Experimental and clinical studies on peritoneal physiology and morphology during chronic peritoneal dialysis

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Vascular and interstitial changes in the peritoneum of CAPD patients with peritoneal sclerosis

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Abstract
To analyze morphological changes in the peritoneum of peritoneal sclerosis (PS) patients was the objective of this study. Emphasis was put on vascular abnormalities, because the continuous exposure to glucose-based dialysis solutions could cause diabetiform changes and because longitudinal transport studies suggested the development of a large peritoneal vascular surface area.

Peritoneal biopsies from CAPD patients were investigated in two studies. Diabetic patients were excluded. In study I, 11 PS biopsies were compared to 3 control groups, varying in duration of CAPD treatment: 0 months (n=15), 2-25 months (n=7) and ≥ 25 months CAPD (n=7). The second study was a case-control study, comparing 6 biopsies from the long-term control group to 6 PS biopsies, matched for age and duration of CAPD. All biopsies were scored for presence and type of fibrosis (Pico Sirius Red, type IV collagen, αSMA) and for neoangiogenesis (factor VIII). Thickening of vascular walls by type IV collagen and vasodilation of capillaries was measured by computer aided planimetry.

In study I the presence of sclerosing fibrosis, deposition of interstitial type IV collagen and the number of myofibroblasts (αSMA positive cells) were greater in the PS biopsies vs biopsies from all control groups (p<0.002). Moreover the number of vessels per field was higher in PS biopsies (p<0.01). Vascular wall thickening of small arteries (p<0.008) and a vasodilation of capillaries was found in PS biopsies compared to all control groups (p<0.007). The second study revealed differences in the presence of sclerosis but not in the extent of fibrosis between PS biopsies and their controls. The number of vessels per field in PS biopsies was higher compared to controls (p=0.04). Also thickening of the vascular wall was more marked in PS biopsies (p=0.03). Vasodilation of capillaries was greater in PS biopsies than in controls (p=0.07).

Fibrosis of the peritoneum may precede peritoneal sclerosis. The deposition of type IV collagen and the presence of myofibroblasts in the interstitial layer could be part of a pathological process, similar to the scarring in diabetic nephropathy. The neoangiogenesis and the thickening of the vascular wall by type IV collagen are consistent with glucose-induced microangiopathy. These abnormalities and the vasodilation of capillaries can explain the high D/P ratios or mass transfer area coefficients (MTACs) of low molecular weight solutes that can be found in long-term CAPD patients.

Introduction
Peritoneal sclerosis is a severe complication of long-term CAPD. It can occur during peritoneal dialysis, but also after discontinuation of treatment and is not directly related to the incidence of microbial peritonitis [1-3]. Sclerosing encapsulating peritonitis is the most severe manifestation. A characteristic finding at laparotomy in these patients is a thickened leathery, fibro-connective sheath of marbled appearance that envelopes the small intestine. Besides the clinical symptoms of bowel obstruction and ascites, ultrafiltration failure during peritoneal dialysis is the most important functional abnormality [1]. It is often accompanied by high dialysate/plasma ratios (D/P ratios) or high mass transfer area coefficients (MTACs) of low molecular weight solutes, suggesting the presence of a large vascular peritoneal surface area [1,4].
Alterations in the morphology of the peritoneum in CAPD patients have been described by the groups of Dobbie, Di Paolo and Gotloib [5-14]. These studies focussed especially on the mesothelium and also on mesothelial and interstitial changes related to peritonitis. Characteristic alterations of the peritoneum in CAPD patients with peritoneal sclerosis have mainly been described as a loss of mesothelial cells and a collagenous change, originating from the superficial layer of the peritoneum as an acellular rind of hyalinized collagen [5-7]. So far, little attention has been given to the vascular changes in long-term peritoneal dialysis patients and in patients with peritoneal sclerosis. Gotloib et al. described an irregular thickening and reduplication of the basement membrane of peritoneal capillaries using transmission electronmicroscopy [14]. Honda et al. described thickening of the media of venules with deposition of type IV collagen in three long-term CAPD patients [15].

