4 Chapter

Celecoxib treatment reduces peritoneal fibrosis and angiogenesis and prevents ultrafiltration failure in experimental peritoneal dialysis

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Abstract

Background: Daily peritoneal exposure to peritoneal dialysis fluid (PDF) induces severe morphological alterations including fibrosis and angiogenesis that lead to a loss of peritoneal ultrafiltration (UF) capacity. Since cyclooxygenase (COX)-2 is involved in fibrosis and angiogenesis, we investigated the in vivo effects of a selective COX-2 inhibitor (Celecoxib) in a rat-PD model.

Methods: Sixteen rats daily received 10 ml conventional PDF for 4-5 weeks intraperitoneally. Half of them (n=8) daily received Celecoxib (20 mg/kg BW) via oral gavage, the other half (n=8) received vehicle via oral gavage. The study also included two control groups (no PDF instillations), each consisting of n=8 animals, that daily received Celecoxib or vehicle respectively via oral gavage. Functional, morphological and cellular parameters were analyzed.

Results: PDF exposure induced an inflammatory condition evidenced by increased leukocyte number and synthesis of MCP-1, VEGF and Hyaluronic acid. After PDF exposure, the omentum showed intense angiogenesis and milky spots formation. The prietal peritoneum showed increased angiogenesis, lymphangiogenesis, submesothelial matrix thickness and enhanced expression of mesothelial aquaporin1 (Aqp1). Concomitant PDF and Celecoxib exposure drastically reduced PGE2 levels, angiogenesis, lymphangiogenesis, fibrosis and milky spots formation in studied tissues, but did not modify mesothelial Aqp1 expression nor tissue expression of VEGF and inflammatory markers. PDF exposure induced severe UF failure that Celecoxib treatment completely prevented.

Conclusions: Altogether, Celecoxib treatment improves UF capacity and reduces morphological alterations in our rat PD model.

Introduction

During peritoneal dialysis (PD) the peritoneal membrane is used as dialyzing organ and constantly exposed to peritoneal dialysis fluid (PDF). The daily instillation of PDF creates an intraperitoneal nonphysiological environment that sustains the development of morphological changes. A chronically exposed peritoneal membrane is characterized by interstitial fibrosis, alteration of the mesothelial layer, new vessel formation. Altogether these events are thought to be major culprits in determining ultrafiltration (UF) failure and PD withdrawal[1]. At the molecular level there is increasing evidence that a number of cytokines, growth factors and prostaglandins play key roles in regulating and sustaining these processes[2].

Cyclooxygenase (COX) enzymes catalyze the rate–limiting step in prostaglandin synthesis from arachidonic acid. Their metabolites play a pivotal role in multiple physiological and pathophysiologic processes. Specifically, COX-1 is constitutively expressed in most tissues and is responsible for maintaining physiological processes such as gastric and renal protection and platelet function. In contrast, COX-2 is induced in response to growth factors, cytokines, and tumour promoters and its expression has been demonstrated in immune and non-immune cells, both under inflammatory and proliferative conditions, and is dependent on, mostly pro-inflammatory stimuli[3].

Increased COX-2 expression is a key step to induce pathological angiogenesis in many different tumours[4]. COX-2 can stimulate angiogenesis via the production of vascular endothelial growth factor (VEGF), a potent angiogenic growth factor, by increasing its mRNA transcription[5;6], but also by the production of thromboxane A2, prostaglandin E2 (PGE2) and prostacycline, products of arachidonic acid which are direct stimulants of endothelial cells[7-9]. Furthermore, the effects of COX-2 induced prostanoids on angiogenesis are probably amplified via a feedback loop, because VEGF activates both phospholipase A2-mediated release of arachidonic acid and COX-2 expression, thereby enhancing PGI2 and PGE2 production[10].

Other evidences indicate that COX-2 can also induce fibrosis as shown by effective reduction of carbon tetrachloride–induced liver fibrosis in rats treated with a potent COX-2 inhibitor[11]. Furthermore it has been recently shown[12] that TGF-β and collagen I production in human peritoneal cells exposed to PDF, is mediated by the synthesis of PGE2 and that this process can be completely prevented by the use of a selective COX-2 inhibitor.

Whether COX-2 pathways are involved in PD-related angiogenesis or fibrosis is not known yet. In vitro experiments have demonstrated that under PD-like conditions mesothelial and inflammatory cells and fibroblasts involved in peritoneal healing after

abdominal surgery can express COX-2[13-15]. A recent publication showed increased COX-2 expression in mesothelial cells that underwent epithelial to mesenchymal transition (EMT), although COX-2 inhibition did not prevent EMT. Moreover in a mouse model of PD, COX-2 inhibition reduced fibrosis, but not angiogenesis and partially recovered ultrafiltration[16].

