2 Chapter

Long-term intervention with heparins in a rat model for peritoneal dialysis

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Abstract

Background: Peritoneal dialysis (PD) is associated with functional and structural alterations of the peritoneal membrane, in particular new vessel formation and fibrosis. Besides anti-coagulant effects, heparin displays anti-inflammatory and angiostatic properties. Therefore the effects of administration of heparins have been studied on function and morphology of the peritoneal membrane in a rat PD model.
Methods: Rats received daily 10 ml conventional PD fluid (PDF) with or without addition of unfractionated heparin (UFH) or low molecular weight heparin (LMWH) in the PDF (1 mg/10 ml ip) via a mini access port. Untreated rats served as controls. After 5 weeks, a 90 minute functional peritoneal transport test was performed and tissues and peritoneal leukocytes were taken.
Results: PD treatment induced loss of ultrafiltration ($p<0.01$), a two fold increase in glucose absorption ($p<0.03$), increased urea transport ($p<0.02$) and loss of sodium sieving ($p<0.03$), which was also found in de PDF+heparin groups. Increased peritoneal cell influx and hyaluronan production was observed in PD rats ($p<0.02$) as well as an exchange of mast cells and eosinophils for neutrophils after PD treatment without changes upon the addition of heparin. Mesothelial regeneration, submesothelial blood vessel and matrix formation, as well as accumulation of tissue macrophages was seen in PD animals. Spindle like shaped vimentin positive and cytokeratin negative cells indicated either partial injury and denudation of mesothelial cells or epithelial to mesenchymal transition. Neither heparin could affect any of these morphological changes.
Conclusion: PD treatment induces within 5 weeks a chronic inflammatory condition in the peritoneum evidenced by high transport, leukocyte recruitment, tissue remodeling and induction of spindle like shaped cells in the mesothelium. Addition of LMWH or UFH to the PDF could not restore the adverse PDF induced peritoneal changes.

Introduction

Peritoneal dialysis (PD) is an effective treatment for patients with end-stage renal failure. During this treatment the peritoneal membrane functions as a dialyzing organ and is constantly exposed to PD-fluid (PDF). It is well known that long-term PD induces loss of mesothelial cells, submesothelial thickening and neo-angiogenesis[1]. The non-physiological environment in the peritoneum caused by chronic PDF installation, sustains a low grade of sterile inflammation[1]. This low grade of inflammatory state may on the long-term cause functional deterioration of the peritoneal membrane with increasing peritoneal permeability for small solutes and reduced ultrafiltration (UF) capacity[2;3]. At the molecular level there is increasing evidence that cytokines, chemokines and growth factors play a key role in regulating and sustaining this pro-inflammatory condition[4]. After long-term PD treatment, peritoneal mesothelial cells show a progressive loss of epithelial phenotype and acquire fibroblast-like characteristics, suggesting Epithelial to Mesenchymal Transition (EMT)[5].

Heparin is a highly sulfated polysaccharide belonging to the family of glycosaminoglycans and is synthesized by mast cells. Although it is most known for its anticoagulant effect, heparin has a number of immunomodulatory and anti-inflammatory activities, including the binding of cytokines, chemokines and growth factors[6]. Earlier reports demonstrate that heparin can inhibit leukocyte rolling along the vessel wall, a process which is known to involve selectins[7;8]. The function of selectins in this process depends on recognition of specific glycoprotein ligands. However, a variety of carbohydrate structures, including heparin, may bind these lectin-like molecules and interfere with ligand binding[9]. Besides unfractionated heparin (UFH), low molecular weight heparin (LMWH) is one of the most common used types of heparin in clinical settings. LMWH fractions are prepared from standard unfractionated heparin and are similar to UFH in many aspects. The main advantages of this class of anti-thrombotic agents as compared with UFH are an improved bioavailability, a prolonged half-life (which may permit one single daily subcutaneous injection) and a more improved efficacy[10;11].

