Contribution of lactate buffer, glucose and glucose degradation products to peritoneal injury in vivo

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Abstract

Background. Long-term peritoneal dialysis (PD) is associated with the development of functional and structural alterations of the peritoneal membrane. In this study, we investigated the contribution of low pH lactate buffer, high glucose concentration and glucose degradation products to peritoneal injury in a rat peritoneal exposure model.

Methods. Rats received daily 10 ml of either heat-sterilized (3.86\% glucose, pH 5.2, \(n = 8\)) or filter-sterilized PD fluid (3.86\% glucose, pH 5.2, \(n = 8\)), or lactate buffer (pH 5.2, \(n = 8\)) via a mini vascular access port during a 10 week period. Untreated rats served as controls.

Results. The low pH lactate buffer instillation induced pronounced morphological changes including the induction of angiogenesis in various peritoneal tissues and mild damage to the mesothelial cell layer covering the peritoneum. It also evoked a cellular response characterized by an increased mesothelial cell density on the liver, the induction of milky spots and accumulation of omental mast cells in the omentum, and significant changes in the composition of peritoneal leukocytes. The addition of glucose to low pH lactate buffer (filter-sterilized PD fluid) strengthened most, but not all of the responses described above and induced a fibrogenic response. In addition to glucose and low pH lactate buffer, the presence of glucose degradation products (heat-sterilized PD fluid) significantly induced an additional omental milky spot response (\(P < 0.03\)) and caused profound mesothelial damage. The vessel density in the omentum and the mesentery was significantly correlated to both the number of tissue mast cells and the hyaluronan content in the peritoneal lavage, which might suggest a role for mast cells and hyaluronan in the induction of angiogenesis.

Conclusions. Instillations of low pH lactate buffer, a high glucose concentration and glucose degradation products contribute differently and often cumulatively to peritoneal injury in vivo.

Keywords: CAPD; glucose degradation products; glucose; lactate buffer; peritoneal injury

Introduction

Conventional peritoneal dialysis (PD) fluids alter the function and morphology of the peritoneal membrane during long-term use. The development of ultrafiltration failure is the most important functional abnormality of PD patients. Loss of ultrafiltration is at least partly associated with the presence of a large peritoneal vascular surface area. Another characteristic feature of these patients is the development of fibrosis [1,2].

The bioincompatibility of conventional heat-sterilized PD solutions might be attributed either to the low pH, the lactate buffer, hypertonicity, the high concentration of glucose, glucose degradation products (GDPs) or a combination of these factors [3]. It has been shown that a high concentration of glucose, used as osmotic agent, is cytotoxic to the peritoneal cells [3]. The process of heat sterilization of glucose-based PD fluids leads to the formation of GDPs [4]. It has been suggested that the presence of several aldehydes and 1,2-dicarbonyl compounds is partly responsible for the cytotoxicity of these fluids [5]. GDPs can also promote the irreversible formation of advanced glycation end products (AGEs) which in turn contribute to the toxicity of PD fluids [6]. Besides these fluid constituents, a low pH can affect the biocompatibility of these fluids. There is strong evidence, however, that
bioincompatibility is not related to low pH alone because several negative side effects persisted in neutralized PD fluids [7]. Finally, there is considerable evidence suggesting cytotoxic effects of lactate as the common buffer used in the conventional PD fluids [3].

We have shown previously that the conventional heat-sterilized PD fluid affects the normal cellular composition of the peritoneal cavity and impairs the bacterial killing capacity of the peritoneal cells in *ex vivo* experiments [8]. In addition, the morphology of peritoneal tissues had changed after 10 weeks of PD fluid instillation. More blood vessels and milky spots were found in the omenta of the rats exposed to the heat-sterilized PD solution. The mesothelial cell layer was also severely damaged and the submesothelial extracellular matrix (ECM) was thickened in the treated animals. In the same study, it was found that the neutral, lactate/bicarbonate-buffered PD fluid (Physioneal 3.86%) significantly improved all peritoneal parameters compared with the heat-sterilized PD fluid-treated rats. Both fluids differ in type of buffer, in pH and in the amount of GDPs. In that study, however, we were not able to determine the separate effects of all individual components.