Among the selective COX-2 inhibitors, Celecoxib showed prevention of angiogenesis in several in vitro angiogenesis assays and in vivo cancer models[17]. Recent findings showed that clinical and experimental effects of Celecoxib may be wider than its COX-2 inhibitory capacity, opening new scenarios for its clinical use[18].

Therefore, the aim of our study was to investigate whether the morphological and functional alterations occurring in the peritoneal membrane after PDF exposure can be prevented through oral Celecoxib treatment in our well-established chronic PD rat model.

Materials en methods

Animals

Male Wistar rats (Harlan CPB, Horst,The Netherlands) of the same age were used throughout the study. Animals were maintained under conventional laboratory conditions. The Animal Care Committee of the Vrije Universiteit of Amsterdam approved all animal experiments described and all experiments are in accordance with the NIH guide for the care and use of laboratory animals.

Experimental design

A peritoneal catheter was implanted as described previously[19]. No omentectomy was performed, and rats had normal renal function. During a one-week recovery period, rats received daily 2 ml of saline solution containing 1 U/ml heparin and on day eight daily instillation of 10 ml of standard PDF (Dianeal® PD4, 3.86% glucose, pH 5.2, Baxter R&D, Utrecht, the Netherlands) was started. Control animals (C) were not surgically treated and received no fluid instillation. Throughout the study 8 of the PDF exposed and 8 of the control animals received daily administration of Celecoxib (20 mg/kg)[20] solved in polyethyleneglycol (PEG) via oral gavage. The other 8 PDF exposed and 8 control animals received oral vehicle administration. After 4 to 5 weeks of PDF exposure functional (90 min peritoneal equilibrium test (PET)) and morphological/cellular/immunological parameters were analyzed. Evaluations of various parameters were done independently by two persons on coded material and the averages of both measurements were used. In this study a total of 8 animals in each group were evaluated.

PET test

At the end of the experimental period, a 90 minute PET test was performed by injecting 30 ml of Dianeal 3.86% into the peritoneal cavity. After drainage, in each sample UF net volume was calculated and cell pellet collected, cell number and viability was determined in a haemocytometer on the basis of trypan blue exclusion. Cytocentrifuge preparations were stained with May-Grünwald Giemsa stain, and cells were differentiated. Cell-free PET fluid was stored at -80° C for determination of biomarkers and glucose absorption.

Part of the stored PET effluent was concentrated up to 30 times using a centrifugal filter device (Millipore Amicon Ultra-4). On concentrated PET fluid we measured by enzyme-linked immunosorbent assay IL6 (NIBSC, South Mimms, UK) and VEGF (Quantikine® M, VEGF kit, R&D Systems, Inc. USA) concentrations while on un-concentrated samples we determined hyaluronic acid (HA) (according to Fosang

et al [21]), monocyte chemoattractant protein (MCP-1) (PharMingen, San Diego, CA, USA) and PGE2 (Quantikine®,PGE2 kit, R&D Systems, Inc. USA).

Morphology

Tissue was taken in a standardized fashion in order to obtain the same anatomical portion and to avoid mechanical trauma to mesothelial cells.

Omentum: Omental tissue was dissected and spread on glass slides for light microscopic examination. The whole mount preparations were stained with Toluidine Blue, and the number of blood vessels and the area of milky spots were counted as previously described[22]. The total milky spot surface of every individual omentum was calculated by multiplying the number of milky spots/cm^2 by the area per milky spot.

Parietal peritoneum: Cryostat sections (6 µm) were cut and stained using different antibodies to visualize blood vessels (CD31, PECAM, Serotec, Oxford, United Kingdom), Aquaporin1 (Aqp1) (a kind gift of Dr. Peter Deen, Dept. Physiology, UMC St. Radboud, Nijmegen, The Netherlands), lymphatic vessels (Lyve 1, Cell Signaling Technology, Inc, The Netherlands), VEGF A (Calbiochem Darmstadt, Germany) and VEGF B (R&D systems, USA). Blood vessel number was counted and expressed per mm mesothelial length while lymphatic vessels where quantified via a computer analysis (analySIS® pro, Olympus soft Imaging solutions GmbH) and expressed as percentage of tissue covered area. The same software was then used to measure Aqp1 positive area at the mesothelial level in parietal peritoneum.

The thickness of the submesothelial extracellular matrix layer (ECM) was determined as the average of 10 independent measurements for each animal on Van Gieson stained sections.