In clinical PD settings heparin is used to prevent fibrin formation during peritonitis and at times of catheter obstructions. The results on the effects of intraperitoneal heparin administration on peritoneal transport and inflammation have been contradictory[12-16]. Therefore, we have studied long-term addition of UFH as well as LMWH to the PDF on function, defence and tissue remodelling of the peritoneal membrane to see whether heparin can improve peritoneal transport and whether it can also prevent

morphological changes of peritoneal tissues. In the present study either UFH or LMWH was added to the PDF in a chronic peritoneal exposure model in the rat for five weeks[17;18].

Material and Methods

Animals

Male Wistar rats (Harlan CPB, Horst, The Netherlands) weighing 250-275 gr. at the beginning of the experiment were used throughout the study. They were allowed one week of acclimatization before the start of the experiment. Animals were housed under conventional laboratory conditions and were given food and water ad libitum. The Animal Experimental Committee of the Vrije Universiteit of Amsterdam approved the experimental design.

Experimental design

Fluids were instilled via a peritoneal catheter connected to an implanted subcutaneous mini access port as previously described[19]. Rats that were not surgically treated and received no fluid instillation, served as control group (n=8). The remainder of the animals (n=34) received 2 ml of saline with 1 IU/ml heparin to allow wound healing during the first week after operation. Thereafter, during a five week period[20-23], rats were daily instilled with 10 ml of standard PDF (Dianeal ® PD4, 3.86% glucose, pH 5.2, Baxter R&D, Utrecht, The Netherlands) (PDF-control; n=14), PDF with addition of unfractionated heparin (PDF-UFH; n=10) or PDF with addition of low molecular weight heparin (Fraxiparine ®) (PDF-LMWH; n=10). The concentrations used for UFH as well as LMWH (1 mg/rat/day, equivalent to 175 IU UFH /rat/day or 100 IU LMWH /rat/day) are consistent with the concentrations used in clinical settings and proportional to the dose used in deep venous thrombosis prophylaxis in humans[24]. To check whether the animals were not over-heparinised due to daily heparin administration, blood samples were taken after 4 weeks of treatment three hours after PDF + heparin instillation, to measure APTT clotting times and anti factor Xa levels. At the end of the experiment a functional 90 minute Peritoneal Equilibrium Test (PET) was performed (see below) under a mixture of Hypnorm (0.05 ml/100 g) / Dormicum (0.08 ml/100 g) anaesthesia. After sacrificing the animals, tissues and peritoneal leukocytes were taken to analyse functional, morphological and cellular parameters.

Analysis of peritoneal cells and effluents

After five weeks of treatment a PET test was performed by injecting 30 ml of standard PDF into the peritoneal cavity via a direct i.p. catheter (Venflon Pro, BD Medical, New Jersey, USA). After 90 minutes the PDF was drained and the ultrafiltration capacity was calculated. Cells were isolated by centrifugation and cell number and viability was determined by Trypan Blue exclusion. Cytocentrifuge preparations were stained

by May Grünwald Giemsa, and cells were differentiated. After the PET test, the animals were sacrificed and a cardiac puncture was performed. Glucose, sodium, creatinine and urea concentrations were analysed in serum samples and in the cell free effluents at t=90 minutes of the PET test. At t=0 and t=45, sodium concentrations in the effluents were analysed to measure sodium sieving. The amount of hyaluronic acid (HA) in the supernatant of the peritoneal effluent was determined in an ELISA-based assay according to Fosang et al[25].

Morphological analysis

Mesentery and Omentum: A part of omental and mesenteric tissue was dissected and spread on a glass slide for fluorescence microscopic examination. Both tissues were stained with antibodies for CD31 (PECAM; Serotec, Oxford, UK) to visualize vasculature and with ED2 (Serotec, Oxford, UK) to visualize macrophages. As a negative control, conjugate controls (Invitrogen, Carlsbad, USA) without the first antibody were used. Images were analysed by computerized digital image analysis (AnalySIS, Soft Imaging System). The positive area for CD31 and ED2 was calculated as a percentage of the total area of the tissue.