In the present study, we have compared in a rat model the effects of heat-sterilized PD fluid and filter-sterilized PD fluid, both containing a high glucose concentration and a low pH lactate buffer, and the effects of low pH lactate buffer instillation without glucose. A group of untreated rats served as control. This allowed us to gain more insight into the role of respectively low pH lactate buffer instillation, a high glucose concentration and GDPs in the observed alterations in our peritoneal instillation model in the rat. Using a combination of immunological and morphological parameters, we now show that each of these factors contributes differently and often cumulatively to the peritoneal damage. Furthermore, the various peritoneal tissues respond differently to PD fluid constituents.

### Materials and methods

**Animals**

Male Wistar rats (Harlan CPB, Zeist, The Netherlands) weighing 180–200 g at the beginning of the experiment were used throughout the study. They were allowed to acclimatize for 1 week before the experiments started. Animals were maintained under conventional laboratory conditions and were allowed free access to food and water. The Animal Care Committee of the Vrije Universiteit of Amsterdam approved the animal experiment described.

**Experimental peritoneal dialysis model**

Fluids were instilled via a peritoneal catheter connected to an implanted subcutaneous mini vascular access port as previously described [8]. During the first week after operation, all animals, except the untreated group, received 2 ml of saline with 1 U/ml heparin to allow wound healing. Then, during a 10 week period, 10 ml of each fluid without any addition of heparin or antibiotics was given between 9 and 12 a.m.

**Experimental design**

Rats were divided into four groups. The first group (*n* = 12) received daily 10 ml of heat-sterilized, lactate-buffered PD fluid with a pH 5.2 and containing 3.68% glucose (Dianeal®, Baxter Healthcare, Utrecht, The Netherlands), the second group (*n* = 13) received filter-sterilized lactate-buffered PD fluid with the same electrolyte composition and pH as the heat-sterilized solution, and also containing 3.68% glucose. In the third group (*n* = 12), lactate buffer (same electrolyte composition and pH as the other two solutions but without glucose) was instilled. A 0.22 μm filter (Millipore) was used to filter-sterilize the solutions for groups 2 and 3. The control group (*n* = 5) remained untreated. After 10 weeks of fluid instillation, the animals were first weighed and then sacrificed 24 h after the last fluid instillation. The peritoneal cells were collected from the peritoneal cavity by injection of 10 ml of Hanks’ balanced salt solution (Life Technology, BRL, Grand Island, NY) followed by a gentle massage of the abdomen for 2 min. The cells were recovered and diluted with an additional 10 ml of Hanks’, followed by centrifugation for 10 min at 600 g. Supernatant was controlled for the absence of the bacteria on agar plates and was stored at −20 °C and peritoneal cells were dissolved in 1 ml of RPMI with 0.1% bovine serum albumin (BSA) and counted in a Bürker counting chamber (Fisher Science, Zoetemeer, The Netherlands). The viability was controlled by trypan blue exclusion (always >90%). Portions of the omentum, the mesentery and the parietal peritoneum were dissected for light and electron microscopy. Omental (~4 cm²/rat) and mesenteric tissues (the most distally situated loop entirely) were dissected, spread on an object slide and air dried. The parietal peritoneum was taken at the contralateral side (~1 cm) to the tip of the implanted catheter using a standardized method. Mesothelial imprints of the liver surface of each rat (five per animal) were taken as described before [8]. Blood samples were collected. Evaluation of various parameters was done independently by two investigators on coded material. The averaged value of both measurements was used for statistical analysis.

**Analysis of peritoneal cells and lavage fluid**

Cytocentrifuge preparations were stained by May–Grünewald–Giemsa and were checked for the absence of bacteria, and the cells were differentiated. The percentage of Fc receptor-positive peritoneal cells was determined by rosette formation with IgG-coated sheep red blood cells ([IgG]SRBC) using routine procedures. After incubation of peritoneal cells and [IgG]SRBC for 1 h at 4 °C, peritoneal cells binding three or more erythrocytes per cell were scored as positive.

The percentage of all macrophages in the peritoneal cavity was determined by immunohistochemical staining of cytocentrifuge preparations with monoclonal ED1 (Serotec, Oxfordshire, UK), while the percentage of mature macrophages was determined with monoclonal ED2 (Serotec). The ED2/ED1 ratio was then calculated, since a decrease of this ratio reflects the influx of young macrophages (no ED2 expression).
The expression of complement receptor 3 on the peritoneal cells was determined by monoclonal ED8 (Serotec).

