Encapsulating peritoneal sclerosis and other aspects of long-term peritoneal dialysis
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Chapter 8

A TWO-HIT APPROACH IN THE DEVELOPMENT OF AN EXPERIMENTAL PERITONEAL SCLEROSIS MODEL

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Submitted
Abstract

Background Models of encapsulating peritoneal sclerosis (EPS) are often based on local administration of chemical irritants. Our aim was to develop a clinically relevant “two-hit” model with incorporation of renal failure and exposure to conventional dialysis solutions.

Methods Thirty-six male Wistar rats underwent a catheter implantation and a 70% nephrectomy. They were randomly divided into 3 peritoneal infusion groups. The experimental group was exposed to a 3.86% glucose-based conventional dialysis solution for 8 weeks and then received a second hit of intraperitoneal blood administration. Two weeks later the rats were sacrificed. Two control groups were exposed to the conventional dialysis solution alone or to a buffer without glucose for 8 weeks. All animals underwent a peritoneal function test at the end of the experiment. The number of peritoneal adhesions was counted at autopsy, and omental tissue was obtained for morphometrics.

Results The rats that received blood as a second hit had developed numerous intraperitoneal adhesions as seen in EPS, but without cocoon formation. Microscopically no differences were present in fibrosis scores and vessel counts between the three groups. Also peritoneal function parameters were similar in all groups.

Conclusion The short infusion period could be the reason that we did not find differences between the groups, with the exception of a large amount of intraperitoneal adhesions in the experimental group. Modifications of the described rat model are required to develop a clinically relevant EPS model. Besides renal failure and long-term exposure to bioincompatible peritoneal dialysis solutions, a different second hit or several additional hits could be incorporated in an experimental model of EPS.
A two-hit approach in the development of an experimental peritoneal sclerosis model

Introduction

Encapsulating peritoneal sclerosis (EPS) is the most severe complication of long-term peritoneal dialysis (PD) in which bowel loops are entrapped in a cocoon of fibrous tissue. Because of the devastating consequences of EPS for the patient it remains a topic of great interest. Several rodent models of EPS have been developed over time. In a recently published murine model, EPS was established with a sophisticated technique using helper-dependent adenoviruses that express transforming growth factor-beta (TGF-ß1). Previously described models have frequently been based on intraperitoneal administration of chemical irritants. In a recent study by our group, we attempted to develop a clinically relevant model of peritoneal sclerosis based on renal failure and exposure to a bioincompatible PD solution in combination with a low dose of chlorhexidine gluconate. In this study, the usage of chlorhexidine gluconate overshadowed the possible effects of the bioincompatible PD solution. Functional abnormalities seen in EPS were accomplished but without encapsulation of the bowels. We concluded that experimental models of simple peritoneal sclerosis and EPS should not involve the usage of chlorhexidine gluconate.

Long-term exposure to bioincompatible glucose-based dialysis solutions is a risk factor for the development of EPS. The “two-hit theory” described by Kawanishi et al. provides a sensible basis for the development of an EPS model. In this theory, exposure to a bioincompatible PD solution is considered the first hit. One of the clinical signs of EPS is the presence of blood-stained ascites. Blood can enhance the formation of adhesions and could therefore serve as a second hit. The concept of using blood in experimental models to induce EPS is not new. In one study, an intraperitoneal injection with bleach to cause a chemical peritonitis followed by an injection of blood one week later resulted in cocoon formation of the bowels in rats. Another model in which intraperitoneal blood was administered, 5 mL of whole blood in combination with TGF-ß1 after exposure to a PD fluid for 5 weeks, showed the development of adhesions, but not EPS. Modification of this model, by prolonging the PD duration and increasing the volume of blood that is administered as a second hit, could potentially lead to the development of a new EPS model. The incorporation of renal failure in the model, because uremia itself can induce both fibrotic and vascular changes of the peritoneum in animals and patients, could bring new insights in the mechanisms leading to EPS. The aim of our study was to develop such a model.
Subjects and methods