Parietal peritoneum: The parietal peritoneum was taken at the contra lateral side to the tip of the implanted catheter using a standardized method. Cryostat sections (7 µm) were cut and stained for CD31 and ED2. Images were analysed by computerized digital image analysis and the positive area for CD31 and ED2 was calculated as a percentage of the total area of the submesothelial matrix layer. Frozen sections were also used to quantify fibrosis formation. The thickness of the submesothelial extra-cellular matrix (ECM) was determined after Van Gieson staining (Merck, Darmstadt, Germany) as the mean of 10 independent measurements for each animal.

Liver imprints: Imprints of the mesothelial monolayer of the liver were taken with 3% gelatin coated slides according to the method described before[26] and stained by May Grünwald Giemsa. Mesothelial cell density per 0.1 mm^2 area was counted using a 'scored eyepiece' and the mean of 15 areas was calculated for each slide and expressed as cells/mm^2. Liver imprints were also stained for Vimentin (Serotec, Oxford, UK) and Cytokeratin (DakoCytomation, Glostrup, Denmark) to determine spindle-like shaped cells that underwent epithelial to mesenchymal transition (EMT).

Statistical analysis

All data are presented as median and inter quartiles. The Kolmogorov-Smirnov test for normality showed that the groups were not normally distributed, therefore differences between groups have been analysed using the nonparametric Mann-Whitney U-test. We made three comparisons, namely: Control vs. PDF-control, PDF-

control vs. PDF-UFH and PDF-control vs. PDF-LMWH. According to a Bonferroni correction, probability values of $p<0.03$ were considered significant. Correlation analysis was performed using the non-parametric Spearman's Rho test. Survival analysis was performed by Kaplan Meier.

Results

During the experiment, the well-being of all animals was monitored daily, but no apparent abnormalities were observed. Throughout the experiment there was a dropout of a number of animals due to abdominal fat or omental tissue wrapping around the tip of the catheter, which was consistent with earlier experience[17;27]. Although it was expected that heparin would impair fibrin formation and may prevent adhesion and omental wrapping, Kaplan Meier survival analysis showed no differences in drop out rate (neither in time, nor in number) between the three treated groups. The development of peritonitis during the experiment is very unlikely since there was no loss of bodyweight, the animals did not show any other clinical signs and there were no abnormalities in neutrophil counts in peritoneal dialysates or in peritoneal tissues. After 5 weeks of fluid instillation 8, 5, and 5 rats in the PDF-control, PDF-UFH and PDF-LMWH group, respectively, had remained for analysis. All 8 control rats were used for the PET and morphological analysis. APTT and anti-Xa measurements showed that daily i.p. treatment with clinical concentrations of 1 mg/day of heparin did not significantly influence clotting times 3 hours after heparin injection. Plasma anti-Xa levels were higher in the PDF-LMWH group compared to the control groups (p=0.048). Although this difference was just not significant, it indicates that heparin was administered in an adequate dose without over-heparinization of the animals.

Transport parameters and cellular composition of peritoneal cells

Table 1 shows the transport parameters measured for all 4 groups in the PET effluents at t=90 minutes.