Liver: Imprints of the mesothelial monolayer of the liver were obtained according to the method described by Whitaker *et al*.[23] and then stained according to May-Grünwald Giemsa. Changes in the mesothelial cell density were assessed by light-microscopic analysis, using a scored eyepiece as previously described[19].

Statistical analysis

All data are presented as median and range and have been analysed using the non-parametric Mann-Whitney U test. Probability values of $p<0.03$ were considered significant, according to Bonferroni correction for multiple comparisons.

Results

Inhibition of COX-2 by Celecoxib

PgE2 levels in cell-free PET fluids in PDF+Celecoxib animals (33.1 pg/ml (0.6 to 90.3)) were significantly reduced compared to PDF treated rats (79.3 pg/ml (49.3 to 142) $p<0.03$) indicating effective COX-2 inhibition by Celecoxib treatment. In the control group PgE2 levels were as high as in the PDF group (70.1 $p>0.3$) indicating a sudden production of PgE2 after a single PDF exposure. In most of C+Celecoxib animals PgE2 was below detection limits (7 pg/ml) indicating a suppression of PgE2 production by Celecoxib treatment.

Functional parameters

The PET in control animals resulted in 9.6ml (9.0-10.8 ml) and 9.5 ml (8.5-12.5 ml) of net UF in the control and in the C+Celecoxib group respectively. PDF exposure reduced peritoneal performance with a significant fall of net UF volume to 6.1 ml (3.2-8.1 ml) in the PDF group. PD animals undergoing COX-2 inhibitor treatment did not loose peritoneal performance and showed a net UF volume of 9.2 ml (8.1-11.9 ml) (PDF vs PDF+Celecoxib: $p<0.05$; C+Celecoxib vs PDF+Celecoxib: NS). A glucose absorption test showed 30.2% (26.4-33.7%) absorption in the control group and 30.1 % (27.5-32.8%) in C+Celecoxib group (no differences between controls), while PDF exposed animals had a significant increase in glucose absorption to 48.0% (34.3-57.6%) which was slightly improved in Celecoxib treated animals (42.0%, range 36.2-54.9%, $p>0.05$ vs PDF).

Inflammatory markers

No differences between control groups were present either in cell number or in cell subpopulations (Table 1). Both PDF exposed groups had a significantly higher influx of inflammatory cells (with an increase in neutrophils and lymphocytes and a reduction in eosinophils and mast cells) compared to controls. No differences were observed between the PDF and the PDF+Celecoxib groups.

Dialysate IL6 was not detectable in any of the four groups. VEGF concentrations did not differ in control groups and increased ~2-5 fold in both PDF exposed groups. No differences were present between PDF and PDF+Celecoxib animals. The same holds true for MCP-1 and HA: both significantly increased under PDF exposure conditions but no differences were found after Celecoxib treatment (Table 1), although a tendency towards reduced values of MCP-1 and VEGF could be seen in the PDF+Celecoxib group.

Table 1: Inflammatory parameters

	Group C	Group C + Celecoxib	Group PDF	Group PDF + Celecoxib
PET test cell parameters				
Total cell count (x 10^6)	24,1 [13,2-6,9]	19,1 [13.0-23.1]	49,6 [a] [26.2-182.5]	56,0 [a] [21.4-114.1]
Macrophages (%)	77 [71-79]	75 [71-78]	79,0 [66-89]	78,5 [74-80]
Lymphocytes (%)	2 [1-6]	2 [1-3]	8,0 [a] [6-19]	11,5 [a] [10-16]
Neutrophils (%)	0 [0-0]	0 [0-0]	6,0 [a] [0-14]	5,5 [a] [3-9]
Eosinophils (%)	11 [8-12]	11 [4-14]	6,0 [a] [0-13]	4,5 [a] [1-6]
Mastcells (%)	10 [7-14]	13 [8-17]	0,0 [a] [0-1]	0,0 [a] [0-1]
PET test biomarkers				
IL6 (pg/ml)	Undetectable	Undetectable	Undetectable	Undetectable
MCP1(ng/ml)	0.4 [0.2-0.5]	0.3 [0.3-0.5]	1.7 [a] [0.8-13.0]	1.2 [a] [0.9-2.4]
HA (ng/ml)	19,8 [11.5-30]	15,6 [15-20.9]	583.4 [a] [220.5-3057.6]	590.2 [a] [115.4-1374.6]
VEGF (pg/ml)	16.1 [7.8-21.1]	15.7 [9.4-21.9]	59.6 [a] [29.5-86.8]	37.7 [a] [19.5-43.6]

All data are expressed as the median (and range). PDF, peritoneal dialysis fluids.
[a] p<0.05 vs group C and C+Celecoxib.