Experimental groups
Thirty-six male Wistar rats underwent a catheter implantation and nephrectomy as described in the next paragraph. They were randomly divided into three groups (n = 12 per group). The experimental group was exposed to Dianeal (3.86% glucose; Baxter Healthcare, Castlebar, Ireland). This glucose-based bioincompatible dialysis solution was the first hit in the model. As a second hit, we administered blood obtained by heart puncture of male Wistar donor rats intraperitoneally after the infusion period. Two weeks later they were sacrificed. One control group received Dianeal alone, and the other control group received a biocompatible, glucose-free buffer (Physioneal without glucose; Baxter, Nivelles, Belgium). All three groups received 30 mL preheated and heparinized (5 IU/mL) dialysis solution on normal week days, 10 mL in the morning and 20 mL in the afternoon, and only one infusion of 20 mL during the weekends.

Peritoneal infusion model in rats with chronic renal failure
As described previously in detail\textsuperscript{11,14}, the basis of our study was a peritoneal infusion model in rats with chronic renal failure. In short, the model consists of a closed catheter system attached to an access port in the neck (Access Technologies; Norfolk Medical, Skokie, IL, USA), a one-step 70% nephrectomy to induce renal failure, and, in this case, 8 weeks of peritoneal exposure. The rats received antibiotic prophylaxis (enrofloxacin 0.02 mL/100 g body weight subcutaneously) perioperatively during both procedures to prevent peritonitis\textsuperscript{15}. They were housed in groups under standardized conditions and fed a nephroprotective diet ad libitum (20% casein, 0.7% calcium, 0.42% phosphorus, 0.57% potassium, and 0.29% sodium; Hope Farms, Woerden, The Netherlands) to avoid too rapid decline of residual renal function\textsuperscript{16,17}. Blood samples were drawn under isofluran anesthesia every 2 weeks by a tail vein punction to monitor renal function.

After 8 weeks of exposure, a standard peritoneal permeability analysis adapted for the rat (SPARa) was performed\textsuperscript{18}. A volume marker, dextran 70 (Hyskon; Medisan Pharmaceuticals AB, Uppsala, Sweden) was added to the test solution, a 3.86% Physioneal solution (Baxter Healthcare, Dublin, Ireland). All SPARa’s were performed during a 4-hour dwell. This peritoneal permeability test is comparable to those in humans and provides information on peritoneal transport characteristics\textsuperscript{19}. Prior to the SPARa, urine was collected in a metabolic cage for 24 hours to calculate residual renal function. Afterwards, the number of intraperito-
neal adhesions was assessed at autopsy and omental tissue was collected. The protocol was approved by the committee on animal experiments of our center and national guidelines concerning animal research were followed.

**Assays**

Various parameters were determined in dialysate, plasma and urine. Urea was determined with an enzymatic method on an automated analyzer (Hitachi H747; Boehringer Mannheim, Germany). Creatinine was measured with an enzymatic method on another automated analyzer (Hitachi H911; Boehringer Mannheim, Germany). The glucose concentration was assessed by the glucose oxidase-peroxidase assay (SMA II; Technicon, Terrytown, NJ, USA). Sodium was measured with an indirect ion selective electrode (Hitachi H747).

**Calculations**

Renal function was assessed by creatinine and urea clearances using 24-hour urine samples obtained in the metabolic cage, and plasma samples obtained prior to the SPARa. Peritoneal small solute transport was expressed as the mass transfer area coefficient (MTAC) of urea according to Waniewski et al.\(^2\), and the percentage of glucose absorption. To assess glucose absorption, the amount of glucose in the dialysate at the end of the SPARa was subtracted from the amount of glucose at the start of the SPARa, relative to the instilled quantity of glucose. Peritoneal function parameters (net ultrafiltration rate and transcapillary ultrafiltration rate) were calculated as described previously\(^1\). Free water transport was expressed as the dip of D/P sodium during the SPARa\(^1\).

