Understanding and preventing the peritoneal damage caused by conventional dialysis solutions
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Chapter 4

**ALPHA-2-MACROGLOBULIN AND ALBUMIN ARE USEFUL SERUM PROTEINS TO DETECT SUBCLINICAL PERITONITIS IN THE RAT**

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Abstract

Background

In experimental PD studies the occurrence of peritonitis is a confounder in the interpretation of effects of chronic peritoneal exposure to dialysis solutions. Since in most experimental PD models in the rat fluid cannot be drained, it is impossible to diagnose peritonitis based on dialysate white blood cell counts. To study the value of serum markers for the presence of peritonitis, alpha-2-macroglobulin (a2M) and albumin were measured in rats with and without peritonitis after chronic exposure to dialysis solutions. To further investigate the time course of these markers in relation to the severity of peritonitis, non-dialyzed rats were challenged with increasing numbers of bacteria and followed for 28 days.

Methods

In the first study a2M and albumin were measured in rats exposed to glucose/lactate based dialysis fluid before sacrifice. A comparison was made between animals with peritonitis, as judged from the presence of extensive infiltrates after sacrifice (gold standard) and/or clinical signs of peritonitis, or absence of peritonitis and infiltrates. In the second study rats were intraperitoneally (i.p.) injected with three different concentrations of Staphylococcus aureus and serum a2M and albumin were measured at various time points.

Results

In the first study serum a2M was higher and serum albumin was lower in animals with peritonitis compared to animals without peritonitis (both p<0.05). In the second study induction of a2M was clearly dependent on the inoculum concentration. Peak values of a2M were found at day 1 and 3. At all time points after inoculation a2M was higher in all injected groups compared to the control group. Serum albumin values decreased in the highest inoculum group and remained decreased until 28 days after i.p. injection. Despite a low sensitivity, serum a2M>40 mg/L and albumin <32 g/L had a specificity for peritonitis of 100%.

Conclusions

Measurement of a2M and albumin once a month is an additional tool in the diagnosis of silent peritonitis in the chronic peritoneal exposure model in the rat. a2M>40 mg/L and albumin <32 g/L are strong indicators for peritonitis. However, normal values do not exclude infectious peritonitis.
INTRODUCTION

Animal models of peritoneal dialysis (PD) are essential to study the effects of experimental interventions, during exposure to dialysis fluids, on structural and functional changes in the peritoneum [1-4]. Long-term PD in patients, as well as chronic peritoneal exposure to dialysis fluids in animals, are associated with structural changes, including angiogenesis and fibrosis [5]. These changes however, can be influenced by peritonitis, although the effect of peritonitis on long-term changes of the peritoneum is controversial [6]. Effects of drug interventions and exposure to different dialysis solutions in chronic peritoneal exposure models in the rat are mostly investigated in animals without peritonitis [7]. If a rat develops clinical signs of peritonitis during the experimental period, like an obstructed catheter, weight loss, failure to thrive, porphyrin staining or ocular discharge, the animal is euthanized and removed from the experiment. However, sometimes after completion of the experiment, peritoneal biopsies show extensive infiltrates even though there were no clinical signs of peritonitis during the whole experimental period. In our chronic peritoneal exposure model PD fluid cannot be drained, which makes it impossible to perform white blood cell counts as in the human situation to diagnose peritonitis. The aim of the study was therefore to investigate whether serum proteins could be useful to detect subclinical episodes of peritonitis in rats and thereby prevent useless continuation of chronic peritoneal exposure. Two studies were performed. In the first study serum alpha-2-macroglobulin (a2M) and albumin were measured in animals chronically exposed to dialysis fluid, with and without peritonitis. In the second study rats were intraperitoneally (i.p.) injected with various concentrations of *Staphylococcus aureus* and serum a2M and albumin were measured at various time points.
SUBJECTS AND METHODS