Table 1: Peritoneal transport parameters determined by PET

	Control	PDF-control	PDF-UFH	PDF-LMWH
Net Ultrafiltration volume (ml/ 90 min)	8.6 +/- 1.8 [a]	6.5 +/- 3.0	5.0 +/- 3.9	3.8 +/- 3.1
Glucose t=90 (mmol/l)	113.4 +/- 32.0 [b]	68.5 +/- 26.9	73.8 +/- 12.5	70.3 +/- 13.5
Glucose absorption (%)	27.9 +/- 19.8 [b]	58.5 +/- 19.3	59.4 +/- 10.8	60.4 +/- 12.5
Creatinine t=90 (µmol/l)	48.5 +/- 9.5	46.0 +/- 7.0	45.0 +/- 10.0	48.0 +/- 15.0
Urea t=90 (mmol/l)	3.9 +/- 1.1 [a]	5.0 +/- 1.9	5.6 +/- 1.6	4.8 +/- 0.8
Sodium t=0 (mmol/l)	129.5 +/- 8.8	129.5 +/- 9.5	131.0 +/- 22.0	130.0 +/- 2.0
Sodium t=45 (mmol/l)	121.5 +/- 3.8	121.0 +/- 7.0	120.0 +/- 17.0	122.0 +/- 10.0
Sodium t=90 (mmol/l)	112.0 +/- 20.5 [c]	123.0 +/- 16.5	118.0 +/- 17.0	122.0 +/- 20.5

All data is presented as Median +/- Inter Quartiles
[a] Control vs. PDF-control: p< 0.02; [b] Control vs. PDF-control p< 0.04;[c] Control vs. PDF-control p < 0.03.

The PET in the control animals resulted in a net UF capacity of 8.6 ml per 90 minutes. PDF exposure reduced peritoneal performance significantly to a net UF volume of 6.5 ml. Addition of UFH or LMWH reduced the UF capacity even slightly further from PDF-control group. Besides the reduced net UF capacity, PDF induced significantly enhanced glucose absorption ($p< 0.03$) and an increased urea transport ($p<0.02$). Sodium measurements at t=0, t=45 and t=90 minutes of the PET test, showed a reduction in the sodium dip upon PDF treatment (Control vs. PDF-control at t=90: $p<0.03$). The addition of UFH or LMWH did not restore PDF-induced changes in peritoneal transport (Table 1).

In table 2 the total cell numbers and differentiation of the peritoneal cells recovered from the PET dialysate are shown. Significantly more cells were found in the peritoneal effluents of the three PDF treated groups compared to the control animals ($p<0.02$). No difference in cell numbers was seen between PDF-control and both heparin groups. Cell differentiation revealed an exchange of mast cells and eosinophils for neutrophils after PD treatment. UFH as well as LMWH treatment seemed to partly abolish the PD evoked inflammation by reducing neutrophil recruitment. Furthermore, addition of UFH or LMWH resulted in a slightly reduced hyaluronan synthesis compared to the PDF-control group (Table 2).

Morphological changes

Mesentery and Omentum: All groups exposed to PDF showed a significant increase ($p<0.01$) in accumulation of mature macrophages (ED2) and new blood vessel formation (CD31) compared to the control group (Figure 1). Neither UFH nor LMWH was able to prevent increased density of macrophages of blood vessels seen in the mesentery and omentum. The increased accumulation of macrophages was

Table 2: Composition of peritoneal leukocytes and HA synthesis

	Control	PDF-control	PDF-UFH	PDF-LMWH
Total cell count (10^6)	23.5 +/- 3.6 [a]	214.5 +/- 51.7	186.5 +/- 48.8	214.6 +/- 54.7
% Macrophages	72.8 +/- 6.1	65.6 +/- 35.7[b]	79.8 +/- 9.6	86.25 +/- 13.1
% Lymphocytes	0.5 +/- 0.7	0.8 +/- 0.5	1 +/- 0.6	0.6 +/- 1.2
% Neutrophils	2.4 +/- 1.7 [a]	27.3 +/- 31.4	17.5 +/- 11.3	12.1 +/- 14.9
% Eosinophils	11.5 +/- 3.3 [a]	4.6 +/- 8.1	0.5 +/- 1.9	0.6 +/- 1.2
% Mast cells	12.25 +/- 5.5 [a]	0.1 +/- 0.3	0.3 +/- 0.4	0 +/- 0.2
Hyaluronan (ng/ml)	21.4 +/- 24.6 [a]	2424.9 +/- 2896.8	2171.8 +/- 1643.64	1190.4 +/- 904.2

All data is presented as Median +/- Inter Quartiles

[a] Control vs. PDF-control: $p< 0.02$;[b] PDF-control vs. PDF-LMWH: $p< 0.05$.

positively correlated (r=0.64 and r=0.89 for respectively mesentery and omentum, both p<0.01) to increased blood vessel density when all 4 groups were combined.