Table 2: Morphometric parameters of peritoneal tissues

	Group C	Group C + Celecoxib	Group PDF	Group PDF + Celecoxib
Omentum				
Number of milky spots/cm2	2,5 [2,0-3,5]	2,6 [1.2-3.0]	29,0 [a] [15,3-42,3]	17,1 [ab] [8,9-27,5]
Total milky spot surface (%)	0,3 [0,2-2,9]	0,2 [0,1-2,9]	7.5 [a] [3,8-18,6]	2,7 [ab] [1,6-7,2]
Mast cells number/cm2	20 [10 to 25]	21 [13 to 50]	132 [a] [69-156]	54 [ab] [46-161]
Number of blood vessels/cm2	9.8 [1 to 14]	9.9 [5 to 15]	266 [126- 455]	143 [ab] [60-222]
Parietal Peritoneum				
Number of blood vessels/cm	4,8 [2.1-9.6]	4,2 [0.0-5.6]	33,7 [a] [12,5-48,4]	19,4 [ab] [4.0-38.0]
ECM thickness (µm)	9,7 [9.2-12.9]	10,0 [8.7-13.6]	19,6 [a] [17.8-27.2]	16,3 [ab] [12.6-22.0]
Mesothelial regeneration				
Cell density on liver/mm^2	1060 [560-1210]	1065 [910-1530]	2130 [a] [1690-2960]	1940 [a] [1350-2480]

All data are expressed as the median (and range). PDF, peritoneal dialysis fluids.
[a] p<0.01 vs C and C+Celecoxib; [b] p<0.03 PDF+Celecoxib vs PDF.

Tissue remodelling

Omentum: No differences were observed between the control groups for mast cell number, blood vessel formation and milky spot response (Table 2 and Figure 1). In both groups exposed to PDF a significant increase in these parameters was observed compared to the control groups. Animals exposed to PDF and treated with Celecoxib had a 2-fold less omentum blood vessel formation ($p<0.01$) and a ~3-fold lower percentage of milky spots surface ($p<0.01$) compared to the PDF group. The reduced angiogenesis in the PDF+Celecoxib vs the PDF group was associated and positively correlated ($r=0.84$) to a significantly lower number of omentum mast cells ($p<0.01$) confirming our previous observations[24]. Moreover a positive correlation was found between number of blood vessels in omentum and PgE2 levels measured in the PET effluent ($r^2=0.5083$; $p=0.01$) of PDF exposed animals (Figure 2).

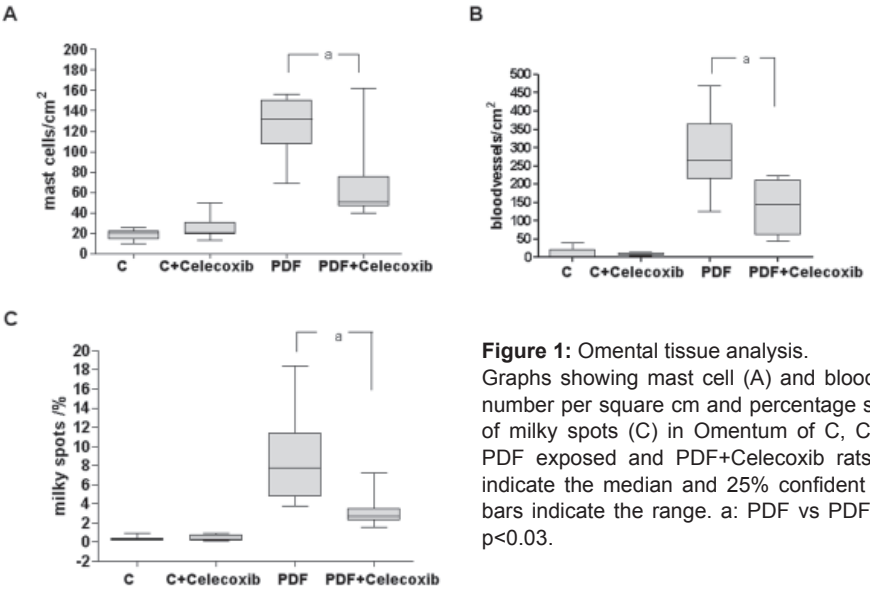

Figure 1: Omental tissue analysis.
Graphs showing mast cell (A) and blood vessel (B) number per square cm and percentage surface area of milky spots (C) in Omentum of C, C+Celecoxib, PDF exposed and PDF+Celecoxib rats. Box plots indicate the median and 25% confident limits, error bars indicate the range. a: PDF vs PDF+Celecoxib, $p<0.03$.