The amount of hyaluronan (HA) in the supernatant of the peritoneal effluent was determined in an enzyme-linked immunosorbent assay (ELISA)-based assay essentially according to Fosang et al. [9], using immobilized HA and competition for the binding of biotinylated HA-binding protein by HA-containing samples.

The amount of interleukin-6 (IL-6 kit, Biosource, USA) was measured in the peritoneal effluents; the detection level was 30 pg/ml. As positive controls, peritoneal effluents of rats (from a separate study) injected with Staphylococcus aureus for 6 h were used.

Light microscopy

Omentum and mesentery. Spread preparations of omental and mesenteric tissues were stained with toluidine blue. Since the omental milky spots (local aggregates of immune cells) are the major route through which leukocytes migrate into the peritoneal cavity [10] and their size and number reflect the activated state of the omentum, we determined the number as well as the size of omental milky spots by light microscopy using a scored eyepiece. Twenty five random areas of 4 mm² were scored (total 1 cm²) and both the milky spot number and the mean surface area of milky spots (in mm²) were determined. Finally, the total milky spot surface (expressed as a percentage of total omental surface) from each individual omentum was calculated by: the number of milky spots/cm² × mean surface area of milky spots × 100%. The number of blood vessels in the omenta and mesenteries was determined similarly; all vessels (usually with a diameter ranging from 10 to 80 μm) were counted and expressed as number of vessels/cm². To determine the number of mast cells in the omenta and mesenteries, 10 randomly selected areas of 0.16 mm² each were counted. Mast cells present in/on the milky spots were differentiated after May–Grünewald–Giemsa staining.

Parietal peritoneum. Cryostat sections of specimens of the parietal peritoneum were cut (8 μm) and stained using a Van Gieson staining kit (Merck KGaA, Darmstadt, Germany); all biopsies were embedded, sectioned and stained in a standardized fashion. The thickness of the submesothelial ECM was determined after Van Gieson staining, as described previously [8]. Frozen sections were also used to quantify the number of submesothelial blood vessels, using anti-CD31 (PECAM) as the endothelial marker and expressed as the number of vessels per mm length of the mesothelial layer.

Liver. Mesothelial liver imprints were dried and stained by May–Grünewald–Giemsa, as described before [8]. The number of cells per 0.1 mm² area was counted and the average of 16 areas was calculated for each slide and expressed as cells/mm².

Electron microscopy

Portions of the dissected omental tissue, mesentery and parietal peritoneum of three animals/group were prepared for electron microscopy according to standard procedures [8]. Overview electron micrographs (15–25 measurements per group) made from mesenteric tissues of all animals were used to determine the thickness of the submesothelial ECM, as fibrotic marker. These measurements were made at those places where no blood vessels were present.

Number and composition of peripheral leukocytes

Blood samples (20 μl) were collected from all animals and were added to Turks solution [0.2 mg of gentian violet in 1 ml of glacial acetic acid, 6.25% (v/v)] in a 1:10 dilution, and leukocytes were counted by a Bürker counting chamber. Leukocytes were differentiated after May–Grünewald–Giemsa staining.

Statistical analysis

All data (presented as median and 25th–75th interquartile ranges) were analysed using the non-parametric Kruskal–Wallis and Mann–Whitney U-tests. Correlation analysis was performed using the Spearman rank correlation test; P < 0.05 was regarded as significant.

Results

During the experiment, the well-being of all animals was monitored daily and no apparent abnormalities were observed. Throughout the experiment, there was a dropout of 30–40% of the animals due to omental wrapping around the tip of the catheter [11], which was not different among the treated groups. After 10 weeks of fluid instillation, eight rats per experimental group (heat-sterilized, filter-sterilized and buffer group) remained, and all (five) untreated animals were used for analysis.

A median weight of 464, 458, 455 and 450 g was found in the heat-, filter-sterilized, buffer and untreated groups, respectively, without any significant differences among groups (P = 0.88).

Cellular composition of peritoneal cells

The total number of peritoneal leukocytes did not differ among the experimental groups (Table 1). No statistically significant differences were found in the percentages of macrophages (P = 0.12), neutrophils (P = 0.72) and lymphocytes (P = 0.32) between various groups. Furthermore, mast cells had almost completely disappeared in all experimental groups compared with untreated rats, whereas the percentage of eosinophils had increased in these groups and might be related to the catheter implantation or the volume loading. These changes are apparently independent of the presence of a high glucose concentration and/or GDPs in the fluids, since similar changes were found in all three fluid-treated groups.

