Nephrol Dial Transplant (2005) 20: 1350–1361
doi:10.1093/ndt/gfh835
Advance Access publication 19 April 2005

Nephrology Dialysis Transplantation

Original Article

Immunopathological changes in a uraemic rat model for peritoneal dialysis

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Abstract

Background. Peritoneal dialysis (PD) is a treatment modality for patients with renal failure. Both the uraemic state of these patients and chronic exposure to PD fluid are associated with the development of functional and structural alterations of the peritoneal membrane. In a well-established chronic PD rat model, we compared rats with normal renal function with subtotal nephrectomized rats that developed uraemia.

Methods. Uraemic and control rats received daily 10 ml conventional glucose containing PD fluid, via peritoneal catheters during a 6 week period. Uraemic and control rats receiving no PD fluid served as controls. Parameters relevant for peritoneal defence and serosal healing responses were analyzed.

Results. Uraemic animals were characterized by 2–3-fold increased serum urea and creatinine levels, accompanied by a significantly reduced haematocrit. Uraemia (without PD fluid exposure) induced new blood vessels in different peritoneal tissues, accompanied by increased accumulation of advanced glycation end products (AGEs) and elevated levels of angiogenic factors such as vascular endothelial growth factor and monocyte chemoattractant protein-1 (MCP-1) in peritoneal lavage fluid. A much stronger peritoneal response was observed upon PD fluid exposure in non-uraemic rats. This included the induction of angiogenesis and fibrosis in various peritoneal tissues, accumulation of AGEs, immunological activation of the omentum, damage to the mesothelial cell layer, focal formation of granulation tissues and increased MCP-1 and hyaluronan levels in peritoneal lavage fluid. Finally, chronic PD fluid instillation in uraemic rats did not induce an additional peritoneal response compared to PD fluid exposure in non-uraemic rats, except for the degree of AGE accumulation.

Conclusions. Both uraemia and PD fluid exposure result in pathological alterations of the peritoneum. However, uraemia did not induce major additive effects to PD fluid-induced injury. These results substantially contribute to the understanding of the pathobiology of the peritoneum under PD conditions.

Keywords: AGE; angiogenesis; fibrosis; peritoneal dialysis; rat; uraemia

Introduction

Peritoneal dialysis (PD) is a renal replacement therapy for patients with end-stage renal failure. PD is based on the ability of the peritoneal membrane to function as a dialyzing membrane between the blood and the intraperitoneal PD fluid, allowing the transfer of excess water and waste products from circulation to the peritoneal cavity. During PD, fluid is instilled into the peritoneal cavity via a permanent catheter and removed after a period of time, automatically or manually, several times a day. Many studies have investigated the structural and functional alterations of the peritoneal membrane in both animal models and patients undergoing PD [1–4]. Major structural alterations during PD are the induction of angiogenesis, the development of fibrosis [1] and damage to the mesothelial cell layer [5], each of which are thought to contribute to failure in these patients [2]. The bioincompatibility of PD fluids can be attributed to volume loading as a result of fluid instillation, to catheter

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implantation and/or to factors related to PD fluid composition.

Besides the PD procedure, uraemia itself has been reported to induce various structural changes of the peritoneum, including angiogenesis and fibrosis both in patients [1] and experimental animals [6]. Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor have been implicated in structural modifications induced by experimental uraemia in the remnant kidney [7], heart [8] and peritoneum [6]. To date, however, most experimental PD models use non-uraemic animals and thus probably underestimate the PD fluid-induced injury under uraemic conditions.

We have previously described our first experience with a uraemic rat model receiving PD fluid for several weeks [9]. In that experiment we used an 'open catheter system' and studied some biological and transport parameters. A major problem with that approach was a high peritonitis incidence. In the current study we combined the same subtotal nephrectomy rat model with a well established peritoneal exposure model in the rat [4,10] using a peritoneal catheter connected to an implanted subcutaneous mini-vascular access port, which enabled us to instil PD fluid for a prolonged period in a sterile fashion. We investigated the potential additional contribution of uraemia to the PD fluid-induced peritoneal changes. We focused on various aspects of the peritoneal defence and serosal healing, including changes of the peritoneal leukocytes, angiogenic inflammatory mediators in peritoneal lavage fluid and relevant morphological features of the peritoneal membrane-like angiogenesis, fibrosis, advanced glycation end products (AGEs) accumulation and damage to the mesothelial cell layer.

Subjects and methods

Animals

Male Wistar rats (Iffa Credo, Brussels, Belgium) weighing 200–220 g at the start of the experiment were used. Animals were maintained under conventional laboratory conditions and were allowed free access to food and water. The Animal Care Committee of the University Hospital Gent approved the experiment.

Subtotal nephrectomy

Chronic renal failure was induced by subtotal nephrectomy, as previously reported [9]. Briefly, rats were anaesthetized with halothane (Fluothane-Zeneca, Destelbergen, Belgium). The left kidney was exposed through a flank incision, and both the anterior and posterior poles and about one-third of the remaining cortical tissue from the exterior lateral port of the kidney were cryoablated. After 1 week of recovery, the right kidney was removed after ligation of the blood vessels and ureter. Urea, creatinine and haematocrit were analysed periodically on tail vein blood.

Peritoneal dialysate fluid exposure

Four weeks after the subtotal nephrectomy, a peritoneal catheter connected to a subcutaneous mini vascular access port was implanted, as previously described [4,10,11]. After recovery, over a 6 week period, 10 ml of conventional PD fluid (lactate-buffered 3.86% glucose containing fluid; Dianeal®, Baxter Healthcare) was given daily between 9 and 12 am.

Experimental design

Uraemic subtotal nephrectomized rats were randomly divided in two groups. The first group was exposed to the conventional PD fluid for 6 weeks, (U/PD, $n=21$), while the second group received no fluid (U, $n=16$) but underwent a sham operation. Non-uraemic rats were also divided in two groups. The first group was exposed daily to 10 ml of the conventional PDF for 6 weeks (C/PD, $n=13$), while the second group was not exposed to PD fluid but underwent sham operations for catheter implantation as well as for nephrectomy (C, $n=8$). After 6 weeks of fluid instillation, the animals were sacrificed 24 h after the last fluid instillation. The peritoneal cells were collected by a 2 min peritoneal wash and were analysed as previously described [4]. Cell-free supernatants of these peritoneal wash fluids were checked for bacteria on agar plates and aliquots were stored at -20°C to measure interleukin-6, monocyte chemoattractant protein-1 (MCP-1), VEGF and hyaluronan (HA) concentrations. The percentage of all macrophages in the peritoneal cavity was determined by immunohistochemical staining of cytocentrifuge preparations with monoclonal ED1 (Serotec, Oxfordshire, UK), while the percentage of mature macrophages was determined with monoclonal ED2 (Serotec). The ratio ED2/ED1 was then calculated, since a decrease in this ratio reflects the influx of young macrophages (no ED2 expression). The expression of complement receptor 3 on the peritoneal cells was determined by the monoclonal ED8 (Serotec). Large portions of the omentum (two portions of $\sim$4 cm^2/rat) and the mesentery (the entire most distal loop of the ileum) as well as portions of the parietal peritoneum (two portions of $\sim$1 cm^2/rat at the contralateral side of the implanted catheter) were dissected for light microscopy and another portion for electron microscopy. Omental and mesenteric tissues were spread on an object slide and dried by air. Another sample of omentum was fixed in 4% neutral buffered formalin and embedded in paraffin for AGE-staining. Mesothelial imprints of the liver surface (five imprints/rat) of animals from U/PD and U were taken and analyzed as described before [4]. Due to technical failure imprints of C/PD and C rats could not be analysed, therefore in a parallel study the number of mesothelial cells were counted on the liver of both non-uraemic rats exposed to the conventional PDF ($n=7$) and non-uraemic control rats ($n=7$).