The aim of the present study was to investigate vascular and interstitial changes in CAPD patients with peritoneal sclerosis and in patients treated with long-term CAPD. The analysis was especially focussed on abnormalities that could be attributed to exposure to constituents of dialysis solutions, e.g. glucose. Peritoneal biopsies from non-diabetic CAPD patients with peritoneal sclerosis were compared with biopsies from various control-groups. Different (immuno)histochemical staining methods were used to identify the presence and type of interstitial fibrosis (Pico-Sirius Red, type IV collagen, α-smooth muscle actin (α SMA)), thickening of the vascular wall (Pico-Sirius Red, type IV collagen), vasodilation of capillaries and neoangiogenesis (Von Willebrand factor). The severity of vascular wall thickening, vasodilation and of neoangiogenesis was quantitated by histomorphometry.

Patients and methods

Patients
Peritoneal biopsies were obtained from 11 patients with peritoneal sclerosis. The criteria for the diagnosis and the clinical presentation of the patients has been described by us previously [1]. In brief the definition of peritoneal sclerosis was based on a combination of clinical features (bowel obstruction, ascites and blood stained effluent often in combination with ultrafiltration failure) confirmed by either a laparotomy or autopsy. Median age of the peritoneal sclerosis patients was 45 years (range 27-69). Median duration of treatment with CAPD in this group was 63 months (40-131 months). Three different control groups of biopsies from CAPD patients were selected, that were different in the duration of CAPD. Patients with diabetes mellitus and patients suffering from a microbial peritonitis at the moment the biopsies were taken, were excluded. Control-group I consisted of 14 peritoneal biopsies from patients at the start of CAPD treatment (0 months CAPD). Median age of these patients was 46 years (range 21-58). Control-group II consisted of 7 biopsies from patients treated with CAPD from 2 to 25 months (median 13, range 2-20). Median age in this group was 42 years (range 32-59). The third control-group consisted of 7 biopsies from patients treated with CAPD for ≥25 months (median 45, range 25-83). Median age in group III was 61 years (range 30-72). Biopsies were obtained during laparotomy, (re)implantation or removal of the peritoneal catheter, during
autopsy and once during surgical correction of an umbilical hernia. Biopsies measuring 1-2.5 cm were taken partly of parietal peritoneum and partly of omentum. The results were analyzed in two ways. In study I the biopsies from the peritoneal sclerosis patients were compared to those of the three control groups. Study II was a case-control analysis comparing six biopsies from the peritoneal sclerosis group [median age 48 years (range 38-69), median duration CAPD 61 months (range 40-78)] to six biopsies from control group III [median 62 years (range 43-72), median duration of CAPD 51 months (range 25-83)], matched for age and duration of CAPD.

Histopathology
The biopsies were fixed in 4% formalin immediately after sampling. Paraffin embedded tissue was serially sectioned at 5 μm thickness. For histomorphometry sections were stained with haematoxylin & eosin and Pico Sirius Red F3B, providing a brick red staining of all fibrillar collagen. Adjacent sections were used for immunohistochemistry. For the immunohistochemical detection of endothelial cells, myofibroblasts and type IV collagen streptavidin-biotin complex (SABC) was used as previously described [16]. Briefly, sections were deparaffinized in xylene and alcohol (100%), followed by blockage of endogenous peroxidase by incubation with hydrogen peroxide 0.3% in methanol for 20 minutes. Sections stained with anti-collagen IV and anti-Von Willebrand factor required enzymatic pretreatment with pepsin 0.25% (Sigma, St. Louis, U.S.A.) in 0.01M HCl, at 37°C for 15 minutes. The staining sequence was: 10% normal goat serum in PBS for 15 minutes; primary antibodies α-smooth muscle actin (DAKO, Denmark; dilution 1:400 in PBS), anti-Von Willebrand factor (DAKO, Denmark; dilution 1:50 in PBS) and anti-collagen type IV (Organon Teknika, Netherlands; dilution 1:400 in PBS) for one hour; biotinylated rabbit anti-mouse F(ab’)2 fragments against polyclonal antibodies (both from DAKO, Denmark) in PBS containing 10% normal human serum (PBS-NHS) for 30 minutes; horseradish peroxidase (HRP)-conjugated streptABCComplex (DAKO, Denmark) in PBS-NHS for 30 minutes. HRP activity was detected by incubation in 1 mg/mL 3,3-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, U.S.A.) and H2O2 0.015% in 50 mM Tris-HCl, pH 7.6, yielding a brown colour. Finally slides were counterstained with Mayer’s haematoxylin and mounted with Pertsch mounting medium. Negative controls consisted of experimental tissues treated in a similar way but with substitution of the primary antibody with an irrelevant antibody of the same isotype.

