Experimental and clinical studies on peritoneal physiology and morphology during chronic peritoneal dialysis
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Vascular endothelial growth factor in peritoneal dialysis, a longitudinal follow-up

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submitted
Abstract
In a previous study VEGF was found to be locally produced (LVEGF) in peritoneal tissue of peritoneal dialysis (PD) patients treated with glucose containing PD solutions. LVEGF was positively related to mass transfer area coefficient (MTAC) of creatinine and the glucose absorption, representatives of the peritoneal vascular surface area. It was therefore hypothesized that VEGF is involved in the peritoneal neoangiogenesis found in long-term PD. The aim of the present study was to investigate the time course of peritoneal VEGF levels in PD patients treated with glucose based PD solutions during longitudinal follow-up. We also studied the effect of the switch to non-glucose PD treatment on VEGF production. Forty standard peritoneal permeability analyses (SPAs) with 3.86% glucose containing dialysis solution were investigated. The SPAs were performed in 10 PD patients with a median number of 3 SPAs/patient, during a follow-up of 23 months. Duration of PD treatment at the last SPA was 74 months. All patients were initially treated with glucose containing dialysis solutions. Four patients switched after 114 months glucose based PD to glucose free PD and were followed for 7 months. A PD regimen of icodextrin, glycerol and amino acid based dialysis solutions was applied in these patients. Four SPAs were performed per patients in this period. To predict the VEGF dialysate-to-serum ratio (D/S), when diffusion would be the only explanation of the VEGF dialysate concentration, we calculated the power relation between D/S ratios of serum proteins, that are only transported across the peritoneum and their molecular weights. The measured VEGF D/S ratio was higher than expected (p<0.001) in each observation, pointing to local production of VEGF. LVEGF increased with duration of glucose PD, 11.7 ng/L to 23.45 ng/L (P<0.03). LVEGF decreased in all 4 patients on glucose free PD from 57.35 ng/L to 23.10 ng/L. A correlation (r=0.83, P<0.001) was found between the differences in MTACcreatinine between the first and last SPA during glucose based PD, and the difference in LVEGF between these observations. A similar correlation was present between the difference in glucose absorption and the difference in LVEGF (r=0.85,P<0.001). This supports a pathogenetic role of high glucose dialysate concentrations in the development of changes in the peritoneum found in long-term PD. Treatment with non-glucose based PD solutions may inhibit further development of these alterations.

Introduction
Vascular endothelial growth factor (VEGF) is a mediator in the development of neoangiogenesis in proliferative diabetic retinopathy [1-5]. Local production in the peritoneal cavity has recently been reported by us [6] in patients undergoing chronic peritoneal dialysis (PD) with glucose containing dialysis solutions. The results of this study suggested a role for VEGF in the development of peritoneal neoangiogenesis, as found in long term PD patients [7]. Other investigators [8-10] described an increase in D/P ratios or mass transfer area coefficients (MTACs) of low molecular weight solutes with the duration of peritoneal dialysis. These functional parameters are likely to reflect the peritoneal vascular...
surface area [11,12]. We found relationships between the effluent concentration attributed to the local peritoneal production of VEGF (LVEGF) and the MTAC of creatinine, and also with the glucose absorption from the peritoneal cavity. Furthermore, inverse correlations were seen between the LVEGF and the transcapillary ultrafiltration, and the net ultrafiltration. These observations all suggest a causal relationship between prolonged peritoneal dialysis with glucose based dialysis solutions and the development of a large peritoneal vascular surface area, in association with, or mediated by peritoneal production of VEGF. Therefore, the aim of the present study was to investigate the presence of VEGF in peritoneal effluent of patients treated with glucose containing peritoneal dialysis solutions in a longitudinal follow-up in relation to their peritoneal transport parameters. And secondly, to study the effect of a switch to glucose free dialysis treatment on LVEGF and peritoneal permeability characteristics in long-term patients with ultrafiltration failure.

Methods

Patients

Ten non-diabetic patients undergoing peritoneal dialysis (5 men and 5 women) were investigated with standard peritoneal permeability analyses (SPA). Forty SPAs were performed with a median number of 3 per patient (range 2-6) during a median follow-up of 23 months (range 7-37). Median duration of peritoneal dialysis at the time of the last investigation was 74 months (range 16-152). All patients were initially treated with commercially available glucose based dialysis solutions (Dianeal; Baxter, Utrecht, The Netherlands). Four patients switched after 114 months (range 80-149) glucose PD to glucose free dialysis treatment, because of ultrafiltration failure. This was defined as net ultrafiltration less than 400 mL during a 3.86% glucose 4-hour dwell [13]. The glucose free dialysis regimen was composed of a combination of a not commercially available 2.5% glycerol based dialysis solution, and commercially available Nutrineal, a 1.1% amino acid containing dialysis solution, and Extraneal, containing 7.5% icodextrin as osmotic agent (Baxter, Utrecht, The Netherlands). During a follow-up of 7 months (range 2-13) these patients underwent a median of 4 SPAs per patient (range 2-5). None of the patients had peritonitis at the time of the study or in four weeks preceding the investigation. Informed consent was obtained from all patients after an explanation of the purpose and the methodology of the study.