No significant differences were seen in either the ED2/ED1 ratio or the percentages of ED8-positive cells among the four groups (Table 1). However, a significantly higher percentage of cells carrying the Fc receptor was found in the heat-sterilized group.
compared with the other groups, indicating GDPs to be instrumental in this respect.

**Morphological changes of the omentum**

Macroscopic inspection showed that in all fluid-instilled groups, the surface area of the whole omentum had increased, whereas the transparency had decreased compared with the untreated rats, which indicates fibrosis. Light microscopic analysis of the omenta revealed that the number and size of milky spots were significantly enhanced in the rats exposed to the heat-sterilized PD fluid compared with the other three groups (Table 2). The amount and size of milky spots were also enhanced in the filter-sterilized and the buffer group compared with untreated rats. These data indicate that both low pH lactate buffer instillation and GDPs, but not glucose itself, evoke a milky spot response. The number of omental blood vessels was significantly increased in the rats exposed to glucose-containing fluids (heat- and filter-sterilized groups) compared with both the lactate buffer group and the untreated group (Figure 1). The angiogenic response in the buffer group was intermediate between the glucose-containing groups on the one hand and the untreated group on the other hand (untreated < low pH lactate buffer < filter-sterilized = heat-sterilized). In addition, the number of mast cells was increased in all treated groups compared with untreated rats (P < 0.03), although to a lesser extent in the buffer-treated group. When all animals were combined, irrespective of their groups, a significant positive correlation was found between the number of vessels and the amount of mast cells in the omentum (r = 0.70, P < 0.0004).

Electron microscopic observation of the omenta confirmed the induction of new blood vessels in rats exposed to heat- and filter-sterilized PD fluid; however, the formation of new vessels was also seen in the buffer group, albeit to a lesser extent (Figure 2). In some cases, the endothelial cells lining the blood vessels seemed to be activated, as determined by the appearance of the high endothelium in the heat-sterilized group, whereas endothelial cells were flat in the other groups, as reported previously [8]. Furthermore, the mesothelial cell layer covering the omentum was greatly damaged in the heat-sterilized group compared with the other groups, as seen by a loss of mesothelial cells. Another feature of this damage was the adhesion of macrophages to lesions where mesothelial cells were no longer present (Figure 2A). The mesothelial damage was also found in the filter-sterilized group but to a lesser extent, while no damage was observed in the untreated group.

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**Table 1. Composition of peritoneal leukocytes**

<table>
<thead>
<tr>
<th></th>
<th>Heat</th>
<th>Filter</th>
<th>Buffer</th>
<th>Untreated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cells (10^6)</td>
<td>14 (12–17)</td>
<td>13 (12–28)</td>
<td>13 (12–13)</td>
<td>13 (11–17)</td>
</tr>
<tr>
<td>% Macrophages</td>
<td>72 (65–74)</td>
<td>69 (62–73)</td>
<td>66 (62–69)</td>
<td>75 (73–77)</td>
</tr>
<tr>
<td>% Neutrophils</td>
<td>4.0 (1.0–5.0)</td>
<td>2.5 (0.7–7.0)</td>
<td>2.0 (0.4–7.0)</td>
<td>1.0 (0–2.0)</td>
</tr>
<tr>
<td>% Lymphocytes</td>
<td>3.0 (1.7–4.0)</td>
<td>4.0 (1.7–4.2)</td>
<td>3.0 (0.7–3.7)</td>
<td>0 (0–2.0)</td>
</tr>
<tr>
<td>% Mast cells</td>
<td>1.0 (0–3.2)</td>
<td>1.0 (0–2.0)</td>
<td>1.5 (1.0–2.7)</td>
<td>7.0 (6.7–8.5)</td>
</tr>
<tr>
<td>% Eosinophils</td>
<td>20 (17–23)</td>
<td>24 (18–26)</td>
<td>26 (25–28)</td>
<td>16 (11–16)</td>
</tr>
<tr>
<td>ED2/EDI ratio</td>
<td>0.4 (0.3–0.4)</td>
<td>0.3 (0.3–0.5)</td>
<td>0.4 (0.3–0.4)</td>
<td>0.4 (0.3–0.6)</td>
</tr>
<tr>
<td>% ED8+ cells</td>
<td>63 (54–69)</td>
<td>67 (56–69)</td>
<td>69 (58–72)</td>
<td>74 (70–76)</td>
</tr>
<tr>
<td>% Fc receptor+</td>
<td>84 (82–86)</td>
<td>71 (65–79)</td>
<td>75 (69–80)</td>
<td>67 (62–72)</td>
</tr>
</tbody>
</table>

Data are presented as median and interquartile ranges.