Parietal Peritoneum: Frozen sections of the peritoneum were stained with Van Gieson to observe changes in the ECM underneath the mesothelial layer. Peritoneum taken from control animals showed a thickness of 8.4 +/- 4.1 µm ECM. The thickness was significantly increased (p<0.01) in the peritoneum of animals exposed to PDF-control (14.7 +/-5.2), PDF-UFH (12.4 +/- 3.4) and PDF-LMWH (12.8 +/- 6.3) (Figure 2A). No differences were found between the PDF treated groups. Sections of the

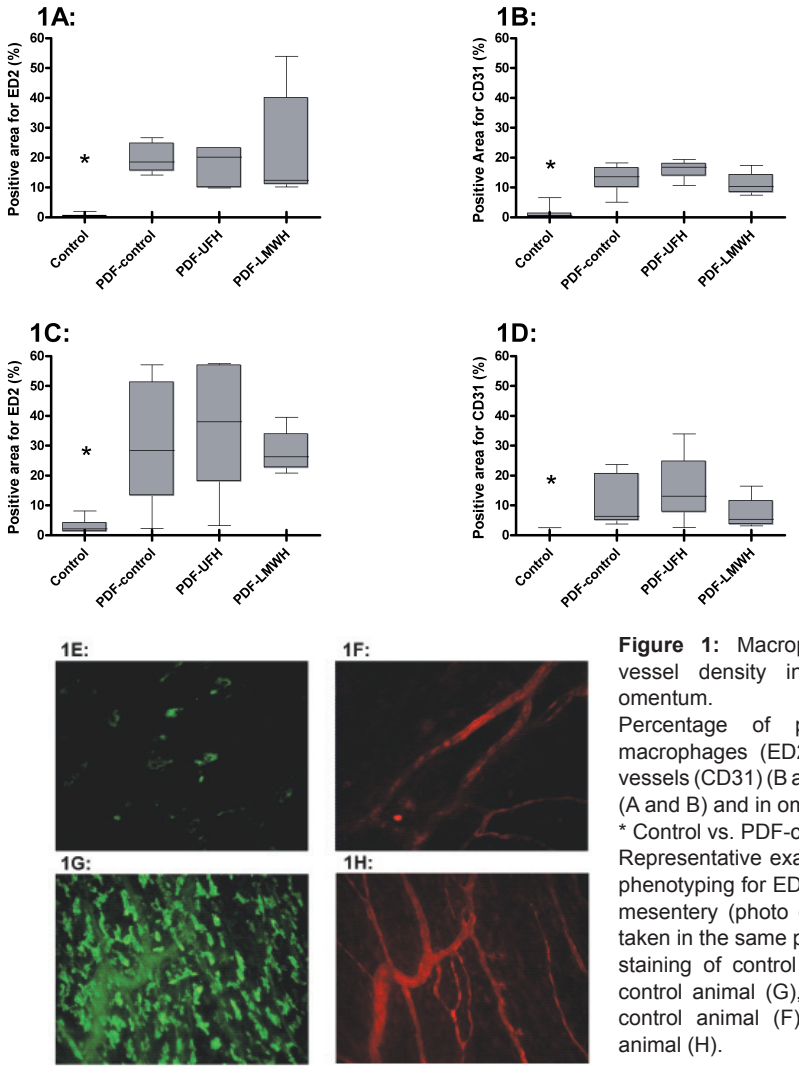

Figure 1: Macrophage and blood vessel density in mesentery and omentum.
Percentage of positive area of macrophages (ED2) (A and C) and vessels (CD31) (B and D) in mesentery (A and B) and in omentum (C and D).
* Control vs. PDF-control: p<0.01.
Representative examples of immunophenotyping for ED2 and CD31 of the mesentery (photo of ED2 and CD31 taken in the same picture frame). ED2 staining of control animal (E), PDF-control animal (G), CD31 staining of control animal (F) and PDF-control animal (H).