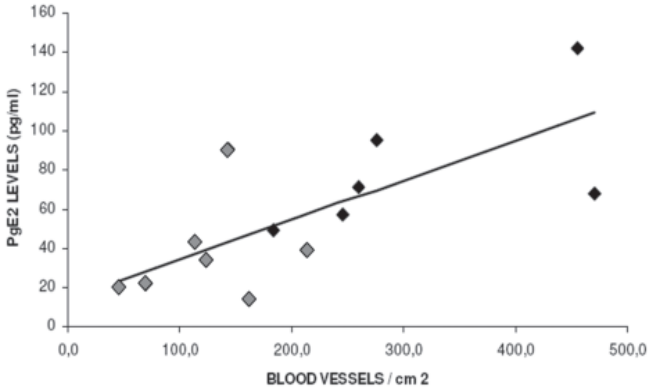

Figure 2: Correlation between blood vessel number and PgE2 levels.
Graph shows positive correlation between omental blood vessels (number/cm2) and PgE2 (pg/ml) levels in dialysates of PDF (black symbols) and PDF+Celecoxib (grey symbols) rats ($r^2=0.54$; $p=0.01$).

Peritoneum: Peritoneum obtained from control animals showed a submesothelial matrix thickness of 10 µm and 9.7 µm in the C and the C+Celecoxib group respectively (Figure 3A and B). This matrix thickness in the peritoneum of PDF animals was significantly increased to 19.6 µm (17.8 to 27.2 µm) (Figure 3C; PDF vs C: $p<0.01$). PDF+Celecoxib animals showed a lower grade of peritoneal fibrosis with ECM thickness reduced to 16.3 µm (12.6 to 22.0 µm), (Figure 3D; PDF vs PDF+Celecoxib: $p<0.03$), although significantly different from control animals ($p<0.01$).

In control animals parietal peritoneum blood vessel number/mm was 4.8 (2.1-9.6) and 4.2 (0.0-5.6) in group C and C+Celecoxib respectively (Figure 3A and B). After PDF exposure a dramatic angiogenic response was observed in the PDF group (Figure 3C) with an 8 fold increase in blood vessels/mm in comparison to control groups (33.7 (12.5 to 48.4) $p<0.001$). Concomitant exposure to PDF and Celecoxib treatment resulted in drastic attenuation of the angiogenic process resulting in a 40% reduction in blood vessel number (19.4 (4.0 to 38.0), $p<0.03$; Figure 3D).

In control animals 4.4% (0.4%-9.9%) of the submesothelial matrix was covered by lymphatic vessels, a small reduction (2.9%) was observed in C+Celecoxib animals (1.3%-5.3%). In the PDF group the extension of lymphatic vessel significantly increased to 12.4% (5.2%-19.5% $p<0.03$ vs control) which was reduced to normal control values (6.2%) in PDF+Celecoxib animals (3.3%-10.4%, $p>0.05$ vs C, $p<0.03$ vs PDF) (Figure 4).

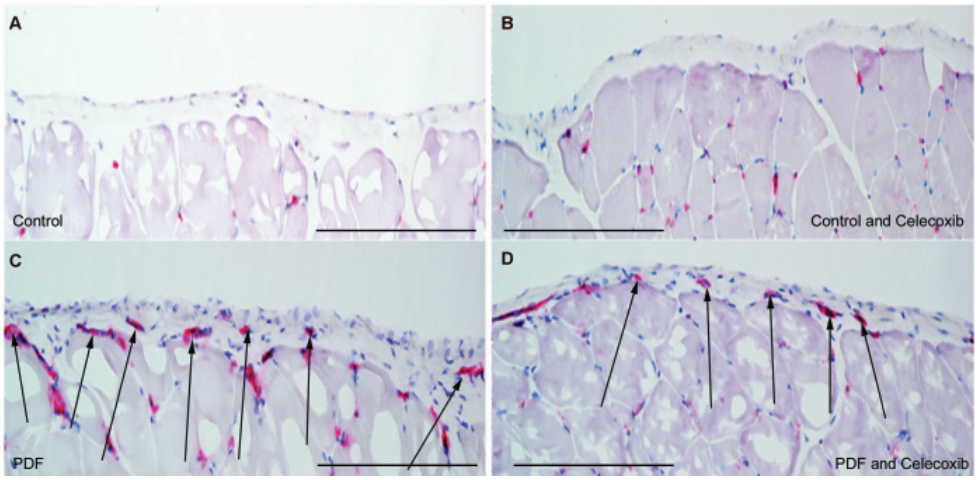

Figure 3: Light micrographs of parietal peritoneum: fibrosis and angiogenesis.
Representative photomicrographs of parietal peritoneum after CD31 (PECAM) staining of C (A), C+Celecoxib (B) rats, PDF (C) and PDF+Celecoxib (D) rats. Red spots (arrows) indicate blood vessels. Magnification A-D: 40x; bar represents 100 µm.