Evaluation of results
I Extent of fibrosis (haematoxylin & eosin, Pico Sirius Red)
All peritoneal biopsies were scored for the same items. To describe the severity of interstitial fibrosis a semiquantitative score was used (0-3), where 0= no fibrosis, 1= mild fibrosis (mild perivascular and interstitial fibrosis), 2= moderate fibrosis (large areas but still of a discontinuous 'patchy' type of perivascular and interstitial fibrosis), 3= severe, non sclerosing fibrosis (extensive confluent diffusive type of interstitial fibrosis). Apart from the severity of fibrosis the presence of a sclerosing type of fibrosis (homogenous, hyaline like type of fibrosis) was scored.
II Collagen IV and αSMA immunoreactivity

In case of interstitial fibrosis, the presence or absence of type IV collagen within the fibrosis was assessed, using a similar semiquantitative score: 0 = absent, 1 = slightly detectable type IV collagen (collagen IV deposition distributed in the interstitial layer), 2 = clearly distinguishable type IV collagen (bandlike collagen IV deposition within the fibrous layer in the interstitium). Finally the presence or absence of αSMA in the interstitial layer was scored.

III Vascularization (anti-Von Willebrandfactor, anti-collagen IV) morphometric analysis

To count the number of vessels per field peritoneal sections were viewed down a light microscope (Leitz, Dialux 20) with a 10x flat field objective (x10). Per section five non-overlapping fields of 3.1 mm² from the upper left to the lower right of the biopsy, stained with von Willebrandfactor, were analysed throughout the length of the biopsy specimen in which all cross sectioned cuts of bloodvessels were counted. Finally the average number of vessels per field was calculated. The thickening of vascular walls was measured using the sections stained for type IV collagen and αSMA. Surface areas of peritoneal capillaries and small arteries were quantified by planimetry using TIM image analysis software on a PC provided with VS-100-AT frame grabber (Data Measuring Systems, Breda, The Netherlands). Sections were projected on a videoscreen and the inner and the outer border of the vessel were outlined manually. Subsequently values of total surface area and luminal area were generated by the above mentioned measuring system in μm². Utilizing these values the total
surface area, the crossectional area of the vascular wall, the wall/total area ratio and the wall/lumen ratio could be calculated. Ten capillaries (diameter 7-8 μm) and 10 arterioles/small arteries (diameter 20-50 μm) were measured per section.

**Statistics**
Analysis of variance was performed. When differences were significant, a Student-t-test was used to compare the PS biopsies with those of other groups, as a normal distribution of the data was present. A correction using the Bonferroni-method was made if necessary. To analyse possible associations between categorical data, the chi square test was used. The paired-t-test was employed to compare six peritoneal sclerosis biopsies with their matched controls.

**Results**

**Study I**
In control-group I (at start CAPD) 7 out of 15 biopsies contained fibrosis. Five out of these 7 contained mild fibrosis, 2 contained moderate fibrosis. In all biopsies from the second control-group (2-25 months CAPD) fibrosis was present. Four out of 7 biopsies in this group showed mild fibrosis, 3 of them showed moderate fibrosis. Fibrosis was more extensive in the third control-group (≥25 months CAPD). Three out of 7 biopsies in this group contained mild, 1 moderate and 2 biopsies contained severe fibrosis. In the peritoneal sclerosis group all biopsies contained a sclerosing type of fibrosis. The presence of interstitial type IV collagen was greater in biopsies with moderate fibrosis and was most marked in biopsies showing severe fibrosis (Figure 1). The number of interstitial myfibroblasts was significantly greater in the long-term CAPD group and was greatest in the peritoneal sclerosis group compared to the different control groups (p<0.04).