peritoneum were also stained for CD31 and ED2. Similar to the morphological changes found in the mesentery and the omentum, significant more macrophages and blood vessels were seen in PDF treated animals compared to the control group, without any differences between the three PDF treated groups (Figure 2B-2G). Correlation analysis showed again a significant positive relation for increased macrophage accumulation and blood vessel density ($r = 0.76$, $p<0.01$). Positive correlations were also found for matrix thickness and CD31 ($r = 0.78$, $p< 0.01$) and for matrix thickness and ED2 ($r = 0.76$, $p< 0.01$).

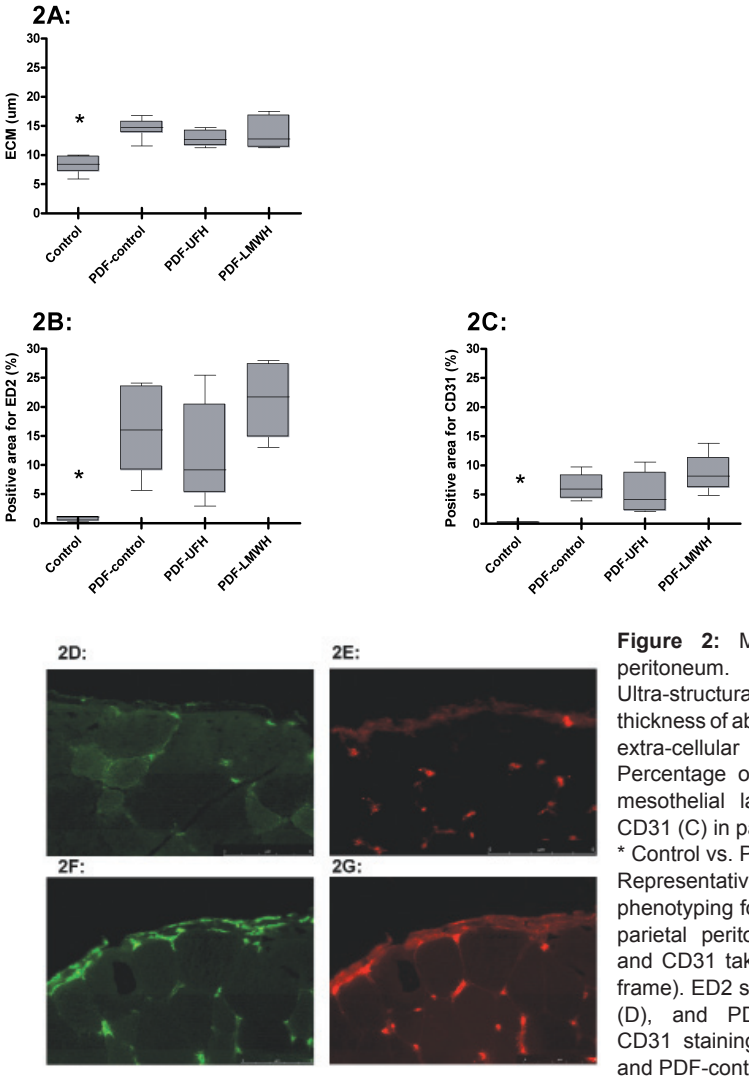

Figure 2: Morphology of parietal peritoneum.
Ultra-structural measurements of the thickness of abdominal submesothelial extra-cellular matrix (ECM) (A). Percentage of positive area of sub-mesothelial layer for ED2 (B) and CD31 (C) in parietal peritoneum.
* Control vs. PDF-control: $p<0.01$.
Representative examples of immuno-phenotyping for ED2 and CD31 of the parietal peritoneum, (photo of ED2 and CD31 taken in the same picture frame). ED2 staining of control animal (D), and PDF-control animal (F), CD31 staining of control animal (E) and PDF-control animal (G).