ANIMALS

STUDY 1
Twenty-nine male Wistar rats (HSD, Harlan, Zeist, The Netherlands) with an average body weight of 300 g were included in this experiment. A peritoneal catheter (Rat-o-Port, MTINC, 71S, Access Technologies, Norfolk Medical, Skokie, IL, USA) was implanted subcutaneously as described previously [7]. Daily intraperitoneal infusion of 20 ml of Dianead 3.86% (Baxter, Castlebar, Ireland) was performed by puncture of the subcutaneous device implanted in the neck of the rat, using a 0.6 mm wing-end infusion set (microperfuseur, Vygon, France). No drainage can be applied in this model. The rats were sacrificed after the experimental period of at least 8 weeks had ended. a2M and albumin were determined in serum samples obtained directly before sacrifice. After sacrifice, the abdomen was opened, cultures from the catheter tips were performed, and peritoneal tissue (the omentum, which is distant from the catheter tip and not in direct contact) obtained. The animals were divided as follows: group 1 (n=10) consisted of rats with clinical (weight loss, obstructed catheter) and histological (extensive infiltrates in all peritoneal biopsies) signs of peritonitis. Group 2 (n=7) consisted of rats without clinical signs but with definite histological signs of inflammation, and group 3 (n=12) consisted of rats without clinical and histological signs of inflammation.

STUDY 2
Thirty-two male Wistar rats (HSD, Harlan, Zeist, The Netherlands) with an average body weight of 200 g were randomly assigned to 4 groups of 8 animals each. Blood was drawn the day after arrival (day -7). After one week (day 0) three groups of rats received a single intraperitoneal injection with 0.5 ml of a suspension containing either 1x10^4 colony forming units (CFU, group 1), 1x10^6 CFU (group 2) or 1x10^8 CFU (group 3) of Staphylococcus aureus ATCC 25923 (ATCC, Manassas, USA), as described previously [8]. An untreated control group (group 4) was also included. On days -7, 1, 3, 7, 14 and 28, blood was collected from the tail artery under light ether anaesthesia. Serum samples were analysed for a2M and albumin. The animals were sacrificed after the collection of the last blood sample. Omental tissues were dissected and spread on a glass slide for light microscopical examination.

Rats from both studies received standard chow and water ad libitum. Both studies were performed in accordance with the regulations required by the local ethics committee for animal experimentation.

ASSAYS

Albumin was measured with the bromcresol green method using an autoanalyzer (Modular P, Roche GmbH Mannheim, Germany). a2M concentrations were determined with a peroxidase sandwich enzyme-linked immuno assay (ELISA). ELISA plates (Maxisorp immunoplate,
NUNC, Roskilde, Denmark) were coated with rabbit anti-rat-a2M. Biotin labelled rabbit anti-rat a2M was applied as conjugate. Streptavidin horseradish peroxidase (CLB, Amsterdam, The Netherlands) was used for peroxidase labelling and o-phenylenediamine dihydrochloride (Sigma, St Louis, MO, USA) as substrate. Absorbance was read at 490 nm. Wistar rat purified a2M was used as a standard [9]. Sensitivity for a2M was 5 µg/L. With regard to cross reactivity the polyclonal antibodies reacted non-specific with a2M in serum and liver homogenates and inter- and intra-assay variation was less than 5%.

Histopathology

Omental tissue and parietal peritoneum were stained with haematoxylin-eosin and toluidine blue in study 1 and assessed for the presence of infiltrates and the number of mast cells by conventional light microscopy. Omental stretch preparations from study 2 were stained with toluidine blue. The number and the size of the omental milky spots, reflecting the activation state of the omentum, were determined by light microscopy, using a scored eyepiece (Figure 3). Twenty-five random areas of 4 mm² (total 1 cm²) were scored and the number and mean surface area of milky spots were determined.

Statistical Analyses

Medians and interquartile ranges are given unless stated otherwise. Analysis of variance was performed where after Mann-Whitney U tests were done to investigate differences between groups. Sensitivity and specificity were calculated based on literature cut off points of 40 mg/L for a2M [10] and 32 g/L for albumin [11].

Results

Study 1

Serum a2M was higher in group 1 and 2 compared to the non-peritonitis group: group 1: 33.8 (19.2-289) mg/L, group 2: 27.9 (20.6-200) mg/L, group 3:15.5 (12.7-18.4) mg/L, p<0.01). No difference was present between groups 1 and 2 (Figure 1a). It appeared that high serum a2M levels were predictive of peritonitis, even in the absence of clinical signs. However, a normal value did not exclude peritonitis (Figure 1a). Animals in group 1 had slightly lower albumin values than the controls: 35 (29-38) vs 38 (36.5-40.5) g/L, p<0.05. The sensitivity and specificity of a2M and albumin for the prediction of peritonitis is given in Table 1. Both proteins had low sensitivity but the specificity was 100%. All animals in group 1 and 2 had positive cultures, all rats in the control group negative cultures except three animals. These animals had cultures positive for E. Coli and Gram negative rods but had no histological signs of peritonitis. The animals with positive cultures, infiltrates and clinical signs of peritonitis had an average number of 10.5 (1-45) mast cells per omental field, compared to 5 (1-11) in the animals with positive
cultures but no other signs of peritonitis (p=0.05) and 7 (0–36) in the animals without peritonitis (p=0.2).