Study design

Previously described standard peritoneal permeability analyses [14] were performed using 3.86% glucose containing dialysate (Dianeal). In brief, prior to the instillation of the test solution the peritoneal cavity was rinsed with 1.36% glucose dialysate. The volume marker dextran 70, 1 g/L (Hyskon; Medisan Pharmaceuticals, Uppsala, Sweden) was added to the test solution for the calculation of fluid kinetics [15]. Samples were taken from the test solution before inflow and at 10, 20, 30, 60, 120, 180 and 240 minutes after instillation of the test solution. These samples were obtained after temporal drainage of
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approximately 200 mL to avoid a dead-space effect. Subsequent to the total drainage at 240 minutes, the peritoneal cavity was rinsed again with 1.36% Dianeeal. A sample from this rinse was used to calculated the residual volume. Blood was drawn at the start and at the end of the test. After the first blood sample was obtained, dextran 1 (20 mL, Promiten, NBPI, Emmer-Compascuum, The Netherlands) was given intravenously to prevent a possible anaphylaxis to dextran 70 [16].

Assays
Glucose concentrations in plasma and dialysate samples were assessed by the glucose oxidase-peroxidase method with an autoanalyzer (SMA-II; Technicon, Terrytown, NY, USA). Urea and creatinine were measured with enzymatic methods by an automated analyzer (Hitachi H747; Boehringer Mannheim, Mannheim, Germany). Total protein in plasma was determined by biuret methodology (Roche, Almere, The Netherlands) also with the use of an automated analyzer (Hitachi 747, Boehringer Mannheim, Mannheim, Germany). β2-Microglobulin, was determined with an Imx system applying a microparticle enzyme immunoassay (Abbot Laboratories, North Chicago, IL, USA). Albumin, IgG and α2-macroglobulin were measured by nephelometry (BN 100; Behring, Marburg, Germany). VEGF was measured in dialysate and serum as previously described [6]. In brief, a commercially available enzyme-linked immunosorbent assay (human VEGF, Quantikine; R&D Systems, Minneapolis, MN, USA) was applied, with a lower detection limit of 15.0 ng/L. Serum was allowed to clot for 4.5 hours. Before determination of the growth factor in dialysate, the effluent samples were concentrated 9.5 times (range 7.6-12.4) by positive pressure ultrafiltration using a 250 mL cell and a YM-10 membrane with a molecular cutoff point 10 kDa (Amicon Corp, Danvers, MA, USA). The concentration factor was defined as the quotient of the albumin concentration in the concentrate divided by the albumin concentration in the original effluent sample. Total dextran 70 was determined by high-performance liquid chromatography [17].

Calculations
Peritoneal fluid and solute kinetics were calculated as reported previously [14,15]. Briefly, fluid transport across the peritoneal membrane is influenced by opposing mechanisms. The transcapillary ultrafiltration volume increases the intraperitoneal volume, and fluid loss from the peritoneal cavity is assumed to occur through transcapillary back filtration and uptake into the lymphatic system. The resultant of these is the net ultrafiltration. The transcapillary ultrafiltration was calculated from the dilution of the volume marker. The convective disappearance of the volume marker from the peritoneal cavity can be used as an indirect measure to quantify the contribution of the peritoneal lymphatics in the absorption of fluid from the peritoneal cavity [18]. These calculations of the effective lymphatic absorption include all pathways of uptake into the lymphatic system, both interstitial and subdiaphragmatic. The change in intraperitoneal volume during the dwell can be assessed from the dilution of dextran 70 after correction for incomplete recovery. The net ultrafiltration is the
difference between the transcapillary ultrafiltration and the effective lymphatic absorption.