*Different from untreated group: P < 0.03.
*Different from filter group: P < 0.03.
*Different from buffer group: P < 0.03.

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**Table 2. Morphometric parameters of peritoneal tissues**

<table>
<thead>
<tr>
<th></th>
<th>Heat</th>
<th>Filter</th>
<th>Buffer</th>
<th>Untreated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Omentum</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Milky spots/cm²</td>
<td>29 (23–34)</td>
<td>14 (13–16)</td>
<td>16 (12–23)</td>
<td>4 (4–6)</td>
</tr>
<tr>
<td>Area/milky spots (mm²)</td>
<td>0.7 (0.6–0.7)</td>
<td>0.4 (0.4–0.8)</td>
<td>0.3 (0.3–0.4)</td>
<td>0.2 (0.2–0.2)</td>
</tr>
<tr>
<td>Milky spots surface</td>
<td>21 (17–24)</td>
<td>7 (6–7)</td>
<td>6 (5–9)</td>
<td>1 (1–1)</td>
</tr>
<tr>
<td>Mast cells/mm²</td>
<td>101 (43–138)</td>
<td>119 (69–200)</td>
<td>52 (44–90)</td>
<td>12 (9–13)</td>
</tr>
<tr>
<td>Mesentery</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mast cells/mm²</td>
<td>61 (57–76)</td>
<td>56 (51–58)</td>
<td>66 (60–78)</td>
<td>47 (45–59)</td>
</tr>
<tr>
<td>Peritoneum</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mast cells/mm²</td>
<td>2.6 (1.5–9.9)</td>
<td>1.3 (0.7–1.8)</td>
<td>1.5 (0.9–2.2)</td>
<td>1.7 (1.4–1.9)</td>
</tr>
<tr>
<td>ECM thickness (µm)</td>
<td>31.7 (29.4–35.3)</td>
<td>27.1 (26.5–28.8)</td>
<td>30.3 (20.4–32.2)</td>
<td>25.7 (23.1–27.8)</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mesothelial cells/mm²</td>
<td>1368 (1253–1423)</td>
<td>1422 (1297–1531)</td>
<td>1203 (1203–1250)</td>
<td>891 (844–1094)</td>
</tr>
</tbody>
</table>

Data are presented as median and interquartile ranges.

*Different from untreated group: P < 0.03.
*Different from filter group: P < 0.03.
*Different from buffer group: P < 0.03.
The mesothelial cell loss was not found in the buffer group but mesothelial microvilli had partly disappeared. In addition, a substantial widening between collagen bundles was observed in the heat-sterilized group (data not shown), which strongly suggests deposition of non-collagenous ECM. This alteration was also found in the filter group, but again to a lesser extent. When comparing the buffer group with the untreated group, this observation was more profound in the buffer group.

**Morphological changes of the mesentery**

The number of blood vessels was significantly increased in all fluid-instilled groups compared with untreated rats (Figure 1), suggesting the contribution of low pH lactate buffer instillation via a catheter to the induction of neovascularization in the mesenteric tissue. No significant differences were found among heat-, filter-sterilized and buffer-treated groups. Despite a higher number of mast cells in the buffer group compared with the filter group ($P < 0.04$), no other significant differences were found in the number of mast cells in the mesenteric tissues among the four groups (Table 2). When all animals were combined, irrespective of their groups, a weak correlation was found between the number of blood vessels and the number of mast cells in the mesentery ($r = 0.44$, $P < 0.05$).

Electron microscopic observation of the mesothelial cell layer covering the mesenteric tissues revealed similar results to those of the omentum (data not shown). Thus, strong mesothelial damage was noted in the heat-sterilized group compared with the other groups, as seen by vacuolization or even complete loss of mesothelial cells. Partial mesothelial damage was seen in the filter-sterilized and buffer groups, while the mesothelial cell layer was intact in the mesenteric tissues of untreated rats. When comparing the filter-sterilized and buffer groups, damage to the mesothelial cell layer was more profound in the filter-sterilized group. Unlike omentum, no adherent macrophages were observed at damaged sites and more fibroblasts were seen in the mesentery (data not shown). Electron micrographs were used further to quantify fibrosis in the mesentery.
The thickness of the submesothelial ECM was significantly increased in both heat- \((P < 0.0001)\) and filter-sterilized \((P < 0.0002)\) groups compared with buffer and untreated rats (Figure 3), which was due primarily to deposition of collagenous ECM. No significant differences were found between either heat- and filter-sterilized groups \((P > 0.99)\) or between buffer and untreated groups \((P = 0.11)\).