Figure 1: The upper panel shows serum alpha-2-macroglobulin values, the lower panel shows serum albumin values from rats exposed to peritoneal dialysis fluid (study 1). Group 1: rats with clinical and histological signs of peritonitis (n=10), group 2: rats without clinical signs but with histological signs of inflammation (n=7), group 3: rats without clinical signs and histological signs of inflammation (n=12). Data are expressed as box and whisker plots: the boxes represent the median, and the 25th and 75th percentiles, the whiskers the extremes. Significant differences (p<0.05) with group 3 are indicated by an asterisk.
Table 1: Sensitivity and specificity (%) of serum albumin for the presence of peritonitis.

<table>
<thead>
<tr>
<th>Albumin cut-off</th>
<th>Day 14</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>26 g/L</td>
<td>sens 0</td>
<td>sens 5</td>
</tr>
<tr>
<td></td>
<td>spec 100</td>
<td>spec 100</td>
</tr>
<tr>
<td>28 g/L</td>
<td>sens 0</td>
<td>sens 25</td>
</tr>
<tr>
<td></td>
<td>spec 86</td>
<td>spec 100</td>
</tr>
<tr>
<td>30 g/L</td>
<td>sens 9.5</td>
<td>sens 30</td>
</tr>
<tr>
<td></td>
<td>spec 71</td>
<td>spec 100</td>
</tr>
<tr>
<td>32 g/L</td>
<td>sens 33</td>
<td>sens 62</td>
</tr>
<tr>
<td></td>
<td>spec 71</td>
<td>spec 100</td>
</tr>
<tr>
<td>34 g/L</td>
<td>sens 71</td>
<td>sens 75</td>
</tr>
<tr>
<td></td>
<td>spec 43</td>
<td>spec 43</td>
</tr>
</tbody>
</table>

Study 2
Three rats in group 3 did not survive the high load of bacteria. Two of them died overnight and one was sacrificed the day after the injection. In all groups weights increased during the experiment. The increase was less steep the first days after i.p. injection with S. aureus and a temporary decrease was present in the highest dosage group. The increase in weight during the experiment was not different between groups 1 and 4, but it was different between group 2 and 4: 135 (129–153) vs 154 (146–158), p=0.03, and between group 3 and 4 (group 3: 138 (130–149), p=0.03).

Table 2: Sensitivity and specificity (%) of serum alpha-2-macroglobulin for the presence of peritonitis.

<table>
<thead>
<tr>
<th>A2M cut-off</th>
<th>Day 14</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>36 mg/L</td>
<td>sens 90</td>
<td>sens 81</td>
</tr>
<tr>
<td></td>
<td>spec 75</td>
<td>spec 75</td>
</tr>
<tr>
<td>38 mg/L</td>
<td>sens 71</td>
<td>sens 62</td>
</tr>
<tr>
<td></td>
<td>spec 75</td>
<td>spec 75</td>
</tr>
<tr>
<td>40 mg/L</td>
<td>sens 43</td>
<td>sens 14</td>
</tr>
<tr>
<td></td>
<td>spec 100</td>
<td>spec 100</td>
</tr>
<tr>
<td>42 mg/L</td>
<td>sens 71</td>
<td>sens 52</td>
</tr>
<tr>
<td></td>
<td>spec 88</td>
<td>spec 88</td>
</tr>
<tr>
<td>44 mg/L</td>
<td>sens 62</td>
<td>sens 48</td>
</tr>
<tr>
<td></td>
<td>spec 88</td>
<td>spec 88</td>
</tr>
</tbody>
</table>

The serum concentrations of a2M and albumin are summarised in Table 2. At all times after inoculation a2M was higher in all injected groups compared to the control group (Figure 2a). The values were clearly dependent on the dose of the inoculum. The peak concentrations were found at day 3. In groups 1, 2, and 4, serum a2M levels were higher at day -7 than at day 28. This might have been caused by a stress response brought about by transportation of the
animals, because day -7 was one day after the animals arrived at the laboratory. Therefore data are presented as percentages of the mean values of group 4 at various days in Figure 2. Serum albumin only showed a decrease at day 3 and 7 in the highest inoculum group (Table 2, Figure 2b). The sensitivity and specificity of a2M and albumin are shown in Table 1. Despite low values for sensitivity, the specificity of the two proteins for the presence of peritonitis was 100%, except for albumin in study 1 at day 14 (71%).

