Chapter 5

Sunitinib treatment prevents angiogenesis in a rat model for peritoneal dialysis

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Abstract

Background: Long term exposure to peritoneal dialysis (PD) fluids leads to the formation of new blood vessels, which is thought to be one of the dominant factors to contribute to ultrafiltration failure. In order to prevent angiogenesis we studied the effects of the tyrosine kinase inhibitor sunitinib, known to inhibit VEGF and PDGF receptor signalling, in a rat PD model.

Methods: Rats were daily instilled with 10 ml PD fluid via an i.p. access port. Half of the animals received oral sunitinib treatment. Animals without catheter, with or without sunitinib treatment, served as controls. After 5 weeks, a peritoneal transport test was performed and tissues were taken for blood vessel analysis.

Results: During the third week of treatment animals developed severe diarrhoea and suffered from weight loss. After five weeks of sunitinib treatment, PD induced angiogenesis was evidently prevented in omentum and mesentery, however ultrafiltration did not significantly improve.

Conclusion: This study indicates that during PD vascular surface area is not directly correlated to ultrafiltration and suggests that other processes like fibrosis and inflammation are involved in ultrafiltration failure.

Introduction

Long term exposure to peritoneal dialysis (PD) fluids induces morphological changes, often seen in combination with loss of ultrafiltration capacity[1;2]. As a result, the peritoneal membrane loses its capacity to function as dialysis membrane. The formation of new blood vessels is thought to be one of the most major causes of ultrafiltration loss and technique failure[3;4].

A well known regulator of angiogenesis is vascular endothelial growth factor (VEGF)[5]. In addition, the importance of platelet derived growth factor (PDGF) is increasingly recognised[6]. These growth factors bind to cell surface receptors, activating intracellular signalling pathways such as tyrosine kinases. Tyrosine kinase receptor inhibitors are able to reduce activated growth factor signals, and have been found to have effective antitumor activity due to the blocking of endothelial cell migration and new blood vessel formation[7]. Sunitinib is a tyrosine kinase inhibitor involved in the inhibition of VEGF and PDGF receptor signalling[8], and may therefore be a possible candidate in the prevention of new vessel formation during PD which may lead to an improved ultrafiltration capacity.

Material and methods

Animals

Male wistar rats (Harlan CPB, Horst, The Netherlands) weighing 275-300 gram at the beginning of the experiment were used throughout the study. Animals were housed under conventional conditions and the experimental design was approved by the animal care committee of the Vrije Universiteit Amsterdam.

Experimental setup

A peritoneal catheter was implanted in 30 male rats (Harlan CPB, Horst, The Netherlands). Rats received daily conventional PD fluid for five weeks as described previously[9]. Untreated rats (n=16) served as controls. Half of the PD group and half of the control group received daily sunitinib (20 mg/kg, Sutent malate, a kind gift of Pfizer, New York, USA) via oral gavage. After 5 weeks, in all animals a 90 minute peritoneal equilibrium test (PET) test was performed by injecting 30 ml of PD fluid into the peritoneal cavity via a direct i.p. catheter (Venflon pro, BD Medical, New Jersey, USA).

Analysis of peritoneal effluents

After the 90 minute PET test the PD fluid was drained and the ultrafiltration capacity was calculated. VEGF levels were measured by ELISA (R&D systems, Abingdon, United Kingdom) in 10-15 times concentrated PET effluents.

Histological analysis

For histological analysis, a part of the omentum and mesenteric tissue was dissected and spread on a glass slide. To visualize vasculature the tissue was stained using toluidine blue staining and CD31-antibody (PECAM; Serotec, Oxford, United Kingdom) in combination with a fluorescently labeled secondary antibody (Invitrogen, Carlsbad, CA, USA) for detection. Images were analyzed blinded by digital image analysis (AnalySIS; Soft Imaging System, Olympus, Hamburg, Germany), and positive CD31 staining was calculated as a percentage of the total tissue area.

Statistics

The non-parametric Mann-Whitney-U-test with Bonferroni correction for multiple comparisons was applied and probability values of $p<0.05$ were considered significant. We made three comparisons, namely: control vs control+sunitinib, control vs PD and PD vs PD+sunitinib.