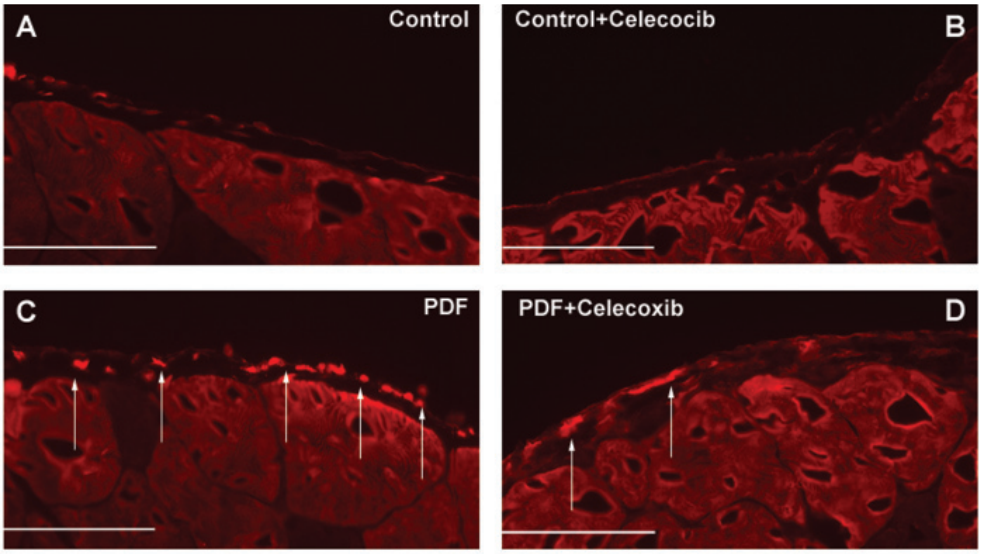

Figure 4: Visualization of lymphatic vessels in parietal peritoneum by immunofluorescence. Representative photomicrographs of parietal peritoneum after Lyve 1 (lymph vessel) staining of C (A), C+Celecoxib rats (B), PDF (C) and PDF+Celecoxib (D) rats. Red spots (arrows) indicate lymphatic vessels. Magnification A-D: 40x; bar represents 100 µm.

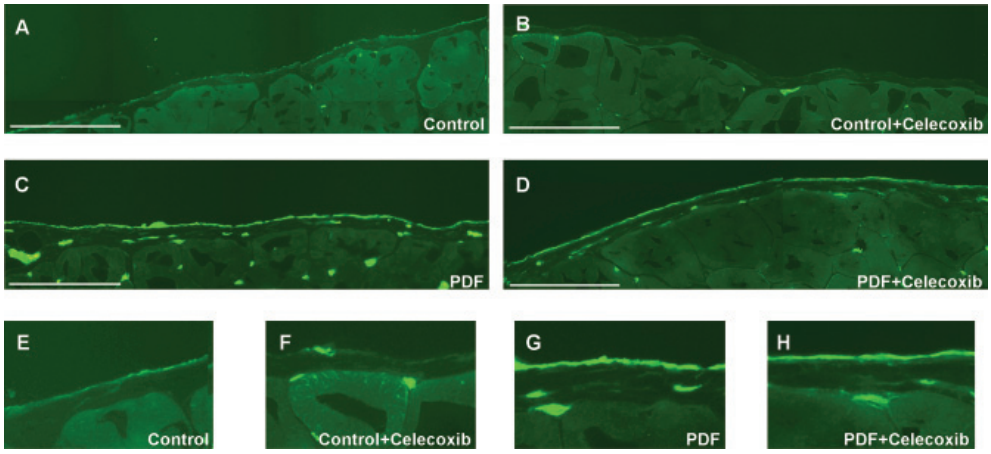

Figure 5: Visualization of aquaporin1 in parietal peritoneum by immunofluorescence.
Representative photomicrographs of parietal peritoneum after Aqp1 staining of C (A+E), C+Celecoxib (B+F) rats, PDF (C+G) and PDF+Celecoxib (D+H) rats. Increased Aqp1 localization at mesothelial (mesothelial cells) and submesothelial (blood vessels) level is shown in circled areas. Figures A-D: Overview of parietal peritoneum: magnification 40 x, bar represents 450µm, E-H: high power insets, 3x magnification of A-D.

