal biopsy and the last exposure to peritoneal dialysate. Separation of the groups into patients with a shorter (<8 h) and longer (>8 h) time after the last exposure to PD solution did not result in significant differences. Therefore, we cannot answer this question at present, but we will perform a future study with defined timeframes of PD dialysate exposure before surgical intervention and biopsy procedures.

Chemokines, particularly CCL2, play pivotal roles in the inflammatory response of the peritoneal membrane. A previous study (36) demonstrated an osmolality-dependent induction of NFAT5, which is known to be a potential driving force for increased CCL2 expression in human mesothelial cultures. Long-term PD leads to a loss of the mesothelial cell layer and accumulation of FBs in the submesothelial cell layer, which is associated with an increased production of the extracellular matrix and thickening of the submesothelial fibrosis zone (1, 4, 10, 32, 33, 53). Therefore, we were interested in the response of human peritoneal fibroblasts to hyperosmolality. In the static cell culture system, peritoneal fibroblasts demonstrated the expected early induction of NFAT5 upon glucose exposure. In contrast to CCL2 mRNA expression, which was upregulated after 96 h, no induction of NFAT5 was detectable at later time points. As our experiments were not flanked by measurements of glucose and osmolality, we cannot exclude a significant decline over time. Furthermore, the static cell culture system does not reflect the human situation, where regular cycles of fresh dialysate expose cells to a rapid increase of osmolality followed by a slower change, depending on the peritoneal transport characteristics.

CCL2 demonstrated kinetics with an early induction by high glucose concentration, which, however, was most prominent after 96 h. Hence, we suggest that there might be an early induction of CCL2 by NFAT5. In contrast, the late induction might be related to other, most likely nonosmotic, mechanisms. A variety of stresses might be involved in chemokine induction in patients on PD. It starts with the “surgical injury” and implantation of a foreign body (catheter) (20). The mechanical stress might induce chemokines in stretched cells (e.g., as shown for endothelial cells) (48). During the PD procedure, bioincompatibility of PD fluids (e.g., low pH, glucose toxicity, glucose degradation products, advanced glycation end-products, and lactate) might lead to membrane injury (30). Finally, intra- and extraperitoneal infects result in tissue injury. Osmolality, as discussed above, is another piece of the puzzle. Our study revealed increased CCL2 expression in peritoneal biopsies of oPD patients compared with pPD patients. Although

Fig. 4. A–G: Immunoreactivity against NFAT5 of vessels and mesothelial cells, NF-κB p50 and NF-κB p65 in FBs, CCL2-positive cells, and CD68-positive cells in pPD and oPD patients. H–K: expression of NFAT5- and CCL2-positive cells in control (cholecystectomy) patients, pPD patients, oPD <8 h patients, and oPD >8 h patients. A: NFAT5 expression of vessels was equal in both groups; no differences could be detected. B: expression of NFAT5-positive mesothelial cells was higher in the pPD group compared with the oPD group. C: CCL2-positive cells were increased in the oPD group compared with the pPD group; however, the difference was not statistically significant. D: CCL2-positive FBs were increased in oPD patients. In pPD patients, no CCL2-positive FBs could be detected. E and F: both NF-κB p50 (E) and NF-κB p65 (F) expression in FBs were increased in the oPD group compared with the pPD group. G: CD68-positive cells were increased in oPD patients compared with pPD patients. H: NFAT5 expression of vessels was equal in all three groups; no differences could be detected. I: expression of NFAT5-positive mesothelial cells was higher in the pPD group compared with the oPD >8 h group and was similar to the oPD <8 h group without reaching significance. J: CCL2-positive cells were increased in both groups of patients on PD compared with the pPD group. K: CCL2-positive FBs were increased in oPD patients without differences in the oPD <8 h and oPD >8 h groups. In pPD patients, no CCL2-positive FBs could be detected.
this induction might still be caused by repeated exposure to
hypertonic solutions, it seems more likely that this reflects
other mechanisms.

Therefore, we focused on NF-κB as a possible inducer for
CCL2 expression and measured two subunits of NF-κB
(NF-κB p50 and NF-κB p65) via immunohistochemistry. Ex-
pression of NF-κB p50 and NF-κB p65 in FBs was signifi-
cantly higher in the PD group compared with the non-PD
group. Recently, Kueper et al. (36) showed NF-κB-dependent
CCL2 expression in human peritoneal mesothelial cells incu-
bated with hyperosmolar glucose, consistent our findings of
significant upregulation of NF-κB in peritoneal biopsies of
PD patients compared with biopsies in pPD patients. Both
NF-κB p50 and NF-κB p65 were predominantly expressed by
MCs and FBs. CCL2 may increase the synthesis of collagen,
fibronectin, and TGF-β, at least in cell cultures (25, 49). Fur-
thermore, inhibition of CCL2 leads to potent antifibrotic
effects in inflammation-induced tissue fibrosis models (21). Beside this, there is evidence that CCL2 and TGF-β have a
mutual interaction (39, 52, 61).