**Morphometric analysis**

Omental tissue obtained after the SPARa was fixed in 4% buffered formalin and embedded in paraffin. Omental tissue was stained with picro-sirius red (PSR; Gurr, BDH, UK) to assess the amount of fibrosis. Submesothelial, intersegmental, and perivascular areas were judged on the presence of fibrosis (0 = normal presence of fibrous tissue, 1 = mild fibrosis, 2 = moderate fibrosis and 3 = severe fibrosis). The maximum fibrosis score possible was 9. A second staining of omental tissue was a vessel staining with platelet endothelial cell adhesion molecule-1 antibody (PECAM-1, goat-anti-rat CD31, Santa Cruz, California, USA). With help of computer-aided morphometrics, the vessels were counted in five microscopic fields, of which the average was calculated. A digital camera (Leica DFC500, Leica Microsystems,
Wetzlar, Germany; software Image Pro Plus, version 5.01, Media Cybernetics, Silver Spring, MD, USA) was connected to a light microscope (Leica 5000MB) with a $\times$ 20 flat-field objective ($\times$ 10 ocular). One computer-aided field represented $440 \mu m \times 331 \mu m$ of omental tissue. Five non-overlapping fields from the upper left to the lower right of the section were scored. Hematoxylin stainings that colour nuclei of cells blue were also performed on omental tissue (HE; Klinipath, Duiven, The Netherlands).

**Statistical analysis**

Data are presented as medians and ranges, unless stated otherwise. Possible differences between the three groups were assessed with a Kruskall-Wallis test.

**Results**

In each group, two rats dropped out of the study due to catheter or nephrectomy related problems. The remaining 30 rats underwent a SPARa. One rat from the buffer group turned out to have worse renal impairment than the others which was a reason to exclude this rat. Renal urea clearance (0.71 (0.30-0.98) mL/min) and creatinine clearance (1.80 (1.18-2.26) mL/min) of the other rats were comparable to our previous studies in rats with renal failure, corresponding to stage III chronic kidney failure in humans. Eight rats did not complete the 4 hour SPARa, probably due to too severe hypovolemia. Therefore, Table 1 shows peritoneal transport characteristics of 21 rats. Free water transport, expressed as dialysate-to-plasma ratio (D/P) of sodium during SPARa, is presented in Figure 1. No differences were present in parameters of peritoneal solute and fluid transport between the groups.

### Table 1

<table>
<thead>
<tr>
<th>Peritoneal transport parameters measured during the SPARa</th>
<th>Dianeal + hit (n = 8)</th>
<th>Dianeal (n = 7)</th>
<th>Buffer (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTAC urea ($\mu$L/min)</td>
<td>340 (305-404)</td>
<td>262 (193-411)</td>
<td>345 (238-405)</td>
</tr>
<tr>
<td>Glucose absorption (%)</td>
<td>63 (58-73)</td>
<td>65 (56-74)</td>
<td>62 (55-67)</td>
</tr>
<tr>
<td>TCUFR ($\mu$L/min)</td>
<td>83 (76-96)</td>
<td>72 (67-82)</td>
<td>80 (74-87)</td>
</tr>
<tr>
<td>NUFR ($\mu$L/min)</td>
<td>64 (46-78)</td>
<td>59 (33-76)</td>
<td>64 (46-77)</td>
</tr>
</tbody>
</table>

SPARa = standard peritoneal permeability analysis adapted for the rat; buffer = Physioneal without glucose; MTAC = mass transfer area coefficient; TCUFR = transcapillary ultrafiltration rate; NUFR = net ultrafiltration rate. All solutions were provided by Baxter. Data are expressed as median (range).
TABLE 2

Macroscopic adhesions, microscopic omental fibrosis and the number of blood vessels

<table>
<thead>
<tr>
<th></th>
<th>Dianea + hit (n = 10)</th>
<th>Dianea (n = 10)</th>
<th>Buffer (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macroscopic adhesions</td>
<td>4 (2-7)</td>
<td>1 (0-2)</td>
<td>1 (0-1)</td>
</tr>
<tr>
<td>Over-all fibrotic score</td>
<td>5 (2-7)</td>
<td>5 (1-7)</td>
<td>4 (3-6)</td>
</tr>
<tr>
<td>Number of vessels per field</td>
<td>34 (14-61)</td>
<td>37 (16-48)</td>
<td>31 (17-47)</td>
</tr>
</tbody>
</table>