**Morphological changes of the parietal peritoneum**

Three out of eight animals of the heat-sterilized group and one rat from the filter-sterilized group showed the formation of granulation tissues characterized by the accumulation of several cell types, including mast cells and possibly fibroblasts, many vessels and ECM deposition (Figure 4). This phenomenon was not seen in either the buffer or the untreated group.

In untreated rats, capillaries were found at the border of the submesothelial ECM and the peritoneal muscles, but not within the ECM layer itself (Figure 5). In fluid-instilled animals, however, newly formed blood vessels were observed throughout the whole width of the submesothelial ECM layer. Quantification of these vessels revealed a gradual increase from untreated < low pH lactate buffer < filter-sterilized < heat-sterilized PD fluid-exposed animals (Figure 1). Furthermore, no significant differences were found in the number of mast cells within the ECM between various groups \((P = 0.20)\). When all animals were combined, irrespective of their groups, no correlation was found between the number of vessels and the amount of mast cells within the submesothelial ECM \((r = 0.30, P = 0.15)\).

Despite a trend towards an increased thickness of the submesothelial ECM in the treated groups, no statistically significant differences were found in the thickness of the submesothelial ECM in the parietal peritoneum among different groups (Table 2). It is worth noting that the granulation tissue had been excluded from the quantification of vessel density, mast cells and the thickness of ECM in the parietal peritoneum.

Electron microscopic observation of the mesothelial cell layer covering the parietal peritoneum revealed less mesothelial damage in comparison with omental and mesenteric tissues, especially in the heat-sterilized group, although microvilli were partly lost and mesothelial cells had disappeared focally. In contrast to omentum, no adherent macrophages were seen at focal sites of damaged mesothelium (data not shown).

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**Fig. 3.** Ultrastructural measurements of the thickness of mesenterial submesothelial ECM. Data are presented as median and interquartile ranges. \(^a\) Heat vs buffer and untreated, \(P < 0.0001\); \(^b\) filter vs buffer and untreated, \(P < 0.0002\).
Morphological changes of the mesothelial cell layer on the liver

The number of mesothelial cells on the liver was significantly increased in all experimental groups compared with the untreated animals (Table 2). A higher number of these cells was also found in the filter-sterilized group than in the buffer group, indicating a contribution of both low pH buffer instillation and high glucose concentration to this regenerative response. In addition, more activated mesothelial cells were found in all injected groups, as determined by the bone-shaped nuclei of these cells. The increased number of mesothelial cells was accompanied by the adherence of non-mesothelial cells such as fibroblast-like cells, macrophages and neutrophils to the liver of the fluid-instilled animals compared with the untreated rats (data not shown).

Peritoneal effluents

A basic level of ~250 ng/ml HA was found in the effluents obtained from untreated rats, while the amount of peritoneal HA was significantly increased upon instillation of all three fluids (Figure 6). Although Figure 5 suggests a higher HA content in the glucose-containing groups, these values are not significantly different from the buffer group, indicating a major contribution of low pH lactate buffer instillation via an implanted catheter to an increased HA release and/or production. Statistical analysis revealed that the amount of HA in peritoneal effluents was significantly correlated with the number of blood vessels in the omentum ($r = 0.48$, $P < 0.02$) as well as in the mesentery ($r = 0.59$, $P < 0.003$); however, no correlation was found in the ECM of the parietal peritoneum ($r = 0.41$, $P = 0.10$).

The levels of peritoneal IL-6 were not detectable in any animal. The total number of peritoneal cells and the percentages of neutrophils did not differ among the groups. In addition, no bacteria in the cytocentrifuge preparations or cell-free supernatants of the peritoneal cells were observed. No IL-6 could be detected in the peritoneal effluents in the four groups. These data indicate that changes found in the treated groups were not related to peritonitis.