Light microscopy

Omentum and mesentery. Spread preparations of omental and mesenteric tissues were stained with toluidine blue. Omental tissues were checked for the presence of bacteria and were consistently negative. Since the omental milky spots are the major route through which leukocytes migrate into

the peritoneal cavity [12] and their size and number reflect the activatation state of the omentum [13], we determined the number, size and total occupied area of omental milky spots by light microscopy, as previously described [4]. The number of mast cells in the omenta and mesenteries were determined after toluidine blue staining, as previously described [11].

Sections of the omentum were stained with anti-AGE antibody 6D12 that recognizes carboxymethllysine-like structures, carboxyethyllysine and several unidentified AGE epitopes [14].

Parietal peritoneum. Cryostat sections of specimens of the parietal peritoneum were cut (8 mm) and stained with Van Gieson (Merck KGaA, Darmstadt, Germany), toluidine blue and anti-CD 31 (PECAM) to visualize submesothelial extracellular matrix, mast cells and blood vessels, respectively [11].

Fibrosis in the parietal peritoneum was quantified by measuring the thickness of the submesothelial extracellular matrix after Van Gieson staining (Merck KGaA), and the average of 10 independent measurements was calculated for each section and expressed in microns [11].

Liver. Mesothelial liver imprints were stained by May Grünwald Giemsa, and analysed as described before [4].

Electron microscopy

Portions of the dissected omental tissue, mesentery and parietal peritoneum of three animals/group was prepared for electron microscopy according to standard procedures [4].

Overview electron micrographs made from mesenteric tissues were used to determine the thickness of the submesothelial extracellular matrix, as a fibrotic marker [11]. We thus measured the distance between both mesothelial cell layers at various places on each electron micrograph and the mean was calculated for each photo. We analysed three rats/group, ~10 photomicrographs/rat, thus ~30 values/group.

Peritoneal lavage fluid

Interleukin-6 [11], MCP-1 (PharMingen, San Diego, California), VEGF (samples were 10× concentrated; Quantikine® M, VEGF kit, R&D Systems, Inc. USA) and HA [15] concentrations were determined in the cell-free peritoneal wash fluids by ELISA according to the manufacturer's instructions. The detection levels were 30 pg/ml, 0.6 ng/ml, 5.1 pg/ml and 50 ng/ml, respectively.

Statistical analysis

Evaluation of various parameters was done independently by at least two investigators on coded material. The average value of measurements was used for statistical analysis. All data (presented as median and 25–75th interquartile ranges) were analysed using the non-parametric Kruskal–Wallis and Mann–Whitney U tests; $P < 0.05$ was regarded as significant. All animals were combined for the correlation analysis (Spearman rank correlation test), irrespective of their group or their uraemic condition. For correlation analysis the *P*-value was adjusted for multiple comparisons according to the formula; $P < 0.05/\sqrt{n}$.

Results

Clinical data from animals

Body weight was significantly lower in both uraemic groups compared to non-uraemic animals before and after PD fluid instillation (Table 1). The body weight of U rats was significantly higher than U/PD animals before PD fluid instillation, probably due to the additional surgery (catheter implantation) in the latter group. Chronic renal failure secondary to subtotal nephrectomy was characterized by 2–3-fold higher serum urea and creatinine levels in uraemic rats compared to non-uraemic rats, before and after the PD fluid instillation for 4 or 6 weeks. In addition, haematocrit was significantly lower in the U/PD and U rats than in the C/PD and C animals respectively, before and after PD fluid instillation. However, haematocrit of U rats was significantly higher than U/PD rats before and after PD fluid instillation, most likely due to peritoneal fluid absorption with haemodilution in the U/PD group. Alternatively, rats in the U/PD group could have a higher degree of uraemia than the U group. These data indicate that at the start of the PD fluid instillation U/PD and U rats were uraemic and remained stable for the whole PD fluid instillation period.

Drop-out

Subtotal nephrectomy was performed in 48 rats. Throughout the experiment, 27% of uraemic animals were excluded from the study due to excessive urea and creatinine levels. During the PD fluid instillation period, drop-out was 48 and 38% in the U/PD and C/PD, respectively, due to omental wrapping around the tip of the catheter. Thus, after 6 weeks of PDF instillation, 11 rats in the U/PD, eight rats in the C/PD and 14 rats in the U-group as well as all eight animals in the C-group remained for analysis. To exclude peritonitis, peritoneal cell-free supernatants were checked for the presence of bacteria and no bacteria were found. In addition, we could not detect interleukin-6 in peritoneal effluents.

Cellular alterations

The total number of peritoneal cells and their compositions are shown in Table 2. Neither cell count nor cellular composition, the ED2/ED1 ratio (reflecting the influx of young macrophages; $P = 0.36$), the percentage of complement receptor 3 (ED8) positive cells ($P = 0.11$) changed among the four groups. In contrast, PD fluid instillation had a profound effect on the percentage of mast cells, accompanied by a tendency towards an increased percentage of neutrophils and lymphocytes.