Staining of parietal peritoneum with Aqp1 antibody showed an increased intensity of Aqp1 in PDF treated animals, both at blood vessel and mesothelial cell level (Figure 5). A comparison between the PDF and PDF+Celecoxib group did not show apparent differences in mesothelial expression of Aqp1, while its expression in vessels was significantly reduced in PDF+Celecoxib group confirming PECAM staining results (see Figure 5).

VEGF A was increasingly expressed in a cellular expression pattern within the parietal peritoneum of PDF exposed animals in comparison to the control group (5.6% (2.2% to 9.6%) and 2.9% (8.7% to 1.0%) respectively, $p<0.05$). Celecoxib treatment induced a non significant reduction of tissue expression (4.5% (1.5% to 8.2%) $p>0.05$ vs PDF). The same tendency was observed for VEGF B, expressed in vessels and mesothelial cells, which was increased after PDF exposure (5.6% (2.0% to 11.2%) $p<0.03$ vs control), but slightly reduced after Celecoxib (4.8% (0.6% to 9.0%) $p>0.05$% vs PDF).

Mesothelial liver imprints: Mesothelial cell density (Table 2) was measured as a marker of mesothelial cell regeneration. No differences were present in control animals while PDF exposure induced a significant increase in mesothelial cell density which Celecoxib treatment could not reduce. Spindle shaped cells were found between mesothelial cells in PDF treated animals. An average of 5.9% (range 4.4% to 13.8%) of the cells found on the imprints of the PDF group showed this phenotype, whereas these cells were not found in the two control groups ($p<0.01$). Celecoxib treatment could not prevent PD-induced induction of these fibroblast-like cells (PDF+Celecoxib: 6.0%, (2.0% to 9.1%), $p>0.05$ vs PDF).

Discussion

Many clinical and preclinical data suggest a potential role for cyclooxygenase-2 inhibitors in reducing fibrosis and angiogenesis both in *in vitro* and *in vivo* settings. In the present study we investigated the possible modulation of peritoneal fibrosis and angiogenesis related to PDF exposure through Celecoxib treatment in a well-described experimental peritoneal dialysis model.
After PDF exposure (4 to 5 weeks) and Celecoxib treatment, fibrosis was clearly reduced in the parietal peritoneum, but more strikingly the angiogenic process (both in omentum and parietal peritoneum) was extremely slower in progression and new lymphatic vessel formation was completely prevented. The improved morphological appearance of the peritoneal membrane resulted in completely preserved membrane UF capacity. Peritoneal inflammatory cell numbers, biomarkers and mesothelial cell regeneration were not affected by Celecoxib treatment. Our data are consistent with the hypothesis that COX-2 activity plays a crucial role in the peritoneal membrane fibrotic and (lymph-)angiogenic processes after PDF exposure.

The mechanisms behind the multiple actions of COX inhibitors are very complex, largely redundant and partially unknown as recently reported by Claria *et al*[25]. Nevertheless, PgE2 still appears to be a major determinant in most of COX-2 mediated actions. In our study we focused on the PGE2 and VEGF pathways since many *in vitro* data reported a correlation between these two molecules and angiogenic processes[5;6;8]. As expected, PgE2 was significantly reduced in PET effluents of PDF+Celecoxib animals, but was only associated to a slight VEGF reduction in the effluent fluid and at tissue level. This might be explained considering that PgE2 production in inflammatory condition is mainly related to COX-2 activity and hence reduced during Celecoxib treatment, while PD-induced VEGF production can be driven by PgE2-independent stimuli. For example, VEGF secretion is up-regulated by several growth factors including epidermal and transforming growth factor -α and -β, fibroblast growth factor and platelet-derived growth factor[26;27] which we can not exclude to be present in our model of peritoneal exposure. Moreover, prostaglandins can influence angiogenesis by promoting vascular sprouting, migration, and tube formation, inducing matrix metalloproteinases synthesis and enhancing endothelial cell survival via Bcl-2 expression and Akt signalling and through many other VEGF independent mechanisms not investigated in this paper[4] . Coherently we showed a direct and positive correlation between PgE2 levels and angiogenesis in omentum of PDF exposed animals.