Peripheral blood parameters

Systemic leucocyte counts and their distribution over monomorphonuclear (10–15%) and polymorphonuclear cells (85–90%) did not differ among the groups ($P = 0.13$) and were not affected by daily peritoneal fluid instillation (data not shown).

Discussion

In the present study, we investigated the contribution of low pH lactate buffer instillation via an implanted catheter, high glucose concentration and GDPs to damage of various peritoneal tissues after long-term intraperitoneal fluid instillation in a rat model. We focused on a set of parameters reflecting peritoneal damage.

Although animals in this model have normal kidney function and no fluid exchanges were performed, this model is accepted as a valuable tool for evaluating the responses of peritoneal tissues upon exposure to PD fluids [8,11].

In this study, no clinical signs of illness or weight loss were detected in any animal. The total number of peritoneal cells and the percentages of neutrophils did not differ among the groups. In addition, no bacteria in the cyto-centrifuge preparations or cell-free supernatants of the peritoneal cells were observed. No IL-6 could be detected in the peritoneal effluents in the four groups. These data indicate that changes found in the treated groups were not related to peritonitis.

Daily instillation of low pH lactate buffer induced relatively mild changes in the peritoneal membrane. These included fibrosis, altered cellular composition of the peritoneal cavity and damage to the mesothelial cell layer. Most importantly, mild angiogenesis accompanied by increased HA concentrations and increased omental mast cell density were found. In this lactate buffer-treated group, we cannot distinguish between the effects of volume loading, low pH, catheter implantation or the type of buffer (lactate). We know that the catheter implantation has some minor effects on angiogenesis [12], but this effect is not comparable with the effects caused in the three fluid-treated groups as found in this study. Therefore, it is unlikely that effects found in the low pH lactate buffer group might be related to the catheter implantation alone. It is worth noting that lactate buffer in an acidic environment drives cellular metabolism towards the hypoxic state and could thus be responsible for (some of) the observed effects in the buffer group. The presence of a high glucose concentration in the PD fluid (filter-sterilized fluid) appears to strengthen some, but not all effects caused by low pH lactate buffer instillation alone. For example, a more severe angiogenesis, accompanied by an elevated amount of HA and an increased number of omental mast cells, fibrosis and mesothelial cell damage was observed. It should be realized, however, that the changes attributed to glucose could be due to the hyperosmolarity of glucose-containing fluids, as the lactate buffer used in this study was not hyperosmolar. Lastly, the additional presence of GDPs (heat-sterilized PD fluid) evoked even more profound peritoneal damage evidenced by severe mesothelial damage, and increased number and...
size of omental milky spots, increased number of peritoneal Fe receptor-positive cells and development of granulation tissues in the parietal peritoneum. With respect to new vessel formation, in none of the peritoneal tissues examined was a statistically significant difference found between the heat- and filter-sterilized groups, indicating that in acidic conditions GDPs play no dominant role in neovascularization in our rat instillation model. However, we cannot exclude the possibility that GDPs alone might contribute to the induction of angiogenesis but not in the presence of a high glucose concentration and/or low pH lactate buffer in our rat model. In addition, the focal formation of granulation tissue was not found in all treated rats, which, however, was most probably due to a sampling error, because in a separate study on rats exposed to heat-sterilized PD fluid, we inspected the whole parietal peritoneum (and not just a relatively small section) for the presence of granulation tissues and we found that all these animals developed granulation tissues (data not shown).

We determined the contribution of glucose and GDPs in an acidic fluid and in the presence of lactate, since lactate-buffered PD solutions are still the most commonly used fluids. Furthermore, it has been shown that the pH of the acidic solutions would increase rapidly (within minutes) and reach the physiological value after instillation in the rat [13]. In order to determine to what extent the peritoneal damage found in the acidic lactate buffer is related to low pH, in a separate study we investigated the contribution of bicarbonate/lactate buffer with a neutral pH to the peritoneal injury in our rat model. We found that rats exposed to the bicarbonate/lactate buffer (pH 7.4) showed a 40% reduction in the number of new blood vessels as well as in the number of milky spots in the omentum, compared with the conventional heat-sterilized PD fluid (data not shown). These results indicate that the peritoneal damage caused by a neutral buffer is comparable with that induced by acidic lactate buffer, suggesting that low pH plays a minor role in this experiment, and that rather fluid instillation by itself is probably an important factor. Thus, it is unlikely that the acidity of the fluids overshadows the bioincompatibility of high glucose and GDPs in our experimental design. The results of our study clearly indicate that the peritoneal response to conventional PD fluid is often a cumulative damage reaction driven by various determinants. Besides a clear contribution of both high glucose concentration and GDPs, we provide evidence for a rather dramatic response to the instillation of fluid (via an implanted catheter) in itself.