The number of mast cells in the omentum was significantly increased in both PD fluid-instilled groups, compared to both control groups (Table 3 and Figure 1). The number and size of milky spots

Table 1. Clinical parameters

	U/PD	C/PD	U[a]	C[a]
Body weight (g)				
Before PDF	264.5 (253.3–279.0)[b]	353.0 (340.3–360.5)	300.0 (290.0–307.8)[d,e]	374.0 (334.0–384.0)
4 weeks PDF	318.5 (310.0–342.0)[b]	415.5 (398.3–431.3)	349.0 (318.8–370.8)[d]	420.0 (373.8–436.8)
6 weeks PDF	N.D.	N.D.	N.D.	N.D.
Serum urea (mg/dl)				
Before PDF	96.3 (78.1–112.8)[b]	42.2 (39.6–48.7)[c]	90.9 (84.7–99.7)[d]	33.3 (32.2–35.7)
4 weeks PDF	92.6 (79.3–101.0)[b]	36.9 (36.5–37.6)	82.2 (70.4–96.2)[d]	37.9 (37.9–43.9)
6 weeks PDF	105.5 (91.5–114.8)[b]	42.2 (40.3–45.1)	97.2 (81.2–116.1)[d]	41.4 (37.6–53.1)
Serum creatinine (mg/dl)				
Before PDF	0.76 (0.67–0.83)[b]	0.23 (0.19–0.27)	0.69 (0.57–0.75)[d]	0.26 (0.23–0.28)
4 weeks PDF	0.98 (0.92–1.07)[b]	0.27 (0.24–0.32)	0.73 (0.68–0.93)[d]	0.31 (0.26–0.35)
6 weeks PDF	1.04 (0.98–1.28)[b]	0.37 (0.30–0.39)	0.81 (0.68–1.15)[d]	0.32 (0.30–0.46)
Haematocrit (%)				
Before PDF	37.0 (35.0–38.0)[b]	44.0 (42.0–45.0)	39.5 (37.5–42.0)[d,c]	45.0 (44.0–46.0)
4 weeks PDF	39.0 (36.0–40.0)[b]	44.0 (44.0–48.0)	44.5 (41.0–46.0)[e,f]	47.0 (46.0–49.0)
6 weeks PDF	N.D.	N.D.	N.D.	N.D.

[a]Control uraemic (U) and control non-uraemic (C) rats were not exposed to PDF, as explained in Subjects and Methods. Data are expressed as medians with interquartile ranges.
[b]U/PD *vs* C/PD and C: $P < 0.002$.
[c]C/PD *vs* C: $P < 0.004$.
[d]U *vs* C/PD and C: $P < 0.04$.
[e]U *vs* U/PD: $P < 0.03$.
[f]U *vs* C: $P < 0.02$.
N.D., not determined.

Table 2. Cellular composition of the peritoneal cavity

	U/PD	C/PD	U	C
Total cells (10^6)	25.0 (22.0–26.0)	26.0 (22.0–26.0)	24.0 (20.0–28.0)	23.0 (22.0–25.0)
% Macrophages	73.0 (70.0–78.0)	57.0 (56.0–58.0)	58.0 (51.0–63.0)	61.0 (52.0–63.0)
% Neutrophils	1.3 (0.5–3.0)	3.5 (0.5–4.0)	1.5 (0.4–2.0)	0.3 (0–0.8)
% Lymphocytes	4.0 (1.0–7.0)	6.0 (2.0–7.0)	1.0 (0–2.0)	2.0 (1.0–2.0)
% Mast cells	4.0 (3.0–5.0)[a]	5.0 (4.0–15.0)	12.0 (10.0–18.0)	17.0 (12.0–21.0)
% Eosinophils	17.0 (10.0–19.0)	23.0 (20.0–25.0)	24.0 (21.0–30.0)	22.0 (20.0–28.0)
ED2/ED1	0.42 (0.38–0.48)	0.52 (0.45–0.62)	0.48 (0.45–0.59)	0.47 (0.45–0.51)
% ED8	71.0 (64.0–77.0)	71.0 (69.0–76.0)	80.0 (75.0–84.0)	80.0 (78.0–82.0)

Data are expressed as medians with interquartile ranges.
[a]U/PD *vs* U and C: $P < 0.002$.

Table 3. Morphometric parameters of peritoneal tissues

	U/PD	C/PD	U	C
Omentum				
Milky spots/cm^2	26.5 (25.0–30.0)[a]	22.0 (21.0–32.0)[b]	5.0 (5.0–6.5)	6.0 (5.0–8.0)
Area/milky spots (mm^2)	0.7 (0.7–0.8)[a]	0.6 (0.6–0.7)[b]	0.2 (0.2–0.2)	0.2 (0.2–0.2)
% Milky spots surface	19.0 (17.0–20.0)[a]	17.0 (13.0–22.0)[b]	1.0 (0.8–1.2)	1.2 (1.1–1.2)
Blood vessels/cm^2	90.0 (79.0–127.0)[a]	96.0 (69.0–234.0)[b]	39.0 (25.0–50.0)[c]	4.0 (2.0–29.0)
Mast cells/mm^2	54.0 (35.0–66.0)[a]	51.0 (32.0–104.0)[b]	25.0 (10.0–33.0)	11.0 (9.0–16.0)
Mesentery				
Blood vessels/cm^2	692.0 (629.5–740.0)[a]	559.0 (548.5–602.5)[b]	324.0 (251.0–429.0)[c]	28.0 (24.0–32.0)
Mast cells/mm^2	79.4 (70.7–90.0)	94.5 (86.9–99.4)	75.6 (65.3–79.7)	78.1 (77.5–87.5)
Liver				
Mesothelial cells/mm^2	1300 (1200–1500)[d]	1300 (1200–1400)[e]	900 (700–900)	900 (800–1100)[e]

Data are expressed as medians with interquartile ranges.
[a]U/PD *vs* U and C: $P < 0.004$.
[b]C/PD *vs* U and C: $P < 0.04$.
[c] U *vs* C: $P < 0.04$.
[d]U/PD *vs* U: $P < 0.002$.
[e]In a parallel study: C/PD *vs* C: $P < 0.003$.