**Figure 2a:** Serum A2M values from the injected groups, expressed as percentage of the mean value of the animals from group 4 at various days after i.p. injection. Group 1: diamonds, group 2: squares, group 3: triangles.

**Figure 2b:** Serum albumin values from the injected groups (n=8 for group 1 and 2 and n=5 for group 3), expressed as percentage of the mean of the animals from group 4 at various days after i.p. injection. Group 1: diamonds, group 2: squares, group 3: triangles.
Histopathology

Since the number and/or size of omental milky spots (leucocyte aggregates) represents the extent of peritoneal activation, these spots were quantified in toluidine blue stained stretch preparations. Omental tissues of group 3 were highly activated: number and size of the milky spots were enormously increased resulting in unrecognizable individual milky spots. Only in tissues from 2 of the 5 animals individual milky spots could be counted. Therefore quantification was impossible in group 3. The number of milky spots per cm² was not different between groups 1, 2 and 4, but the mean size of the milky spots was lower in group 4 compared to the other groups. Group 1: 0.10 (0.09–0.13) cm², group 2: 0.12 (0.09–0.15) cm², compared to group 4: 0.07 (0.07–0.09) cm², both p<0.05.

Table 3: Serum a2M and albumin levels from rats before (day -7) and after i.p. injection of Staphylococcus aureus. Group 1: injected with 10⁴ colony forming units (CFU), group 2: 10⁶ CFU, group 3: 10⁸ CFU and group 4: controls

<table>
<thead>
<tr>
<th></th>
<th>Day -7</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>a2M (mg/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 1</td>
<td>63 (48-71)</td>
<td>61 (55-65)</td>
<td>65 (54-68)</td>
<td>41 (38-44)</td>
<td>39 (35-41)</td>
<td>26.5 (25-27)</td>
</tr>
<tr>
<td>Group 2</td>
<td>49 (41-67)</td>
<td>255 (48-512)</td>
<td>248 (50-563)</td>
<td>75 (39-163)</td>
<td>33 (30-47)</td>
<td>23 (19-26)</td>
</tr>
<tr>
<td>Group 3</td>
<td>49 (40-73)</td>
<td>3910 (185-4994)</td>
<td>9077 (59-13910)</td>
<td>4707 (58-11161)</td>
<td>832 (29-3129)</td>
<td>46 (22-121)</td>
</tr>
<tr>
<td>Group 4</td>
<td>53 (40-56)</td>
<td>35 (30-37)</td>
<td>32 (27-36)</td>
<td>30 (27-33)</td>
<td>24 (21-27)</td>
<td>17 (15-18)</td>
</tr>
<tr>
<td>albumin (mg/mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 1</td>
<td>39 (38-39)</td>
<td>34 (33-35)</td>
<td>36 (34-37)</td>
<td>34 (33-36)</td>
<td>35 (34-36)</td>
<td>31 (28-34)*b</td>
</tr>
<tr>
<td>Group 2</td>
<td>32 (29-39)</td>
<td>31 (29-33)</td>
<td>33 (32-34)</td>
<td>33 (32-34)</td>
<td>33 (32-34)</td>
<td>34 (28-38)</td>
</tr>
<tr>
<td>Group 3</td>
<td>34 (30-37)</td>
<td>30 (23-32)</td>
<td>26 (21-33)</td>
<td>26 (25-32)</td>
<td>32 (29-33)</td>
<td>31 (30-31)*b 35</td>
</tr>
<tr>
<td>Group 4</td>
<td>33 (32-34)</td>
<td>32 (31-33)</td>
<td>36 (34-37)</td>
<td>33 (32-34)</td>
<td>34 (29-38)</td>
<td>(34-37)</td>
</tr>
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</table>

*: p<0.05 compared to group 4. b: p< 0.01 compared to group 4. *:p<0.05 day -7 vs day 28

Discussion

Exclusion of peritonitis is very important in experimental studies on effects of exposure to dialysis solutions on the morphology of the peritoneal membrane. The present study has shown that the specificity for the presence of peritonitis of an increased serum a2M and a decreased serum albumin concentration is 100%, even in the absence of clinical symptoms.

