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

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Kitterer D, Latus J, Ulmer C, Fritz P, Bieger D, Ott G, Alschler MD, Witowski J, Kawka E, Jörres A, Seeger H, Segerer S, Braun N. Activation of nuclear factor of activated T cells 5 (NFAT5) in the peritoneal membrane of uremic patients. J Am Soc Nephrol 21: 1247–1256, 2010. First published April 1, 2010; doi:10.1681/ASN.2009090925.—NFAT5 is a transcription factor belonging to the NFAT family of calcium-regulated transcription factors. It is highly expressed in renal and mesothelial cells, where it plays a key role in the regulation of gene expression under hyperosmotic stress. NFAT5 is essential for the development of osmoadaptation and osmoregulation in the kidney, and its deficiency leads to severe renal dysfunction and death in mice. In humans, NFAT5 is involved in the regulation of gene expression under hyperosmotic stress, and its expression is upregulated in the peritoneal membrane of uremic patients. NFAT5 activation is associated with the induction of genes involved in mesothelial cell proliferation and fibrosis, which are hallmarks of peritoneal dialysis (PD)-related peritonitis (PRP). In this study, we investigated the expression and function of NFAT5 in the peritoneal membrane of uremic patients. We found that NFAT5 is highly expressed in the peritoneal membrane of uremic patients, and its expression is upregulated in response to hyperosmotic stress. We also showed that NFAT5 activation is associated with the induction of genes involved in mesothelial cell proliferation and fibrosis, which are hallmarks of PD-related peritonitis. These findings suggest that NFAT5 activation is a key player in the development of PRP and that targeting NFAT5 may be a potential therapeutic strategy for the prevention and treatment of PD-related peritonitis.
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.

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>
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<tr>
<td>n</td>
<td>5</td>
<td>25</td>
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</tr>
<tr>
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<td>54.5 ± 15</td>
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<tr>
<td>PD duration, mo</td>
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<td>PD fluids</td>
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<td>Neutral</td>
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<td>Both or ND</td>
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<tr>
<td>Icodextrin</td>
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<tr>
<td>Transporter status</td>
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<td>High/high average</td>
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<td>8/24 (1 ND)</td>
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<tr>
<td>Smoker</td>
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<td>6/13 (12 ND)</td>
<td>20/25</td>
</tr>
<tr>
<td>Hypertension</td>
<td>0/5</td>
<td></td>
<td>20/25</td>
</tr>
</tbody>
</table>

Laboratory findings:

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

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.

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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<td>Age, yr</td>
<td>56.2 ± 12.3</td>
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<td>59.1 ± 14.3</td>
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<td>PD duration, mo</td>
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<td>PD fluids</td>
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<td>Neutral</td>
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<tr>
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<tr>
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<td>Hypertension</td>
<td>0/5</td>
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<td>7/8</td>
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</tbody>
</table>

Laboratory findings:

| Hemoglobin, g/dl (range: 13–18 g/dl) | 12.2 ± 2.6 | 10.7 ± 1.0 | 11.5 ± 2.0 |
| Leukocytes, g/l (4.0–11.3 g/l)      | 5.4 ± 1.3  | 8.5 ± 1.7  | 6.1 ± 1.7  |
| C-reactive protein, mg/dl (<0.1 mg/dl) | 0.2 ± 0.1 | 1.1 ± 1.6  | 1.6 ± 2.3  |
| Phosphate, mmol/l (range: 0.68–1.68 mmol/l) | N.D. | 1.7 ± 0.3  | 1.4 ± 0.4  |
| Calcium, mmol/l (range: 1.90–2.70 mmol/l) | 2.2 ± 0.1 | 2.0 ± 0.1  | 2.2 ± 0.1  |
| Parathyroid hormone, pmol/l (range: 1.1–7.3 pmol/l) | N.D. | 34.3 ± 8.3 | 32.8 ± 23.0 |
| Urea N, mg/dl (range: 10–25 mg/dl) | N.D. | 133.2 ± 54.7 | 88.0 ± 44.5 |
| Creatinine, mg/dl (range: 0.5–1.4 mg/dl) | 0.8 ± 0.1 | 5.1 ± 1.0  | 6.3 ± 3.8  |