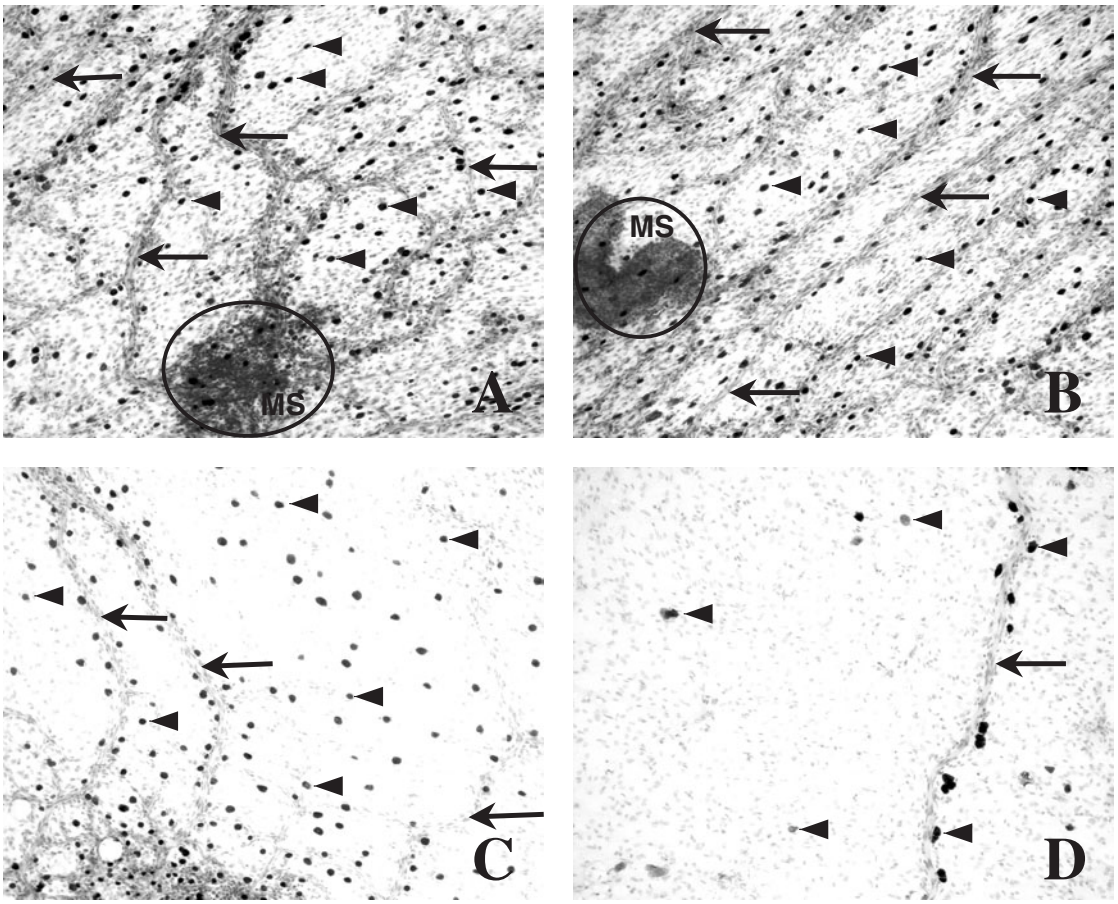

Fig. 1. Representative light micrographs of omental tissues from U/PD (**A**), C/PD (**B**), U (**C**) and C rats (**D**), after toluidine blue staining. The number of milky spots (MS), blood vessels (arrows) and mast cells (arrowhead) were significantly increased in both PD fluid-treated groups. Vessel density was also increased in control uraemic rats compared to control non-uraemic rats.

Table 4. Correlation analysis between blood vessel density and potential angiogenic cells/factors

	Mast cell density	Vascular endothelial growth factor	Hyaluronan	Monocyte chemoattractant protein-1
Omental vessels	$r=0.85$; $P<0.0001$[a]	$R=0.15$; $P=0.517$	$r=0.52$; $P=0.023$	$r=0.40$; $P=0.079$
Mesenteric vessels	$r=0.25$; $P=0.230$	$r=0.72$; $P<0.002$[a]	$r=0.60$; $P<0.016$[a]	$r=0.56$; $P<0.017$[a]

[a]Corrected for multiple comparisons, $P<0.05/\sqrt{n}=P<0.017$ regarded as significant.

were significantly increased in the omentum of rats exposed to PD fluid, irrespective of the uraemia, compared to both controls (Table 3). Unlike the omentum, the mesentery was not characterized by differences in the number of mast cells ($P=0.50$).

Angiogenesis

Analysis of large portions of the omental and mesenteric tissues revealed a pronounced angiogenesis in U rats as well as all rats exposed to PD fluid, compared to C animals (Table 3 and Figure 1), with no significant differences between U/PD and C/PD groups. A significant correlation was found between the number of blood vessels and the mast cell density in the omentum (Table 4; $r=0.85$, $P<0.0001$), but not in the mesentery ($r=0.25$, $P=0.230$).

The induction of angiogenesis in the U/PD, C/PD and U groups was confirmed by electron microscopic examination (data not shown). In contrast to control animals, the endothelial cells lining some of omental vessels were enlarged, similar to high endothelial venules, in rats exposed to PD fluid, suggesting an activated vascular endothelium. Taken together, our data indicate the contribution of both uraemia and PDF instillation in the induction of angiogenesis.

Granulation tissue

Light microscopic observation of the parietal peritoneum showed the focal development of granulation tissues in animals of the U/PD and the C/PD groups (Figure 2), whereas C and U animals showed normal parietal peritoneum. The granulation tissue was characterized by the accumulation of several cell types, including mast cells and possibly fibroblasts, many vessels and pronounced submesothelial extracellular matrix deposition. The precise quantification of granulation tissues was impossible, due to the high number of blood vessels as well as mast cells and the large variation in the thickness of the submesothelial extracellular matrix. The numbers of blood vessels and mast cells were increased 10–20-fold, along with a 5–10-fold increase in the thickness of the submesothelial extracellular matrix in the granulation tissues.

Accumulation of AGEs

Untreated C rats showed barely any AGE staining in the peritoneal membrane, only a few arterioles were slightly (+/−) positive. In control U rats staining was seen in more arterioles, mean intensity was 1+. In the C/PD group a clear increase was observed in arterioles and some newly formed vessels (mean intensity 2+). Interestingly, AGE staining in arterioles and some smaller vessels was more profound in rats from U/PD than C/PD animals (mean intensity 3+), suggesting an additive effect of uraemia to the PD-induced AGE accumulation. The staining of the arterioles was sometimes segmental and predominantly in the vascular smooth muscle layer. Endothelium was always negative, as were most other vessels such as venules and capillaries. The mesothelial cell layer was also always negative. Representative photomicrographs are shown in Figure 3.

Fibrosis

Macroscopic observation of the whole omentum revealed that the surface area of the tissue had increased, whereas the transparency was decreased in all PDF-treated animals compared with animals from the U and C groups. It indicates the development of hypertrophy along with fibrosis in animals from both U/PD and C/PD groups (data not shown).

Electron microscopic observation of the omentum and the mesentery revealed a marked increase in the number of collagen bundles and tissue thickness in rats from U/PD and C/PD groups compared to animals from both control groups (Figure 4). When the thickness of the submesothelial extracellular matrix of mesenteric tissues was measured, a significant ~2-fold increase in the mesenteric thickness was observed in both PD fluid-treated groups compared to both controls ($P < 0.0002$), with no difference between C/PD and U/PD groups ($P = 0.16$, Figure 5A). No significant difference was found between C and U groups ($P = 0.37$).