Various strategies have been used to prevent peritonitis during experimental animal studies. These include the application of prophylactic antibiotics, but this affects the morphology of the peritoneum [12]. Heparin-coated catheters have been applied, but their use did not reduce the incidence of peritonitis in rat models [13]. The use of an aseptic technique as performed by our group allows extension of the duration of peritoneal exposure in rats for 20 weeks without peritonitis [7]. However, as in humans, it is inevitable that some animals will develop peritonitis.
during the experimental period. Unlike in patients, the diagnosis cannot be made on the presence of an increased dialysate cell count, because no drainage is applied in the model. The golden standard used in this model is the presence of extensive infiltrates in peritoneal tissue.

The majority of peritonitis cases in rats is accompanied with clinical symptoms such as weight loss and catheter obstruction. Sometimes however, the diagnosis is only made after completion of the experimental period because of the presence of extensive histological signs of peritonitis. The presence of positive catheter tip cultures was in accordance with the histological signs of peritonitis. However, positive cultures were also found in 3 animals without any clinical and histological signs of peritonitis, as has been described in patients [14].

The results obtained in study 2 showed that clinical symptoms were especially present in group 3, the highest inoculum group. However, also animals in group 2 showed a temporary lag in body weight at days 1 and 3. It is evident that this could easily be missed during chronic peritoneal exposure to dialysis solutions, which makes body weight a difficult to interpret parameter for peritonitis. Also, an increase in the number of milky spots was only found in group 3. In groups 1 and 2 only their size was somewhat increased.

Peritonitis in CAPD patients is accompanied by elevated serum concentrations of acute phase proteins, like C-reactive protein, secretory phospholipase A2 [15] and interleukin-6 [16,17]. Therefore, in the present study it was investigated whether the determination of an acute phase protein would contribute to the diagnosis of peritonitis in the chronic peritoneal exposure model in the rat. C-reactive protein is not an acute phase protein in rats [18], but a2M is one of the most important acute phase proteins in this species [10]. Albumin is considered to be a negative acute phase protein [19] and can be determined easily with colorimetric methods, as used in autoanalyzers.

The results of the first study have shown that both an elevated a2M and a decreased serum albumin were highly specific markers for peritonitis, but not very sensitive. This means that abnormal values, even in the absence of clinical signs, indicate peritonitis. The presence, however, of normal serum values does not exclude peritonitis. The quantitative analysis done in the second study, confirmed the initial observation. All three dosages of CFU cause a non-lethal peritonitis in rats, from which they recover spontaneously [20]. The changes in serum alpha-2-macroglobulin and albumin were most pronounced in group 3 who received the highest inoculum, but were also present in the other groups. Abnormal values were still observed after two and four weeks. However, despite a high specificity the sensitivity was low. Different cut-off points for both albumin and a2M were calculated (table 1 and 2). The specificity should be as high as possible to avoid that healthy animals will be marked as having peritonitis and sacrificed. Based on these calculations a cut-off point of 32 g/L for albumin and 40 mg/L for a2M have highest specificity. After each experiment, histology is used as the gold standard to exclude rats that completed the experiment without clinical signs of infection but appeared to have peritonitis.

Group 3 probably represents a situation that is rather similar to the occurrence of peritonitis in the long-term peritoneal exposure model. When the sensitivity analysis was restricted to this group of animals the values at two weeks were 60% for a2M and 40% for albumin. After four
weeks the sensitivity of a2M for the presence of peritonitis was 60% and of albumin 50%. These results confirm that animals with clinical signs of peritonitis can have normal values of acute phase proteins.

It can be concluded that measurement of serum a2M and albumin every four weeks is an additional tool in the diagnosis of peritonitis in the chronic peritoneal exposure model in the rat. Even in the absence of clinical signs, an a2M value above 40 mg/L and a serum albumin below 32 g/L are strong indicators for the presence of peritonitis and should be followed by termination of the experiment in that particular animal. However, normal values of these proteins do not exclude infectious peritonitis.

Acknowledgements

a2M standard and antibodies were a generous gift from Dr. W Boers, Hepatology Department, Academic Medical Center, Amsterdam The Netherlands.

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