Liver Imprints: Mesothelial cell density was measured as a marker for mesothelial cell regeneration. The number of mesothelial cells on the liver showed a significant 2 fold increase up to 2044.8 +/- 548.4 cells /mm^2 in the PDF-control group compared to the untreated animals (1167.6 +/- 272.6 cells/mm^2 p< 0.01) (Figure 3A and B). Neither of the 2 heparins was able to influence mesothelial cell regeneration. Spindle-like shaped cells were found between mesothelial cells in PDF treated animals (Figure 3B). An average of 7-10% of the cells found on the liver imprints of PDF treated animals showed this phenotype, whereas these cells were not found in control animals (p<0.01). No differences in spindle shaped cell numbers were found between the three PDF exposed groups. A vimentin positive and cytokeratin negative staining (Figure 3C and 3D) identified those cells as fibroblasts-like cells, indicating either that the imprint is picking up submesothelial fibroblasts due to partial injury and denudation of mesothelial cells, or possibly mesothelial cells undergoing EMT (Figure 3D).

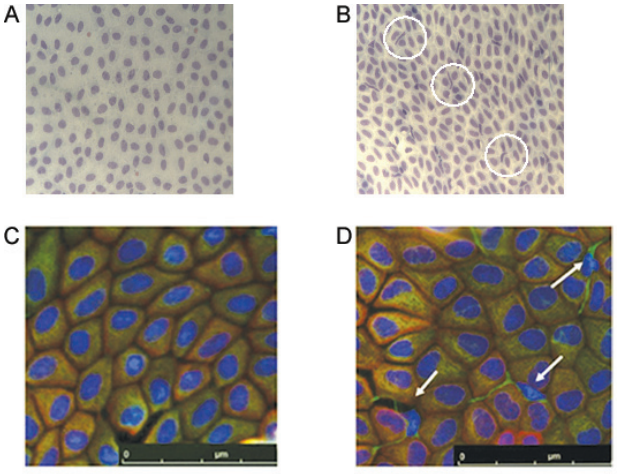

Figure 3: Morphology of the liver imprints.
Representative example of mesothelial imprints of a control (A) and a PDF (B) treated animal. Spindle-like shaped cells (indicated by circles) are present between mesothelial cells on liver imprints of PDF treated animals (B). Representative example of a vimentin (green) and cytokeratin (red) staining of a liver imprint of a control (C) and a PDF treated animal (D), nuclei are stained in blue. Arrows indicate spindle-like shaped cells.

Discussion

In our study either UFH or LMWH was added to conventional PDF, based on the characteristics of heparins to reduce new vessel formation, block leukocyte rolling and adhesion, and the capability to bind cytokines and growth factors[6]. We show significant worsening in peritoneal performance after five weeks of PDF exposure evidenced by high transport characteristics, inflammation and peritoneal tissue remodelling. Moreover mesothelial imprints revealed fibroblast-like cells upon PDF exposure. However, none of these PDF induced events could be diminished or prevented by the addition of UFH or LMWH to the PDF.