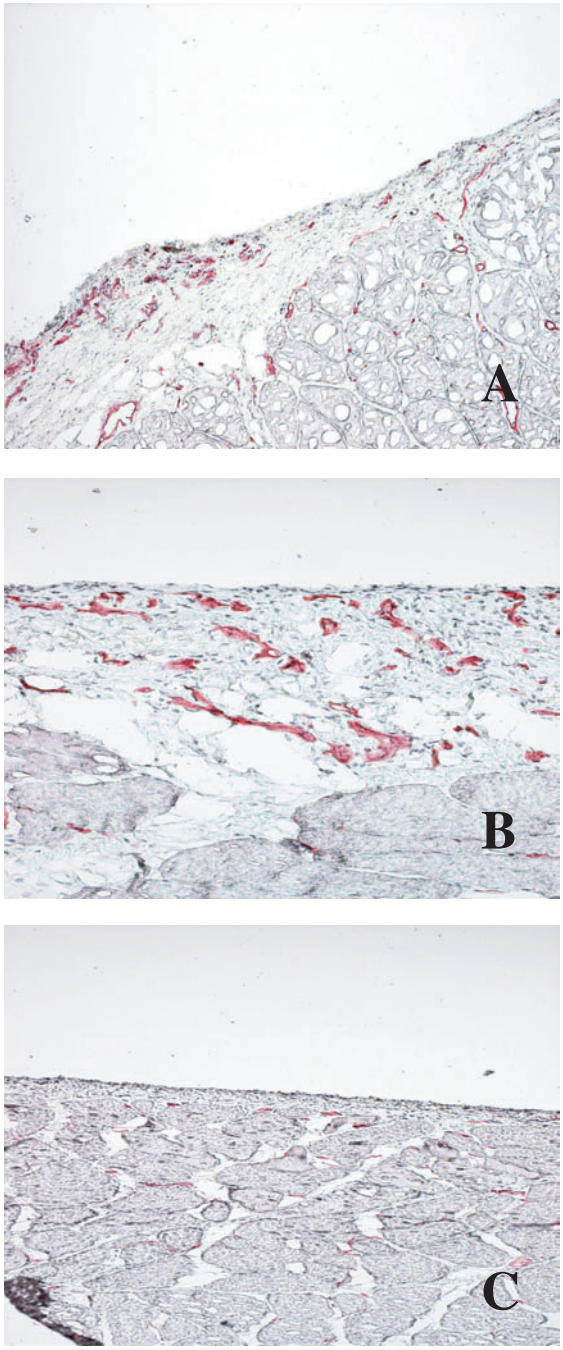

Fig. 2. Light micrographs of the parietal peritoneum from a non-uraemic rat exposed to the conventional PD fluid for 6 weeks, after staining with anti-CD31 (PECAM) for vessel density (in red). Note the heterogeneous development of the parietal peritoneum after exposure to PD fluid (**A**). Also shown are a detailed light micrograph of the granulation tissue (**B**) and the unchanged parietal peritoneum (**C**).

Fibrosis was also quantified in the parietal peritoneum. The thickness of the submesothelial ECM of the parietal peritoneum was markedly increased in both PDF-exposed groups, compared to U and C groups (Figure 5B). No significant differences were found in the degree of fibrosis between U/PD and C/PD ($P = 0.22$) or between C and U groups ($P = 0.93$).

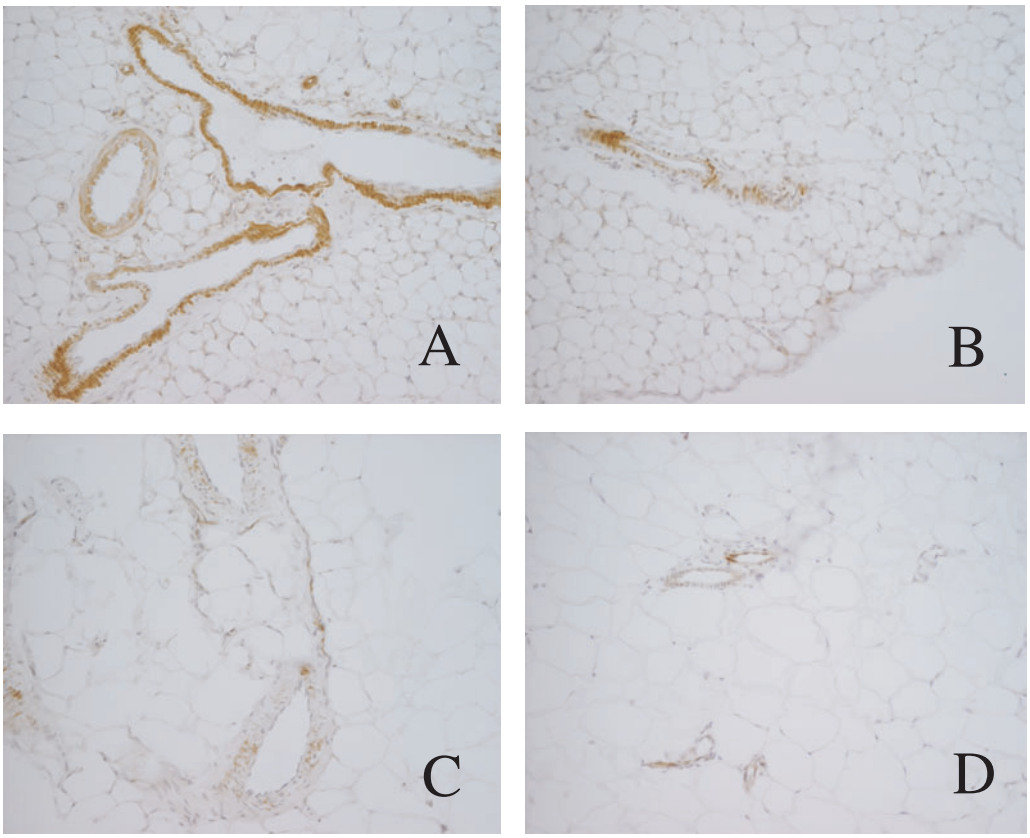

Fig. 3. Light micrographs of the visceral peritoneum (omentum) from U/PD (**A**), C/PD (**B**), U (**C**) and C rats (**D**) after staining with anti-AGE antibody. Both uraemia and PDF exposure resulted in AGE accumulation, especially in the vascular smooth muscle cell layer of arterioles. AGE accumulation was more intense in rats from U/PD group compared to C/PD rats, indicating an additive effect of uraemia to PDF-induced AGE accumulation.

Alteration of the mesothelial cell layer

The mesothelial cell density on the liver was significantly increased in uraemic rats exposed to PD fluid compared to U animals ($P < 0.002$) (Table 3 and Figure 6). Activated mesothelial cells were found in uraemic rats exposed to PD fluid, as determined by the bone-shaped nuclei of these cells. In a parallel study, we found a significant higher number of mesothelial cells in rats exposed to PD fluid compared to control non-uraemic rats ($P < 0.003$, Table 3).

Electron microscopic observation of the omentum, mesentery and parietal peritoneum showed that the mesothelial cell layer covering these tissues was equally damaged in both PD fluid-instilled groups, irrespective of uraemia, whereas both control groups had an intact mesothelium (Figure 6). The mesothelial damage was characterized by loss of microvilli, either vacuolization or focal loss of mesothelial cells and adhesion of macrophages.