There are several shortcomings of our study. As previously
discussed, the primary question (i.e., induction by hyperos-
motic solutions) could not be addressed in our patients as the
time between exposure to dialysate and the peritoneal biopsy
was too long in the majority of patients. The second shortcom-
ing is the relatively low biopsy number included in the mRNA
experiments. As the effects were found to be significant, a
prominent expression difference can be expected. Third, in
vivo, it is not possible to distinguish between upregulation of
NFAT5 caused by osmotic stress or increased NFAT5 expres-
sion caused by various inflammatory stimuli. Finally, the static
cell culture system does not mimic the in vivo situation, as
HPFBs are imbedded in a three-dimensional microenvironment
constantly exposed to diffusive gradients as well as mechanical
stresses. In conclusion, osmotic stress induced an early but not
enduring induction of NFAT5 in HPFBs. CCL2 was increased
early in HPFBs by hypertonic conditions, but the prolonged
induction needs further evaluation. NFAT5 expression was
found to be increased in the peritoneal membrane of uremic
patients irrespective of whether they were treated with PD or
not. pPD patients showed an induction of NFAT5 compared
with healthy control patients. NFAT5 was associated with
increased peritoneal thickening in uremic patients. Upregu-
lation of CCL2 in PD patients, via NF-κB, might promote the
migration of CD68-positive cells. This mechanism could in-
duce and maintain a chronic inflammatory process, leading to
fibrosis and sclerosis of the peritoneal membrane.

REFERENCES

1. Aguilara A, Yanez-Mo M, Selgas R, Sanchez-Madrid F, Lopez-Cal-
brera M. Epithelial to mesenchymal transition as a triggering factor of
peritoneal membrane fibrosis and angiogenesis in peritoneal dialysis

2. Aramburu J, Drews-Elger K, Estrada-Gelonch A, Minguillon J, Mor-
ancha B, Santiago V, Lopez-Rodriguez C. Regulation of the hypertonic
stress response and other cellular functions by the Rel-like transcription


A, Barbiano di Belgioioso G. Changes in peritoneal membrane after
continuous ambulatory peritoneal dialysis--a histopathological study. Adv

Reimold F, Bode-Lesniewska B, Ziegler U, Bieger D, Wuthrich RP,
Segerer S. Podoplanin-positive cells are a hallmark of encapsulating

Reimold F, Thon KP, Dippom J, Segerer S, Alschier MD. Histological
criteria for encapsulating peritoneal sclerosis - a standardized approach.

MD. Fibrogenic growth factors in encapsulating peritoneal sclerosis.

8. Brebrowicz A, Polubinska A, Oropouloos DG. Changes in volume of
peritoneal mesothelial cells exposed to osmotic stress. Perit Dial Int 19:

1983.

10. Chen YT, Chang YT, Pan SY, Chou YH, Chang FC, Yeh PY, Liu YH,
Chiang WC, Chen YM, Wu KD, Tsai TJ, Duffield JS, Lin SL. Lineage
traicing reveals distinctive fates for mesothelial cells and submesothelial
2014.

11. Cheung CY, Ko BC. NFAT5 in cellular adaptation to hypertonic stress-

Impact of icodextrin on clinical outcomes in peritoneal dialysis: a system-
atic review of randomized controlled trials. Nephrol Dial Transplant 28:

13. Cho Y, Johnson DW, Badve SV, Craig JC, Strippoli GF, Wiggins KJ.
The impact of neutral-pH peritoneal dialsates with reduced glucose
degradation products on clinical outcomes in peritoneal dialysis patients.

DG, Page D. Increased peritoneal membrane transport is associated with
decreased patient and technique survival for continuous peritoneal
dialysis patients. The Canada-USA (CANUSA) Peritoneal Dialysis Study

15. Cohen CD, Frach K, Schlonndorf D, Kretzler M. Quantitative gene
expression analysis in renal biopsies: a novel protocol for a high-through-

16. Davies SJ, Bryan J, Phillips L, Russell GI. Longitudinal changes in
peritoneal kinetics: the effects of peritoneal dialysis and peritonitis.

17. Davies SJ, Phillips L, Griffiths AM, Russell LH, Naish PF, Russell GI.
What really happens to people on long-term peritoneal dialysis? Kidney

18. Deshmone SL, Kremlev S, Amini S, Sawaya BE. Monocyte chemoat-

peritoneal membrane during continuous ambulatory peritoneal dialysis.

GRANTS

This work was supported by Swiss National Foundation Grant
32003B_129710, the CKM-Stiftung, and a grant by Fundação Pesquisa e
Desenvolvimento Humanitario (to N. Segerer). D. Kitterer, J. Latus, and N.
Braun were supported by the Robert-Bosch Foundation.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

results of experiments; D.K., S.S., and N.B. prepared figures; D.K., J.L., M.D.A.,
A.J., H.S., S.S., and N.B. drafted manuscript; D.K., J.L., M.D.A., J.W., A.J., H.S.,
S.S., and N.B. approved final version of manuscript; J.L., C.U., P.F., G.O.,
ROLE OF OSMOTIC STRESS IN UREMIA