Results

To prevent the formation of new vessels during PD treatment, rats were treated daily with 20 mg/kg sunitinib, which is seen as the efficacious dose in rats[10;11]. Two weeks after the start of the experiment side effects of the daily sunitinib treatment started to appear. Sunitinib treated animals started to lose weight (up till 10%) and the eyes and fur showed yellow discoloration. During the third week of treatment animals developed severe diarrhoea and overall discomfort (fatigue). Therefore, from week four on the daily sunitinib treatment was reduced to 3 times a week, resulting in a slight recovery of the animals in the last week of the experiment. After sacrificing severe ill animals and drop out due to omental wrapping, 8 control, 7 control+sunitinib, 8 PD and 8 PD+sunitinib animals remained for analysis.

Sunitinib treatment significantly enhanced the filtration capacity in control animals (ultrafiltration/100gr body weight control vs. control + sunitinib; p=0.035). Five weeks of PD treatment did not induce the expected ultrafiltration failure and in PD treated rats. The effect of sunitinib in PD treated animals on ultrafiltration was only minor (~0.3 ml/100 gram body weight, Table 1).
However, after 5 weeks of PD fluid treatment a significant increase in vessel formation was found. Sunitinib treatment completely prevented new vessel formation in omentum ($p<0.05$, Figure 1A and 1C) as well as mesentery ($p<0.01$, Figure 1B and 1D) in PD treated animals. Blood vessel surface area in the mesentery of both sunitinib groups appeared to be even less compared to control rats (p=0.01). Toluidine blue staining of the mesentery confirmed the prevention of angiogenesis and showed a loss of mast cells after sunitinib treatment (Figure 1D).
VEGF levels measured in the effluents on the other hand, were significantly increased upon sunitinib treatment in control (>10 fold) as well as in PD treated animals (> 6 fold) (Table 1).

Table 1: Body weight, ultrafiltration and VEGF levels

	Control	Control+Sunitinib	PD	PD+Sunitinib
Body weight (gram)	442.5 +/-10.8	355.0 +/-14.8[a]	410.0 +/-9.5	346.5 +/-13.4[b]
Ultrafiltration (ml)	8.5 +/-0.7	10.0 +/-0.9	8.5 +/- 0.3	9.0 +/-1.3
Ultrafiltration/100 gr BW (ml)	2.0 +/-0.1	2.7 +/-0.3[a]	2.1 +/-0.1	2.3 +/-0.5
Dialysate VEGF (pg/ml)	0.7 +/-0.	7.1 +/-2.8[a]	2.6 +/-0.8[a]	16.6 +/-7.8[b]

Data given as median +standard error, [a]:p<0.05 compared to control, [b]:p<0.05 compared to PD.
PD=peritoneal dialysis; VEGF:=vascular endothelial growth factor.