Angiogenic factors in peritoneal lavage

The level of VEGF in the peritoneal effluents of both uraemic groups was significantly higher than C animals, with no difference between U/PD and U rats ($P = 0.30$) (Figure 7A). Furthermore, a larger amount of VEGF was found in the U/PD group compared to the C/PD groups ($P = 0.07$), whereas no difference was seen between C/PD and U animals ($P = 0.17$). A significant correlation was found between VEGF concentrations and the number of blood vessels in the mesentery ($r = 0.72$, $P < 0.002$), but not in the omentum (Table 4; $r = 0.15$, $P = 0.517$).

The level of MCP-1 was significantly increased in the peritoneal effluent of both uraemic groups as well as in the C/PD group, compared to C animals (Figure 7B). MCP-1 levels seem to be higher in rats from the U/PD group compared to C/PD animals, although no statistically significant differences were found between U/PD and C/PD groups ($P = 0.23$) or between both the PD fluid-treated groups and the U group ($P = 0.12$). The correlation between MCP-1 concentrations and vessel density was significant in the mesentery ($r = 0.56$, $P < 0.017$), but not in the omentum ($r = 0.40$, $P = 0.079$).

The concentration of HA was increased in both PD fluid-exposed groups compared to both controls (Figure 7C), with no significant differences between U/PD and C/PD groups ($P = 0.20$). No significant differences were found in the HA concentrations between either control ($P = 0.23$). The correlation between

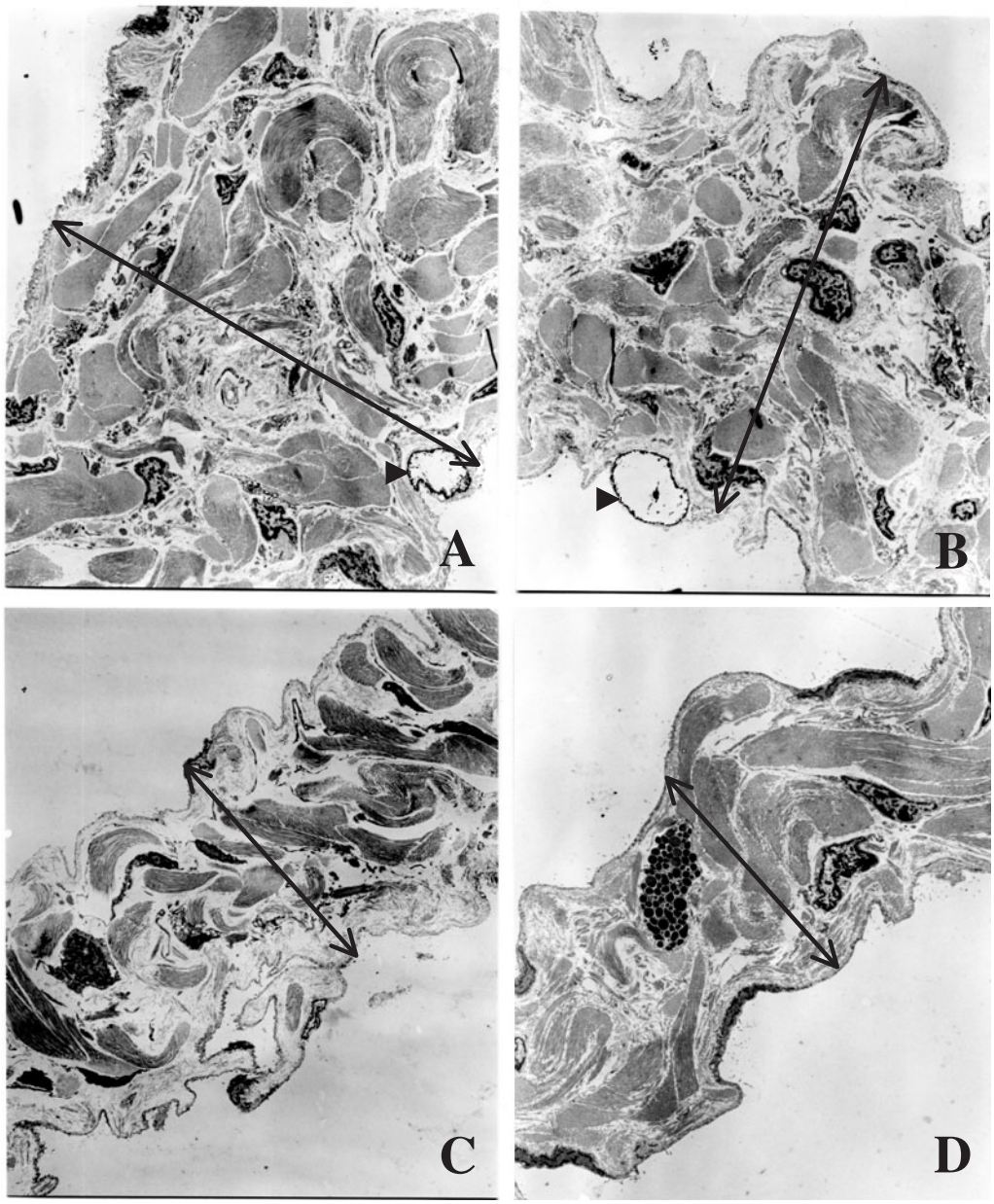

Fig. 4. Transmission electron micrographs of the mesenteric tissues from U/PD (**A**), C/PD (**B**), U (**C**) and C rats (**D**). The thickness of the tissue, indicated by arrows, was increased in both PD fluid-treated groups (52–57 μm) compared to both controls (26–29 μm). Note the profound increase in collagen bundles in (A) and (B). Arrowheads represent vacuolization of mesothelial cells. Magnification (A–D), 1120×.

peritoneal HA concentration and vascular density was significant in the mesentery ($r = 0.60$, $P < 0.016$), but not in the omentum ($r = 0.52$, $P = 0.023$).

Discussion

In the present study we analysed peritoneal changes secondary to uraemia, to chronic exposure to PD fluid, and to a combination of both conditions. Although uraemia in itself clearly induced new vessel formation in peritoneal tissues, accompanied by the release of some angiogenic factors, PD fluid-induced alterations of the peritoneum were more profound including mesothelial injury and regeneration, fibrosis and severe angiogenesis. However, we were unable to demonstrate a statistically significant contribution of uraemia to peritoneal changes in addition to those caused by PD fluid.