Figure 2 shows the number of vessels per microscopic field (x100) in each patient group. The number of vessels increased with the duration of peritoneal dialysis. An example is shown in Figure 3. A significantly higher number of vessels per field was found in the peritoneal sclerosis group compared to all different control-groups (p<0.01). The wall/total area ratio of the small arteries in the peritoneal sclerosis group was higher compared to all different control groups (p<0.001), which is consistent with a thickening of the vascular wall (Figure 4). In all cases the thickening could be seen in the media and was positively stained for type IV collagen (Figure 5). The lumen/wall ratio of peritoneal capillaries was also higher in the peritoneal sclerosis group, compared to the other groups (p<0.007), pointing to vasodilation.

Biopsies positively stained for αSMA (Figure 5C) had more severe interstitial fibrosis (p<0.01), more interstitial type IV collagen (p<0.02) and contained more vessels (p=0.001) than biopsies negatively stained for αSMA (Figure 6). The largest number of vessels was present in biopsies with severe interstitial fibrosis (Figure 7).
Figure 3A (left panel): omental tissue at the start of CAPD, anti-type IV collagen stain, x25. Figure 3B (right panel): omental tissue after 25 months of CAPD, anti-type IV collagen stain, x25.

Figure 4: Wall/total area ratio of small peritoneal arteries in the various patient groups. The values were higher the longer the duration of CAPD. Highest ratios were found in the group with peritoneal sclerosis compared to the various groups (p<0.001).

Study II
No marked differences in the presence or extent of fibrosis were found in peritoneal sclerosis biopsies compared to their controls. However a difference was found in the histological type of fibrosis, i.e. sclerosing fibrosis was more evident in the peritoneal sclerosis group. No interstitial type IV collagen was found in the control biopsies, while 5 out of 6 peritoneal sclerosis biopsies were positive for interstitial type IV collagen. The presence of interstitial myofibroblasts in the peritoneal sclerosis biopsies was not significantly different from their controls. Figure 8 shows the comparison between the number of vessels per field, the wall/total area ratio of the small peritoneal arteries and the lumen/wall ratio of peritoneal capillaries in the two patient groups. (Only five lines can be counted because in one of the tissue specimen of a matched control no small arteries were found. For all parameters peritoneal sclerosis patients had significantly greater values than their matched controls.
Discussion

Vascular and interstitial changes were investigated and quantified in the peritoneal tissues of CAPD patients with peritoneal sclerosis. It appeared that the number of microvessels was increased, as well as their wall thickness. Also capillary dilation was found. Deposition of type IV collagen and the presence of active fibroblasts, myofibroblasts, were the most striking changes in the peritoneal interstitium. All morphological abnormalities occurred in the absence of diabetes mellitus and in none of the cases peritonitis was present.

Loss of ultrafiltration is the major peritoneal transport abnormality in long-term CAPD patients and in those with peritoneal sclerosis [4,17-21]. In long-term PD, it is most commonly associated with high D/P ratios of low molecular weight solutes, leading to a rapid disappearance of the osmotic gradient. In peritoneal sclerosis patients the ultrafiltration loss can either be associated with low or high mass transfer area coefficients of low molecular weight solutes [1,4,22]. High MTACs or D/P ratios of e.g. creatinine suggest the presence of a large peritoneal vascular surface area with many pores available for solute transport. In the present study we focussed especially on morphological abnormalities that could explain these functional changes of the peritoneal membrane.

The vascular alterations found in the present study included an increase in the number of vessels with the duration of PD, which was most marked in the peritoneal sclerosis group. The high number of microvessels and the dilation of capillaries can therefore fully explain the observed functional changes in peritoneal solute transport characteristics. The cause of the capillary dilation described above is unclear. One could speculate whether the interstitial changes might cause some mechanical hindrance to bloodflow, leading to vasodilation.