Furthermore it is widely accepted that inflammation is the most relevant process in determining peritoneal membrane deterioration, but in our study we showed a significant better preservation of peritoneal membrane morphology and function even in absence of a reduced inflammation. In our experiment no differences were measured both in mesothelial regeneration process and in inflammatory cell influx in the peritoneal cavity and coherently no differences in MCP1 and HA were found. These findings indicate that the Celecoxib-reduced fibrosis and (lymph)angiogenesis is not due to Celecoxib-mediated inhibition of inflammation. Recently a study was published in which PDF-treated mice were subjected to Celecoxib treatment[16]. The investigators reported reduction of submesothelial fibrosis, no effects on angiogenesis, along with reduced peritoneal leukocyte numbers upon Celecoxib treatment. Based on these observations, the authors concluded that inflammation is an early response to PDF exposure, which in turn lead to fibrosis, whereas angiogenesis does not seem to be entirely dependent on inflammatory reaction. Their results partially disagree with our findings, which might be related to different animal models and variations in study design. Nevertheless, our finding of a non anti-inflammatory action of the COX-2 inhibitor Celecoxib is an important finding, which warrants further exploration. We speculate PGE2 downstream signalling pathways to be major culprits in this respect. On the other hand several studies are indicating that Celecoxib can harbour additional pharmacological activities at a micromolecular dose that are completely independent of its COX-2 inhibitory activity. The discovery of additional drug targets (such as protein kinase1 (PDK1) and sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA))[28;29] will give new insight in the understanding of Celecoxib role in reducing angiogenesis and its potential role in PD setting.

In our study we measured a completely restored UF capacity after Celecoxib treatment. This finding was associated to a slight reduction in glucose absorption probably due to the global reduction in vessel density. The ultrafiltration capacity of the peritoneal membrane is not only influenced by vascular surface area and glucose absorption but also by Aqp1 function, lymphatic absorption rate and submesothelial fibrosis[30;31]. The role of COX-2 in lymphangiogenesis has been recently investigated in breast cancer where after COX-2 inhibition a decrease of lymphatic vessels has been observed probably due to the effect of PgE2 on VEGF-C production[32]. Accordingly, in our study we measured a reduction in number of lymphatic vessel in the parietal peritoneum that can partially explain the higher UF (lower lymphatic reabsorbtion) measured in the Celecoxib treated PD animals. Rippe and Venturoli[31] showed that an increased deposition of collagen fibers in the interstitium appears to be very efficient in reducing UF capacity in a computer simulated model. Furthermore,

in a recent in vitro study Liu H. *et al*[12] demonstrated a positive effect of COX-2 inhibition in reducing the PgE2-mediated ECM production by human mesothelial cells exposed to PDF. Interestingly, in PDF+Celecoxib rats we observed a preserved UF associated to a significantly thinner submesothelial matrix and significantly lower PgE2 levels. Although fibrosis was clearly reduced, no effect of Celecoxib treatment was seen on the formation of spindle shaped cells within the mesothelial layer. In an earlier study[33] we have shown that these spindle shaped cells likely represent fibroblastic cells as a result of EMT. Our data confirms the findings of Aroeira et al[16] that Celecoxib treatment does not prevent EMT.

The water channel Aqp1 is considered as the ultra small pore of the peritoneal membrane and its pharmacologic modulation might influence water permeability and improve UF during PD. It has been shown that corticosteroids increase the expression of Aqp1 in the peritoneum with a significant increase in net UF[34]. However, no data are available about possible interactions between COX-2 and Aqp1. In our study Aqp1 expression was increased after glucose exposure in both PDF groups, but was not influenced by Celecoxib treatment, which makes it unlikely that a better UF was due to an increase in Aqp1 channels. Notably we localized Aqp1 not only in endothelium but also at mesothelial cell level confirming previous in vitro observations from other authors[35]. Collectively, our data thus suggest that improved UF by Celecoxib is most likely caused by reduced vascular surface area, reduced lymphatic absorption and reduced fibrosis.

Major concerns have been raised on cardiovascular safety of COX-2 inhibitors and the Adenoma Prevention trial with Celecoxib has been halted[36]. This condition seems to be common to all COX-2 inhibitor drugs[28] probably due to a great inhibition of PGI2 (vasodilator and anti-thrombotic prostaglandin) without any effect on platelet production (COX-1 mediated) of the pro-thrombotic prostaglandin tromboxane 2 when administered at high doses. Further studies focussing on the different Celecoxib target proteins will provide more specific interventions with hopefully minimum adverse effects on cardiovascular risk.

In conclusion, our results show that Celecoxib treatment reduced peritoneal tissue remodelling after PD exposure and preserved membrane UF capacity. Since Celecoxib shows many pleiotropic effects we could not identify one single mechanism of action in our study, although its anti-fibrotic and (lymph-)angiostatic properties seem to mediate its relevant effects. In the clinical setting, COX-2 inhibitors showed cardiovascular side effects making them difficult to use. A better comprehension of

the molecular mechanisms that regulate COX-2 induced processes is needed to reveal specific targets of intervention that are of clinical interest in preventing PD related side effects and eventually UF failure.

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