Uraemia, without PD fluid exposure, resulted in a striking neoangiogenesis in the omentum and mesentery. Uraemia also induced some AGE accumulation in the peritoneal membrane. Moreover, peritoneal effluent levels of VEGF and MCP-1 were elevated

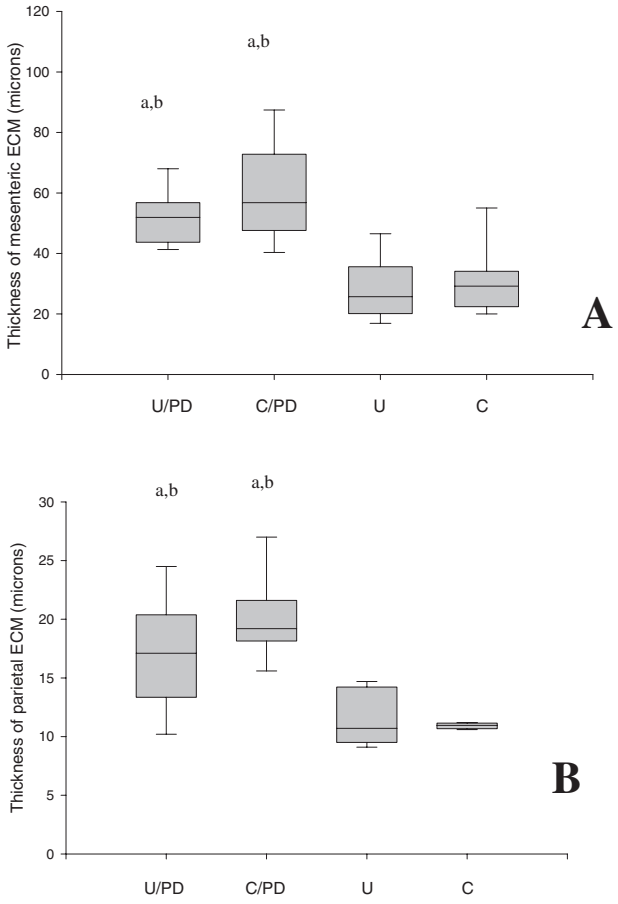

Fig. 5. Thickness of submesothelial extracellular matrix (ECM) in the mesenteric tissues (**A**) and in the parietal peritoneum (**B**). Data are presented as medians with the spread from 25th to 75th percentile (box) and the spread from 10th to 90th percentile. [a]U/PD and C/PD *vs* C: $P < 0.04$; [b]U/PD and C/PD *vs* U: $P < 0.03$.

in control uraemic rats. Our findings are in good agreement with recent literature showing increased levels of MCP-1 [16], along with structural changes of the peritoneum such as angiogenesis [1,6] and AGE accumulation [17] in uraemic patients and in experimental animals. Thus, the present study confirms and extends (by detailed morphometric analysis) our understanding concerning the contribution of uraemia *per se* on pathological changes of the peritoneum.

Compared to uraemia, the daily exposure of non-uraemic animals to PD fluid evoked much more severe changes, namely: (i) lower number of mast cells in the peritoneal cavity; (ii) elevated vascular density in various peritoneal tissues; (iii) increased number of milky spots and mast cells in the omentum; (iv) profound damage to the mesothelial cell layer; (v) the development of fibrosis; (vi) the focal formation of granulation tissues; (vii) increased mesothelial regeneration; and (viii) increased levels of MCP-1 and HA in the peritoneal lavage.

Although (silent) peritonitis might also cause profound changes of the peritoneal membrane, we tried to avoid this as much as possible. Therefore we worked strictly aseptically using a subcutaneously placed vascular access port connected to a peritoneal catheter. During the experiment no obvious clinical signs (weight loss, diarrhoea, abnormal behaviour) were observed. At the end of the experiment cell-free supernatants of the peritoneal dwells were controlled for the presence of bacteria; however, no bacteria were found. In addition, we could not detect interleukin-6 (inflammation marker) in these peritoneal effluents. Omental tissues were consistently negative for adhered bacteria, Furthermore, no clear influx of neutrophils was found in the peritoneal washes (see Table 2). All these findings make it very unlikely that the peritoneal changes, as observed in both PDF-treated groups, are secondary to peritonitis.

In this study, we were not able to show a significant contribution of uraemia to various peritoneal damage, in addition to those caused by PDF exposure, except in case of AGE accumulation. Uraemia appeared to induce an additive effect on PD-induced AGE-accumulation in the peritoneal membrane; however, this condition did not result in the formation of more new blood vessels in the U/PD group compared to the C/PD group. In some parameters analysed in this study, such as the number of mesenteric blood vessels and angiogenic factors in peritoneal effluents, the value in the U/PD group exceeded the value in the C/PD group, which is also suggestive of a minor role of uraemia in PD fluid-related peritoneal injury. At the same time, however, from our detailed immunopathological analysis of the peritoneum, we conclude the uraemia-induced damage was less severe compared to the PD fluid-induced injury. This might be the reason why no statistical differences were found between the U/PD and C/PD groups with respect to many immunological/morphological parameters. Of course, we cannot exclude the possibility that differences would have been found in other parameters related to peritoneal functions such as solute transport, ultrafiltration and glucose absorption, which were not analysed in this study.

Our data suggest different angiogenic factors involved in the formation of new vessels in the omentum and mesentery upon PD fluid exposure. For example, PD fluid exposure increased the number of mast cells in the omentum but not in the mesentery. Consequently, a positive correlation was found between the number of vessels and mast cells in the omentum, but not in the mesentery. Since mast cells can produce angiogenic factors such as tryptase [18] and basic fibroblast growth factor [19], future work should address the contribution of mast cells in PDF-induced neoangiogenesis. Levels of VEGF, MCP-1 and HA correlated with the number of blood vessels in the mesentery, but not in the omentum. At present we do not know the reason for this differential response, but strongly suggest different induction routes of new vessel formation in these tissues. Several studies have implicated VEGF as an important mediator of neovascularization in the peritoneal membrane [20]. In addition, VEGF blockade prevented neoangiogenesis