The neovascularisation in peritoneal sclerosis patients bears similarity to the situation in diabetic retinopathy. Vascular endothelial growth factor (VEGF) has been demonstrated to play a key role in its pathogenesis [23-25]. Recently we found evidence for local peritoneal production of VEGF in CAPD patients [26]. The locally produced effluent VEGF showed a good correlation with the MTAC of creatinine. This suggests that VEGF is also involved in the peritoneal neoangiogenesis found in the present study. The pathological thickening of the vascular wall of small peritoneal arteries, consisting of type IV collagen, is in accordance with findings in a limited number of CAPD patients [15] and with alterations found in diabetic nephropathy [26-30]. In the latter extracellular matrix proliferation mainly consists of type IV collagen [31]. In vitro studies of Danne et al. and Woodrow et al. [32,33] showed an increased expression of type IV collagen in the glomerular basement membrane in diabetic nephropathy. Furthermore exposure of cultured calf glomerular mesangial cells to 25 mM D-glucose led to a stimulation of type IV collagen synthesis [32]. In a study from Phillips et al. the exposure of human renal tubular cells to glucose caused accumulation of type IV collagen [34]. All these findings support a pathogenetic role of glucose as a mediator in the pathological deposition of type IV collagen. Aging has been described to contribute to the deposition of type IV collagen in the basement membrane [28]. However the results of the present study are not likely to be caused by the effect of aging as the patients did not differ significantly in age in study I and in study II the controls were matched for age. A
significant difference in the vascular parameters still existed when peritoneal sclerosis patients were compared to controls, matched for age and duration of CAPD. The neovascularisation, the thickening of the media in small peritoneal arteries and the capillary dilation were most extensive in the peritoneal sclerosis group. This suggests an increased susceptibility to these alterations in the patients with peritoneal sclerosis. The interstitial abnormalities in long-term CAPD patients and in the peritoneal sclerosis group consisted of an increase in the severity of fibrosis in the interstitial layer. The peritoneal sclerosis group showed a diffuse type of sclerosing fibrosis as well. These findings are consistent with those of others [4-7, 9-11]. The presence of type IV collagen in the interstitial layer was also investigated. The collagen deposition in idiopathic peritoneal sclerosis mainly consists of type I and type III collagen. In the present study also an increase of interstitial type IV collagen was found in long-term CAPD biopsies and in peritoneal sclerosis. The presence of myofibroblasts in the peritoneal interstitium was another finding consistent with the development of peritoneal fibrosis. Myofibroblasts are activated fibroblasts that can express cytoskeletal proteins like αSMA [35] and are likely to be involved in the pathogenesis of interstitial fibrosis. The presence of myofibroblasts in the renal interstitium has been described to correlate with the deposition of interstitial collagen, arteriolar density and progression of renal failure in patients with diabetic nephropathy [36]. In a study from Essawy et al. interstitial αSMA in the kidney proved to be a predictor of progressive diabetic nephropathy [37]. The presence of myofibroblasts in the interstitium (positive staining for αSMA) in the present study was associated with an increase in the severity of fibrosis, an increase in the presence of interstitial type IV collagen as well as with an increase in the number of vessels per field. It could be speculated that all features are part of one pathogenetic process similar to the scarring in diabetic nephropathy, although myofibroblasts are not absolutely specific for diabetic nephropathy. Furthermore a relationship was present between the number of vessels and the degree of interstitial fibrosis. This makes the existence of a common pathogenetic factor in the development of these abnormalities in the peritoneum and the kidney not unlikely.