To express the transport of low-molecular-weight solutes urea and creatinine, mass transfer area coefficients (MTACs) were calculated according to the model of Wanieński [19]. The solute concentrations in plasma were corrected for plasma water [20]. Glucose absorption was estimated as the difference between the instilled and the recovered amount of glucose, relative to the instilled quantity of glucose in the dialysate. The peritoneal handling of the macromolecules β2-microglobulin, VEGF, albumin, IgG and α2-macroglobulin was expressed as dialysate-to-serum ratios (D/S). A peritoneal transport line was computed for all investigations performed in each patient, based on the least squares regression analysis of the D/S ratio of β2-microglobulin (mol wt = 11.8 kDa), albumin (mol wt = 69 kDa), IgG (mol wt = 150 kDa), and α2-macroglobulin (mol wt = 820 kDa) and their molecular weights when plotted on a double logarithmic scale [21]. The slope of this line represents the size selectivity of the peritoneal membrane as these proteins are transported from the circulation to the peritoneal cavity. By interpolation of the molecular weight of VEGF (mol wt = 34 kDa) in the regression equation, the expected D/S ratio was calculated, assuming the dialysate concentration of VEGF would be determined only by transport from the circulation. The concentration of the growth factor attributed to local production was defined as the difference between the measured and expected dialysate concentration.
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Statistics
The results are presented as median values and ranges. The differences between the measured and expected dialysate concentrations of VEGF were tested by a modified t-test to determine whether the deviation from the regression line was significant. This test takes the variability of the regression lines into account [22]. To investigate the absolute difference between the measured and expected dialysate concentrations of VEGF, Wilcoxon matched pairs rank sum test was applied. The differences in the effluent concentrations attributed to local production in the longitudinal follow-up of glucose based PD, were investigated with repeated measurement analysis of variance. Spearman rank correlation analysis was used for the calculation of correlation coefficients.

Results
Parameters of peritoneal permeability obtained from the last SPA during glucose peritoneal dialysis treatment are presented Table 1. Regression lines were calculated for each individual patient for each investigation, based on the power relationship between the D/S ratio of the various serum proteins and their molecular weights (see Methods). The r-values of the regression lines all exceeded 0.91 (p<0.05). Based on these regression lines, the D/S ratio of VEGF could be predicted when the concentration in the effluent would only be determined by transport from the circulation to the peritoneal cavity (Figure 1). The measured D/S ratio of VEGF was significantly greater (p<0.001) than could be expected based on the peritoneal transport line, indicating local production of VEGF (LVEGF). The follow-up of glucose peritoneal dialysis in 9 patients, in relation with LVEGF is plotted in Figure 2 (left panel). Nine patients are presented because 1 patient underwent only 1 SPA during glucose dialysis treatment before switching to the none glucose treatment. LVEGF increased with prolonged treatment of glucose peritoneal dialysis from 11.7 ng/L (2.0-19.0) to 23.5 ng/L (5.4-75.9), p=0.06. A comparison between the first and last LVEGF showed a significant rise (p<0.03, Figure 3, left panel). The magnitude of the increase in LVEGF was independent from the initial LVEGF, and independent from the duration of peritoneal dialysis at the time of the first investigation. The effect of the switch to glucose free PD on LVEGF production is presented in Figure 2, right panel. Comparing the LVEGF obtained from the last SPA during glucose based PD and LVEGF from the last one during glucose free PD showed a decrease from 57.4 ng/L (8.2-225.3) to 23.1 ng/L (4.4-61.7).

A correlation (r=0.83, p<0.001) was found between the difference of the MTAC of creatinine in the initial and last SPA during glucose PD, and the difference in LVEGF in the same investigations (Figure 4). A similar correlation (r=0.85, p<0.001) was present between the difference in peritoneal glucose absorption and the difference in LVEGF (Figure 4). The change in these parameters obtained in the patients after the switch to glucose free PD are shown in the same figure.
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Figure 2. The individual data of the longitudinal follow-up of VEGF attributed to local production in the peritoneum obtained in 9 patients during glucose based PD is presented in the left panel. These data obtained in 4 long-term PD patients before and after the switch to glucose free PD treatment are presented in the right panel. Time point t=0 is the last observation during glucose based PD, thereafter the patients were treated with glucose free dialysis solutions.

Table 1. Parameters of peritoneal permeability.