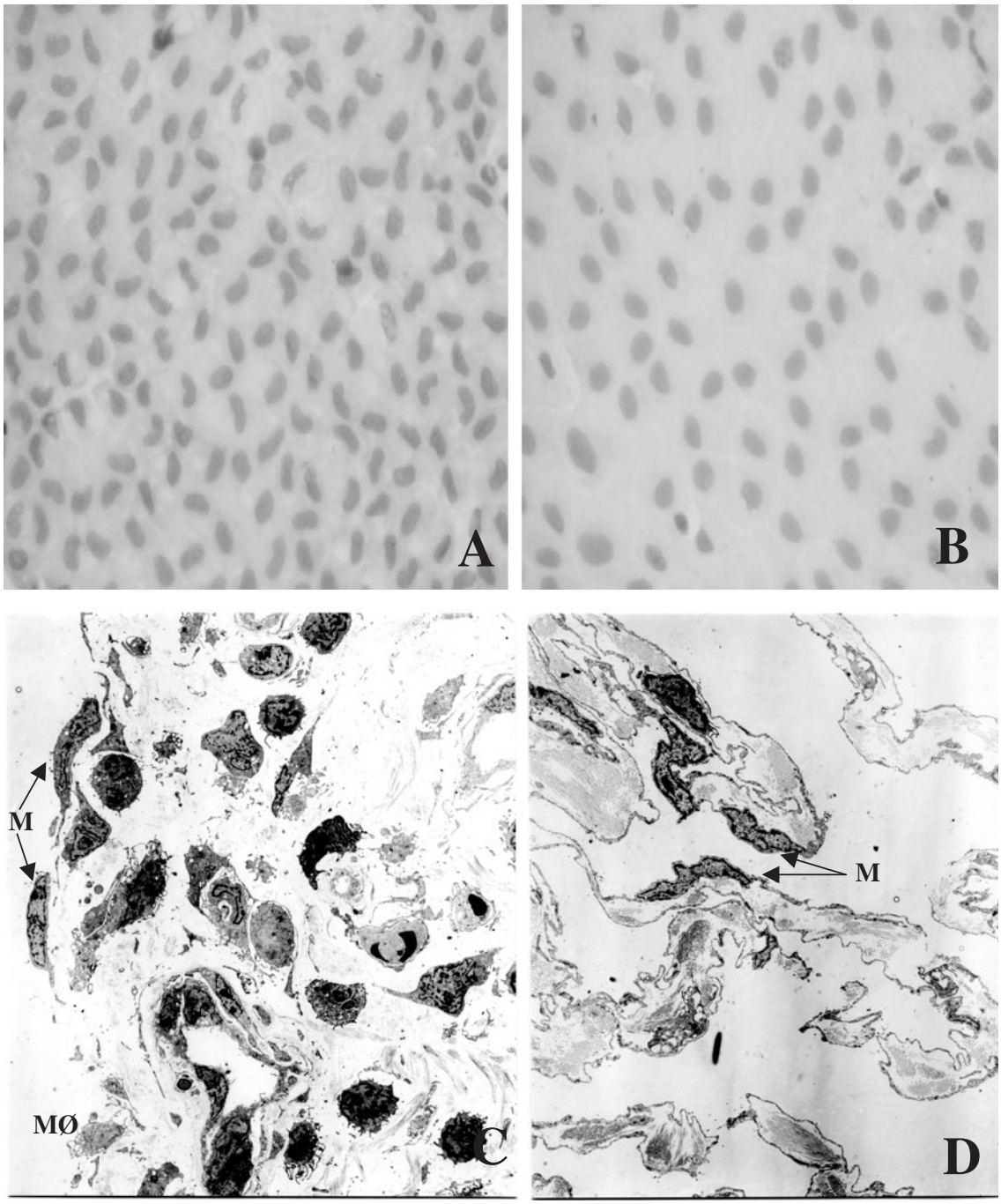

Fig. 6. PD fluid-induced changes in the mesothelial cell layer. Liver imprints of uraemic rats exposed to PD fluid (**A**) and control uraemic rats (**B**), indicating a significant increase in the density of mesothelial cells on the liver by PD fluid treatment. Electron micrographs of the omentum revealed that the mesothelial cell layer covering the tissue was damaged and partly covered by adherent macrophages in uraemic rats exposed to PD fluid (**C**), while the cell layer was intact in control uraemic rats (**D**). M, mesothelial cell; MØ, macrophage. Magnification (C and D), 2100×.

in the peritoneum of experimental animals with diabetes [21]. Although VEGF is a very potent enhancer of microvascular permeability, we have previously reported an unchanged vascular permeability in rat mesenteric vessels exposed to PD fluid for 5 weeks [22]. Thus, the precise role of VEGF in the formation of new blood vessels during PD is presently unclear. MCP-1 has been found to be involved both in peritoneal fibrosis [23] and in angiogenesis [24], and is induced by hyperglycaemia [23]. HA is a major component of interstitial tissue and is involved in various biological processes including fluid homeostasis, response to inflammation and wound healing. Several studies have highlighted the angiogenic properties of HA fragments [25,26]. Our data suggest a contribution of VEGF, MCP-1 and HA in peritoneal

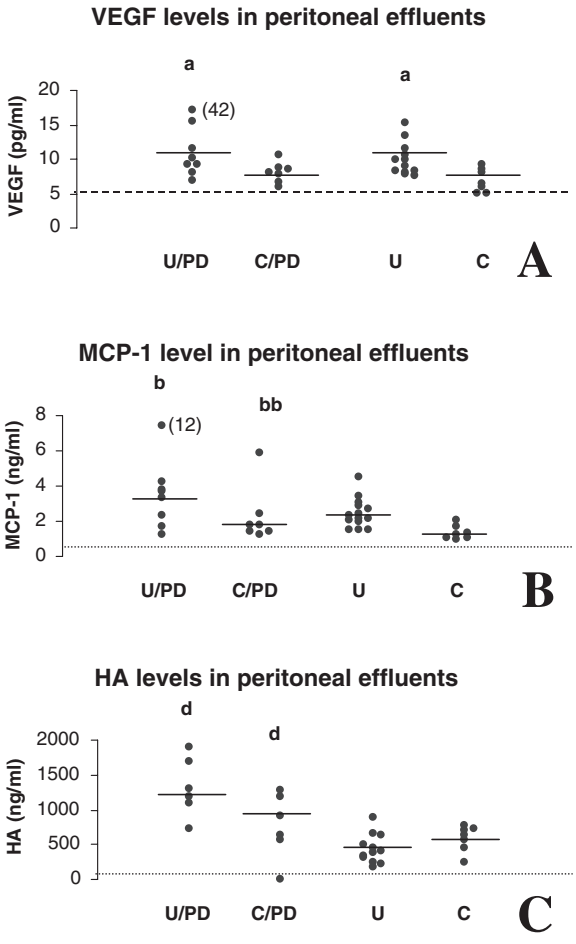

Fig. 7. Peritoneal effluent concentrations of VEGF (**A**), MCP-1 (**B**) and HA levels (**C**) of all experimental animals. Individual animals and median values (–) are represented. Dashed lines represent the detection levels. [a]U/PD and U *vs* C: $P < 0.03$; [b]U/PD, C/PD and U *vs* C: $P < 0.04$; [c]U/PD and C/PD *vs* U and C: $P < 0.05$.

new vessels formation upon uraemia and/or PD. Experiments aimed at blocking individual factors, or their receptors, have to prove this suggestion definitively. Long-term peritoneal dialysis has rarely been applied in a uraemic animal model, thus most studies in this field do not exactly mimic the clinical PD situation. To the best of our knowledge, we are the first to provide detailed data regarding the long-term effect of PD fluid instillation on the immunopathology of the peritoneum in a uraemic rat model. Since structural changes such as angiogenesis and fibrosis as well as damage to the mesothelial cell layer each may contribute to ultrafiltration failure of the peritoneal membrane, we believe that our findings are important for the clinical situation as well as for further basic research in the PD field.

Acknowledgements. This study was supported by grant 97.1705 from the Dutch Kidney Foundation. We thank Tommy Dheuvaert, Mieke Van Landschoot, Julien Dupont, Eelco Dennis Keuning and Inge de Greeuw for technical and supporting help with this experiment.

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Received for publication: 26.4.04
Accepted in revised form: 23.3.05