We decided to add heparins to the PDF in order to obtain maximal local availability and minimize systemic effects of the heparin intervention. Non significant changes in APTT clotting times and anti-factor-Xa levels indicated that the animals were not over-heparinized during the experiment, whereas increased plasma Xa levels after LMWH administration, showed that adequate working doses were administered. The concentrations used for UFH as well as LMWH are clinically relevant doses and were similar or even higher compared to the literature[15;24;28-30]. Higher concentrations of heparin might have led to bleeding complications or heparin induced thrombocytopenia[31]. The normal clotting times in animals which received daily heparin, might in part be due to the fact that i.p. heparin barely reached the circulation or that it already left the circulation within three hours after administration. In line with our results are the unaffected APTT and anti factor Xa measurements of PD patients which received an i.p. injection with LMWH[14;32;33], whereas increased plasma factor Xa levels were seen after subcutaneous injection[33]. These data indicate that intraperitoneal administration of heparin only have minor partial systemic effects[32;33]. Although our intention to avoid systemic effects of heparin treatment was successful, none of the PDF induced peritoneal changes could be (partly) prevented by the local heparin treatment. Since we were especially interested in the long-term effects of heparin treatment and to avoid direct effects of heparin, we treated the animals daily during five weeks and performed the PET tests without heparin one day after the last heparin gift. Identical methodological approach has been successfully applied in the past with i.p. aminoguanidine treatment[34]. This is an important difference with some other studies, and might explain why others reported beneficial effects of glycosaminoglycans such as heparin or hyaluronan that was present in the PDF during the PET procedure[15;35], which is explained by a reduction of the peritoneal fluid reabsorption rate due to the formation of a filter-cake of the glycosaminoglycans chains at the tissue/cavity interface.

The fact that chronic treatment with either of the heparins did not prevent peritoneal membrane remodelling (new vessel formation, fibrosis, macrophage influx and mesothelial EMT) might largely explain why the peritoneal transport parameters in the heparin treated groups were not different from the PDF-control group, since molecular transport is highly dependent on vascular surface area and submesothelial matrix thickness and composition[36]. Positive effects of heparin on peritoneal transport are reported in studies of Pawlaczyc et al[13] and Sjoland et al[14]. However, the first study only showed marginal, non significant, differences of heparin after 30 days of treatment and to our best knowledge the second study has not been confirmed by others.

The absence of any effect of chronic heparin treatment on peritoneal inflammatory markers like cell influx, might be explained by the fact that intraperitoneal cell recruitment occurs at the level of post-capillary venules[22] and via the omentum[23]. We show that micro vascular density in the peritoneal tissues was unchanged by heparin treatment. Moreover, the absence of heparin in the circulation during the PET procedure allows normal selectin–ligand interaction, which is the first step in leukocyte–endothelial interaction. There have been reports of anti-inflammatory properties of heparinoids in other peritoneal inflammatory models. However, in all those studies the heparinoids were given intravenously and present in systemic circulation thereby inhibiting L- and/or P-selectin dependent peritoneal cell influx[8;37-40]. In line with our results, heparin showed no effects on PDF induced neutrophil numbers[15], nor did it show effect on inflammatory cells, fibrosis and angiogenesis in an animal model for abdominal wound healing[30].

Concerning the role of heparins on angiogenesis, opposite results have been described[30;41]. In general, tumor angiogenesis seems to be highly dependent on fibrin formation and tissue factor pathway, processes that are highly modulated by heparin[42]. However, not all forms of new vessel formation are fibrin and/or tissue factor dependent. An impressive number of factors and mediators might be involved, highly dependent on the model system used[43]. The fact that chronic heparin treatment did not prevent neo-angiogenesis suggests that intraperitoneal fibrin formation is not involved in our experimental PD model.

Another intriguing finding was the appearance of vimentine-positive, cytokeratin-negative, spindle like shaped cells within the mesothelial monolayer, which suggests EMT of the mesothelial cells. An alternative explanation would be that the spindle like shaped cells might be submesothelial fibroblasts which are picked up by the

imprint and indicate partial injury and denudation of mesothelial cells. Addition of either of the heparins did not modify this process and could neither affect mesothelial regeneration, evidenced by increased mesothelial cell density on the liver imprints.

In conclusion, we have confirmed and extended that PD induces significant worsening of the peritoneal tissues within 5 weeks. Moreover, we have shown an induction of spindle like shaped cells in the mesothelium, indicating either mesothelial damage or EMT. Secondly, we conclude that based on our findings intraperitoneal addition of UFH or LMWH to PDF does not improve peritoneal transport, nor does it influence the remodelling of peritoneal tissues during PD treatment. Therefore, long term peritoneal administration of heparin during chronic PD treatment is not supported by our data.

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