<table>
<thead>
<tr>
<th>Fluid Transport</th>
<th>Glucose PD</th>
<th>Non Glucose PD</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCUF (mL/4 hour)</td>
<td>667 (185-1059)</td>
<td>225 (126-796)</td>
</tr>
<tr>
<td>ELA (mL/4 hour)</td>
<td>250 (89-523)</td>
<td>132 (108-194)</td>
</tr>
<tr>
<td>NUF (mL/4 hour)</td>
<td>579 (81-1126)</td>
<td>41 (-16-602)</td>
</tr>
</tbody>
</table>

Solute Transport

- MTAC urea (mL/min): 17.7 (13.4-20.9) vs 15.5 (8.5-19.1)
- MTAC creatinine (mL/min): 9.0 (7.0-18.0) vs 10.6 (6.6-16.6)
- glucose absorption (%): 60 (44-75) vs 64 (44-76)
- effluent VEGF attributed to local production (ng/L): 17.3 (6.5-225.3) vs 23.1 (4.4-61.6)

Median and ranges are presented of data obtained from the last SPA during glucose PD, and the last glucose free investigation. TCUF: transcapillary ultrafiltration, ELA: effective lymphatic absorption, NUF: net ultrafiltration, MTAC: mass transfer area coefficient.

Discussion

In this study the presence of VEGF in peritoneal effluent of patients treated with glucose peritoneal dialysis was investigated during longitudinal follow-up, in relation with peritoneal permeability parameters. Furthermore, the effect of the switch to glucose free dialysis treatment on VEGF production and the characteristics of peritoneal permeability was studied in patients with ultrafiltration failure.

The concentration of VEGF in peritoneal effluent found in each patient in each observation, was higher than could be explained by diffusion from the circulation. This points to local peritoneal production of VEGF as was suggested.
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Figure 3. The left panel presents the first and last LVEGF determination during glucose based PD (left panel, p<0.03). The right panel shows the last LVEGF determination during glucose based PD compared to the last LVEGF observation during glucose free dialysis treatment.

by us previously [6]. In the present study VEGF was measured in serum. Recently platelet-mediated secretion of VEGF during clotting processes have been described by Webb et al. [23]. As a consequence serum levels are on average twice the concentration measured in EGFA plasma. We used a standardized clotting time of 4.5 hours before centrifugation. If VEGF would have been measured in EDTA plasma, the dialysate-to-plasma ratio would have been higher. Therefore, the calculated local production of VEGF in the present study may have been an underestimation of the actual LVEGF.

The production of VEGF attributed to local production increased with prolonged duration of glucose peritoneal dialysis. LVEGF decreased in all four patients with ultrafiltration failure after the switch to glucose free dialysis treatment. This points to a role for the high glucose concentrations in dialysis solutions to trigger VEGF production and the development of peritoneal neoangiogenesis [7], in analogy with VEGF production induced by hyperglycemia in diabetic retinopathy [1,2,5].

Low molecular weight solutes as creatinine and glucose have been shown not to be size selectively hindered by the peritoneal membrane [11,12], therefore their transport to the peritoneal cavity mainly depends on the peritoneal vascular surface area. Consequently, changes in MTACs or glucose absorption reflect changes in the peritoneal vascular surface area. In our crossectional study [6] MTAC creatinine and the glucose absorption correlated with LVEGF suggesting involvement of VEGF in peritoneal neoangiogenesis. The relationship that was present between the difference in MTAC creatinine between the first and the last observation on glucose PD, and the difference in LVEGF between these investigations, emphasizes a possible role for VEGF mediating the peritoneal vascular surface area during glucose PD. Especially because the difference in these parameters obtained in the last SPA during
Figure 4. The difference between the first and last MTAC creatinine, and the difference between the first and last observation of LVEGF during glucose based PD (●), and the last LVEGF determination during glucose based PD and the last one during none glucose based PD (■) are shown (upper panel r=0.83, p<0.001). A similar relationship was present between the difference in glucose absorption and the difference in LVEGF (lower panel, r=0.85, p<0.001).

glucose PD and those obtained in the last glucose free investigation showed the same relationship. LVEGF decreased after the switch to glucose free dialysis treatment, and the parameters of the peritoneal vascular surface area also decreased in 3 out of 4 patients. These finding support a causal relationship between the peritoneal exposure to extremely high glucose concentrations and the development of peritoneal angiogenesis, through a pathogenetic mechanism with involvement of VEGF.

The magnitude of the increase in LVEGF during glucose PD was however, independent from the initial LVEGF and the duration of peritoneal dialysis at the start of the follow-up during glucose based PD. Some patients showed a steep rise in LVEGF during less than 3 years follow-up of glucose based PD, where in others LVEGF increased only marginally during the same period of follow-up. This explains why no relationship between LVEGF and the duration of peritoneal
dialysis was found in our cross sectional study [6]. The great inter-individual variability suggests a role for individually determined factors that may influence the effect of the prolonged duration of glucose peritoneal dialysis on the slope of and the extent of the local production of VEGF in each PD patient. These factors may involve VEGF and other polymorphisms. However, these speculations require further study.

References