In addition to clarifying the contribution of various individual factors in conventional PD fluid, we also found that various peritoneal tissues responded differently to the low pH lactate buffer instillation, a high glucose concentration or GDPs. Angiogenesis, for instance, was induced to a different degree in the omentum and in the mesentery. Furthermore, the number of blood vessels correlated strongly to the number of mast cells in the omentum, but weakly in the mesentery, suggesting a role for mast cells in the induction of neovascularization in the omentum. Indeed, many mast cells, but not all, were found to be closely situated around blood vessels in this study. Unlike in omentum, angiogenesis most probably occurs mast cell independently in the parietal peritoneum and probably in the mesentery. The possibility that the accumulated mast cells in omentum, by secreting angiogenic factors, might be responsible for the induction of new blood vessels in the mesentery and parietal peritoneum cannot be excluded. Interestingly, different PD fluid constituent(s) were found to be instrumental in the induction of angiogenesis in various peritoneal tissues. Whereas low pH lactate buffer instillation by itself was enough to induce new blood vessels in the mesentery, glucose seems to be an additional factor responsible for the increased vessel density in the omentum. A difference in the response of the various peritoneal tissues was also found for the mesothelial cell layer. For either fluid, the damage to omental and mesenteric mesothelium was always more pronounced compared with parietal peritoneum. Also, the fact that the mesothelial cells on the omentum and mesentery were partially lost but these numbers were increased on the liver may indicate a differential mesothelial response of various peritoneal tissues to the same environment, which might be related to different mesothelial phenotypes. Since various peritoneal tissues respond differently, there is thus no representative peritoneal tissue that reflects overall the morphological and structural changes during long-term PD.

HA is a major component of interstitial tissue that is involved in a wide range of biological processes including fluid homeostasis, response to inflammation and wound healing. On one hand, there is a large body of evidence suggesting a beneficial role for HA during PD. HA supplementation in the PD fluid was found to improve the ultrafiltration capacity in the rat [14]. On the other hand, other studies indicated an angiogenic property of HA. A recent study showed that the inhibition of HA synthesis resulted in a reduction in tumour vascularity of up to 70–80% [15]. We have now found that the number of omental and mesenteric blood vessels is correlated to the amount of HA in the peritoneal lavage. Although correlation data do not prove a causal relationship, we suggest a supporting role for HA in the induction of angiogenesis. Several studies suggested vascular endothelial growth factor (VEGF) to be an important mediator of neovascularization in the peritoneal membrane, as VEGF blockade prevented neoangiogenesis in the peritoneum of experimental animals with diabetes [16]. However, in a separate study, we could not detect increased VEGF levels in the peritoneal effluents of rats exposed to PD fluid for 10 weeks (detection level of 5.1 pg/ml; data not shown). Furthermore, VEGF is known to be a very potent enhancer of microvascular permeability. We have, however, previously reported an unchanged vascular permeability in mesenteric vessels of rat exposed to PD fluid for 5 weeks [12]. Taken together, the precise role of VEGF in the formation of new blood vessels during PD presently is unclear.
In addition, the present study identified glucose as the main factor involved in the development of fibrosis, at least under acidic conditions. This finding is in agreement with other studies reporting an increased proliferation of peritoneal fibroblasts and increased expression of laminin by the supernatant of mesothelial cells cultured in high glucose medium [17,18].

Various clinical [19] and experimental [8] studies clearly demonstrate the superior biocompatibility of the new generation PD fluids. These new PD fluids differ from the conventional fluids in their acidity, buffer type and GDP content, which all may contribute to the described improvements. Up to now, only a single experimental study unravelled the individual contribution of GDPs, glucose and acidity and/or buffer choice [20]. That study, however, focused exclusively on transport parameters. To the best of our knowledge, in the present study we provide the first detailed description of the individual contribution of the acidic lactate buffer, high glucose concentration and GDPs to several immunological and morphological parameters after long-term intraperitoneal instillation in a well-established animal model for CAPD. The results of the present study help to explain the harmful effects of conventional PD fluids and might contribute to the development of more biocompatible PD fluids.

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Conflict of interest statement. None declared.

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