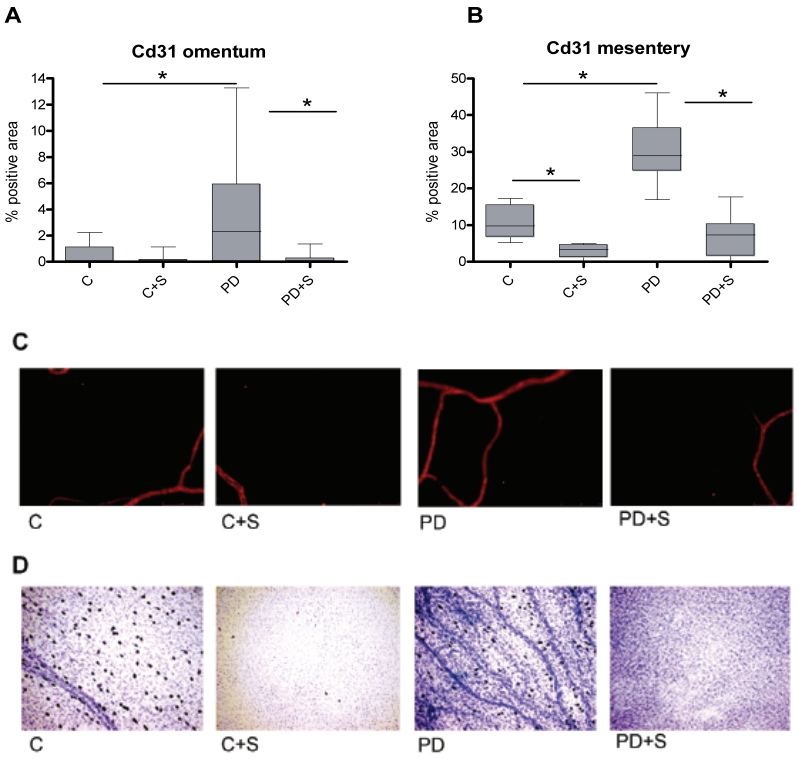

Figure 1: Prevention of angiogenesis by sunitinib treatment.
Percentage of vessel surface area in mesentery (2A, *p<0.01) and omentum (2B, *p<0.05) measured by CD31. Box plots indicate the median and 5-95% level. Representative examples of CD31 staining of omentum (2C). Toluidine blue staining of mesentery confirms CD31 measurements (2D). Magnifications 10x. C=control, C+S=control+sunitinib, PD=peritoneal dialysis, PD+S=PD+sunitinib

Discussion

These data show that sunitinib is very potent in inhibiting angiogenesis in our rat PD model. However, the treatment at the given dose induced severe side effects. This dose was based on literature describing long-term rat experiments using a dosage of 20 mg/kg/day for 30-40 days, which is equivalent to the efficacious dose of 40 mg/kg in the mouse based on allometric scaling[10;11]. Several long-term mouse studies have been performed, in which 40 mg/kg/day was used without reporting side effects[10;12;13]. In line with our results, after this study was finished, Pfizer reported similar side effects of sunitinib at dosages of 5 and 15 mg/kg/day[14].
Intriguingly, the fact that in control animals significantly less vessels were found after sunitinib treatment compared to control rats, suggests that even mature existing vessels may be somehow affected by the treatment. Initially tyrosine kinase inhibitors are not expected to target normal vasculature, because most blood vessels remain quiescent during adulthood[15]. However, under normal physiological circumstances, growth factor signalling in endothelial cells seems to be important for the survival and maintenance of vascular integrity. Inhibitors of angiogenesis are capable of affecting signalling pathways in endothelial cells and might elicit toxicities as a result of decreased endothelial renewal capacity[16].
Along with the prevention of blood vessel formation, a loss of mast cells was seen in the mesentery of sunitinib treated animals. The loss of mast cells in peritoneal membranes was indeed previously associated with a reduction in vessel formation[17].
The high dialysate VEGF levels possibly reflect an increased VEGF production or lack of degradation due to blocking of the VEGF receptor signalling. Sunitinib induced increases in plasma VEGF levels have also been reported by previous studies[18;19], in which the increased VEGF levels restored to near baseline levels after a two week off-treatment period, during which sunitinib levels are cleared, indicating that the increased VEGF levels were due to sunitinib activity[19].

Unfortunately, the standard PD treatment did not induce loss of ultrafiltration as seen in our previous studies[20;21]. The improvement of biocompatible solutions but also of conventional dialysis fluids, makes that we have to prolong our experiments and expose rats longer than 5 weeks to PD fluid. However, due to the severe side effects of sunitinib this experiment was ended after 5 weeks.
Nevertheless, treatment with PD fluid for 5 weeks resulted in a significantly increased vascular surface area, which was completely prevented by sunitinib treatment. Interestingly, the vascular surface area did not influence the ultrafiltration capacity in the PD groups. This indicates that during PD the vascular surface area is not directly

correlated to ultrafiltration and that other processes like inflammation and fibrosis are likely to be involved in ultrafiltration failure.

The prevention of angiogenesis during PD is gaining increasing interest. Animal studies have shown that anti-VEGF antibody therapy prevents microvascular alterations in the peritoneal membrane[22]. Also octreotide was shown to markedly reduce angiogenesis by reducing VEGF production[23]. However, clinical trials using specific anti-angiogenic therapies showing prevention of ultrafiltration failure are still lacking[24]. Therefore, further research on the inhibition of angiogenesis in combination with prevention of fibrosis and inflammation during PD is warranted, focussing on treatment regimens with new inhibitors without undesirable side effects.

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