Buffer = Physioneal without glucose. All solutions were provided by Baxter. The number of macroscopic adhesions was counted at autopsy. The scoring of fibrosis was done in submesothelial, intersegmental and perivascular areas in picro-sirius red-stained omental tissue. Maximum score per area was 3, leading to a maximum over-all score of 9. Also, the mean amount of vessels of vessels of five microscopic fields in platelet endothelial cell adhesion molecule-1 antibody-stained omental tissue was assessed. Data are expressed as median (range). Significant difference is marked: *p < 0.001, Dianea + hit versus both control groups.
Figure 2 – An example of macroscopic adhesions seen at autopsy of a rat that was first exposed to Dianeal for 8 weeks and then received blood of a donor rat intraperitoneally as a second hit.
A two-hit approach in the development of an experimental peritoneal sclerosis model

Autopsy and peritoneal tissue collection was performed in 29 rats. The groups showed similar fibrosis scores and very high vessel counts, but the experimental group that received blood intraperitoneally had more intraperitoneal adhesions (Table 2). Figure 2 shows an example of these adhesions seen at autopsy. The adhesions in the experimental group were also thicker than those observed in both control groups. Actual encapsulation of the bowels did not develop. The presence of inflammation was arbitrarily judged on hematoxylin stained omental tissue. In all three groups, inflammatory cells and infiltrates were frequently observed but tissue sections without signs of inflammation were also seen (Figure 3).
Discussion

This attempt to develop a new experimental model in which clinically relevant risk factors for EPS are incorporated did not lead to full-blown EPS. Although the severe adhesions that were seen in the experimental “two-hit” group might cause bowel obstruction, no cocoon formation was present. Moreover, no differences were found in peritoneal transport, fibrosis scores, and vessel counts between rats that were exposed to a biocompatible glucose-free buffer and rats that were exposed to Dianef for 8 weeks. We were especially puzzled by the high vessel counts in the buffer group, since these results differ from previous studies by our group in rats that were exposed to a glucose-free buffer for 16 weeks.

The most likely explanation is an inflammatory reaction provoked by the presence of a peritoneal catheter as suggested by Flessner et al. in two studies. In the first study, rats were injected daily for 8 weeks with a solution containing a low concentration of glucose degradation products via needle injection (NI) or via an intraperitoneal catheter injection (CI). The CI group showed more angiogenesis than the NI group. Also other inflammatory changes in peritoneal structure and function were found. A similar effect of the peritoneal catheter was observed in a second, very recent study by the same group. This effect was still detectable after 20 weeks of exposure, but much less pronounced than after 4 weeks. This is in line with unpublished findings by our group, also showing an acute inflammatory reaction after 4 weeks administration of various dialysis solutions. A temporary effect of catheter implantation has been found in PD patients as well. In contrast, after long-term (at least 16 weeks) exposure to dialysis solutions through a catheter in our experimental studies, marked differences in peritoneal morphology have been found between conventional and more biocompatible dialysis solutions, favouring the latter.

In the present study, we think that the duration of 8 weeks of exposure was too short to find a difference between the conventional dialysis solution and the buffer because the presence of a peritoneal catheter induced an inflammatory state in all three groups. We acknowledge that it is possible that a catheter effect is still present after long-term exposure to dialysis solutions. Nevertheless, the presence of a peritoneal catheter mimics the clinical situation of a PD patient who might also suffer from a chronic foreign body reaction. Of course biocompatibility of peritoneal access devices should be strived for, not only for experimental use but also for clinical purposes.

We induced renal failure in all animals, since previous research has shown that uremia per se leads to functional and morphological alterations of the peritoneal membrane.
To better understand mechanisms leading to EPS, further modifications of the described rat model with renal failure and exposure to conventional PD solutions are required. We speculate that future studies should be performed in long-term peritoneal infusion models to avoid results from being influenced by an acute catheter effect. Perhaps a different second hit, or several additional hits when regarding the development of EPS as a multifactorial process, should be applied.
References

A two-hit approach in the development of an experimental peritoneal sclerosis model