The diabetiform vascular and interstitial alterations in peritoneal tissues of long-term PD patients and PD patients with peritoneal sclerosis all point to a pathogenetic role of glucose or its degradation products in the pathogenesis of this process. This is further supported by the accumulation of advanced glycosylation end products (AGE) in the vascular wall of PD patients as described by Nakayama et al. [38]. These authors found a positive relationship between the amount of peritoneal AGEs and D/P ratios of various solutes. Another finding pointing to a pathogenetic role of glucose is the greater cumulative glucose exposure we found in peritoneal sclerosis patients compared to controls who were matched for the duration of CAPD [1]. In vitro studies [39,40] and studies in an animal model [41] also found that glucose was toxic to mesothelial cells, although the effect of low pH and lactate was more pronounced. However, in vivo the effect of low pH may be less important because the pH of the instilled solution increases rapidly and exceeds 7.0 already 7 minutes after instillation [42]. In contrast to the in vitro studies, the exposure to high glucose concentrations in CAPD is continuous.
Peritoneal sclerosis: vascular and interstitial changes

Figure 5: Figure 5A (left panel): Thickened vascular wall of a small artery in a parietal peritoneum section of a patient with peritoneal sclerosis stained with Pico Sirius Red. In this staining method, all fibrillar types of collagen are positively stained. The inner vascular wall is not stained. A diffuse type of sclerosing fibrosis can be seen at the side of the peritoneal cavity, the upper part of the picture (x25). Figure 5B (middle panel): same section, stained for type IV collagen. The inner vascular wall is stained positively for interstitial type IV collagen at the upper part of the picture (x25). Figure 5C (right panel): same section, stained for αSMA. Especially the layer of sclerosing fibrosis is stained positively for αSMA, representing the presence of myofibroblast.

Figure 6 (left panel): Fibrosis in biopsies stained positively for interstitial αSMA (myofibroblasts) was more severe compared to fibrosis in biopsies stained negatively for interstitial αSMA (0.01<p<0.001) (open: no fibrosis, dashed: mild fibrosis, hatched: moderate fibrosis, solid: severe fibrosis). Figure 6B (middle panel): Presence of interstitial type IV collagen in biopsies stained positively for interstitial αSMA was greater compared to biopsies negatively stained for interstitial αSMA (0.02<p<0.05) (open: type IV collagen absent, dashed: type IV collagen slightly detectable, hatched: type IV collagen obviously present). Fig 6C (right panel): The number of vessels per field in biopsies positively stained for interstitial αSMA was greater compared to biopsies negatively stained for interstitial αSMA (p=0.001).
Figure 7. The number of vessels per field in biopsies, according to the severity of fibrosis. The number of vessels per field was highest in the group with severe fibrosis, compared to the other groups (p<0.007).

Previous studies mainly reported on parietal peritoneum. In the present study parietal peritoneum as well as omentum was investigated. This was done because material from the same specified site was not always available for this retrospective analysis. However similar changes were found in both parietal peritoneum and omentum. This is not a surprising finding as the whole peritoneal membrane is likely to be exposed to the same extent. The mesothelial layer was not investigated as this has already been done extensively by others [7-11]. Furthermore the tissue specimens, especially those of the parietal peritoneum, had often lost a major part of their mesothelial layer, probably due to the extraction itself.

The morphological alterations found in peritoneal sclerosis patients were not principally different from those in long-term CAPD patients, but much more extensive. This fits in the hypothesis that peritoneal sclerosis may be the end stage of a more general pathological process in the peritoneal membrane. The morphological abnormalities explain the functional changes in peritoneal transport. Glucose and/or its degradation products are likely to be important in the pathogenesis of these alterations. However, a contributing role of previous peritonitis episodes cannot be excluded [43]. An animal model is needed to provide more insight into the pathogenesis of morphological as well as functional changes in long-term CAPD and peritoneal sclerosis.
Peritoneal sclerosis: vascular and interstitial changes

Figure 8A (upper panel) the number of vessels per field in peritoneal sclerosis biopsies was higher compared to their matched controls (p=0.04). Figure 8B (middle panel) the wall/total area ratio of small arteries in peritoneal sclerosis was higher compared to their matched controls (p=0.03). Only five lines can be counted because in one of the tissue specimen of the matched controls no small arteries were found. Figure 8C (lower panel) the lumen/wall ratio of capillaries was higher compared to their matched controls (p=0.07).
References
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