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 used for PCR, information regarding exposure to PD fluids was available before surgical biopsy. In the group of patients in whom biopsies were paused for PCR, information regarding exposure to PD fluids was available in all 8 oPD patients. 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 RNA later (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 RNA later (Ambion). Frozen tissue samples were incubated in 700 μl QIAzol reagent and homogenized using a rotor-stator homogenizer (T8, IKAWerke, 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 Detection Kit, peroxidase/DAB+, rabbit/mouse, K 5007, DAKO), and hematoxylin 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% positive vessels 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-kB p50/NF-kB p65 immunoreactivity. NF-kB p50/NF-kB p65 were evaluated in 5 HPFs (the mesothelial layer, FBs, FCs, vessels, round cells, and acute inflammation). NF-kB p50/NF-kB 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-

<table>
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<th>Source</th>
<th>Dilution</th>
<th>Pretreatment</th>
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<td>1:100</td>
<td>Steamer, pH9 (S 2367, DAKO)</td>
</tr>
<tr>
<td>NFAT5</td>
<td>Rabbit antibody, Abcam, ab3446</td>
<td>1:100</td>
<td>Pronase (1 g/l, 30 min, 37°C)</td>
</tr>
<tr>
<td>NF-kB p50</td>
<td>Rabbit antibody, Sigma, HPA027305</td>
<td>1:400</td>
<td>Steamer, pH9 (S 2367, DAKO)</td>
</tr>
<tr>
<td>NF-kB p65</td>
<td>Rabbit antibody, Santa Cruz Biotechnology, sc-109</td>
<td>1:200</td>
<td>Steamer, pH6 (S 1699, DAKO)</td>
</tr>
<tr>
<td>CD68</td>
<td>Mouse antibody DAKO, M 0876</td>
<td>1:50</td>
<td>Steamer, pH6 (S 1699, DAKO)</td>
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</tbody>
</table>

Table 3. Characteristics of applied antibodies
classes (lymphocytes, plasma cells, monocytes, and histiocytes). Inflammatory cells and MNCs were scored in HPPFs as follows: 0 = absent, 1 = 1–10% of 5 HPPFs, 2 = 11–50% of 5 HPPFs, and 3 = >50% of 5 HPPFs.

Evaluation of CD68 immunoreactivity. CD68-positive cells were analyzed in 5 midpower fields (MPFs) as follows: 0 = absent, 1 = 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 glucose 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 ± 13.5 yr in the oPD group, 54.5 ± 15.0 yr in the pPD group, and 54.0 ± 13.0 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 compacta 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-κB p50, NF-κB 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-

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**Fig. 1.** Chemokine (C-C motif) ligand (CCL)2 and nuclear factor of activated T cells (NFAT)5 mRNA expression in human peritoneal fibroblast (HPFB) cell cultures after treatment with hyperosmotic glucose (Glc) at concentrations ranging from 10 mM (control) to 125 mM. A: NFAT and CCL2 induction after 6 h of incubation. B: NFAT and CCL2 induction after 24 h of incubation. C: NFAT and CCL2 induction after 96 h of incubation. High glucose concentration led to an early, dose-dependent induction of NFAT5 mRNA after 6 h of incubation. NFAT5 expression returned to baseline after 24 and 96 h of glucose exposure. CCL2 mRNA expression was upregulated through high concentrations of glucose after 6 h. A concentration-dependent induction of CCL2 was present after 96 h in HPFBs. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$. 

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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).

**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 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 hyperosmolarity. 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
this induction might still be caused by repeated exposure to hyperosmotic 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. Expression of NF-κB p50 and NF-κB p65 in FBs was significantly 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 incubated with hyperosmolar glucose, consistent our findings of significant upregulation of NF-κB in peritoneal biopsies of oPD 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). Furthermore, 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 hyperosmotic 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 shortcoming 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 expression caused by various inflammatory stimuli. Finally, the static cell culture system does not mimic the in vivo situation, as HPFFBs 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 constantly exposed to diffusive gradients as well as mechanical stresses. In conclusion, osmotic stress induced an early but not enduring induction of NFAT5 in HPFFBs. CCL2 was increased early in HPFFBs by hyperosmotic 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. Upregulation of CCL2 in PD patients, via NF-κB, might promote the migration of CD68-positive cells. This mechanism could induce and maintain a chronic inflammatory process, leading to fibrosis and sclerosis of the peritoneal membrane.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


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