Soluble Tie2 fusion protein decreases peritoneal angiogenesis in uremic rats

JING XIAO1*, JIA GUO1*, XIN-XIN LIU1, XIAO-XUE ZHANG1, ZHEN-ZHEN LI2, ZHAN-ZHENG ZHAO1 and ZHANG-SUO LIU1

1Department of Nephrology, and 2Key Disciplines Laboratory Clinical Medicine Henan, The First Affiliated Hospital, Zhengzhou University, Zhengzhou, Henan 450052, P.R. China

Received December 26, 2012; Accepted April 23, 2013

DOI: 10.3892/mmr.2013.1478

Abstract. Angiogenesis is considered to be one of the most common mechanisms leading to ultrafiltration failure (UFF) in long-term peritoneal dialysis (PD) patients. The angioptin (Ang)/Tie system was found to play a role in the initiation of pathological neoangiogenesis and is also involved in peritoneal angiogenesis caused by peritoneal fluid. This aim of this study was to investigate the effects of the soluble Tie2 fusion protein (sTie2/Fc) on peritoneal angiogenesis in PD-treated uremic rats. The rats were divided into 6 groups: normal, sham surgery, uremic rats without PD, uremic PD-treated rats, uremic rats treated with PD and sTie2/Fc (0.25 µg/100 g) and uremic rats treated with PD and sTie2/Fc (0.5 µg/100 g). PD rats were treated once a day for 28 days prior to testing. Real-time polymerase chain reaction (RT-PCR) or tissue immunohistochemical staining was used to detect Ang-2 mRNA or protein expression in the peritoneal tissues of each group. The microvessel density (MVD) of the peritoneum was detected and quantified by immunohistochemical staining using the anti-CD34 antibody. Compared with the control group, Ang-2 mRNA and protein expression was significantly upregulated in the uremic and PD groups (P<0.05). MVD in the experimental group increased compared with the control group. sTie2/Fc treatment decreased the levels of Ang-2 mRNA and protein expression (P<0.05) in a dose-dependent manner and decreased PD-induced MVD in the peritoneum. In conclusion, angiogenesis of the peritoneum induced by PD was inhibited using sTie2/Fc in a uremic rat model.

Introduction

Peritoneal dialysis (PD) is one of the most effective ways to treat end-stage renal disease (ESRD) patients. Peritoneal structure and function are essential factors in the maintenance of PD; however, the daily instillation of PD fluid (PDF) leads to morphological and functional alterations of the peritoneal membrane. These alterations ultimately lead to ultrafiltration (UF) failure (UFF), which results in PD patients opting out of treatment, or even in mortality. For long-term PD patients, increasing angiogenesis is one of the significant changes to the peritoneum (1). According to a previous report, peritoneal angiogenesis is important in the mechanism of UFF (2).

Angiopoietin (Ang) is important in the initiation of pathological neoangiogenesis (3,4). Within the Ang family, Ang-2 has been shown to be associated with neoangiogenesis (5). Under hypoxic conditions or in the presence of vascular endothelial growth factor (VEGF), Ang-2 leads to angiogenesis through promotion of the proliferation and migration of vascular endothelial cells, and by increasing the sensitivity of endothelial cells to VEGF (6,7). In the absence of VEGF, Ang-2 may lead to vessel regression (8). Ang-2 binds to the tyrosine kinase receptor Tie2, forming the Ang-2-Tie2 complex, which is involved in angiogenesis. However, there are limited reports with regard to the impact of the Ang/Tie system in peritoneal angiogenesis (9,10). The extracellular Tie2 domain is released by proteases, forming soluble Tie2 (sTie2) that competitively inhibits the binding of Ang-2 to Tie2 (11).

To identify new therapeutic modalities against PD-induced angiogenesis, a uremic PD rat model treated with sTie2 fusion protein (sTie2/Fc) was used in this study.

Materials and methods

Reagents. Silicon-based (GB 18671-2002; Shandong Weigao Medical Polymer Co., Shandong, China) ethylene oxide sterilized PD catheters were manufactured in our laboratory (12,13). Reagents used in this study were as follows: Ang-2 or CD34 polyclonal antibodies, DAB, the ultrasensitive SP kit (Maixin Inc., Fuzhou, China), total RNA extraction reagent TRIzol (Bio Basic Inc., Amherst, NY, USA), reverse transcription kit, RT-PCR amplification kit (Takara Co., Shiga, Japan), recombinant rat sTie2/Fc (R&D
Detection of Ang-2 protein and MVD in rat peritoneal tissue. Tissue specimens were embedded in paraffin and 5-µm sections were obtained. From each rat, one section of the peritoneum was randomly selected for immunohistochemical pathological examination to detect Ang-2 protein expression and MVD.

Specimens were dewaxed and rehydrated, treated with 3% H₂O₂ to block endogenous peroxidase and incubated in 5% goat antiserum for 30 min at 37°C. The sections were marked with Ang-2 antibody (1:80 dilution) and incubated overnight at 4°C. The sections were washed once with PBS (0.01 M, pH 7.4), then a biotin-labeled secondary antibody was added and incubated at 37°C for 35 min. Avidin biotin-peroxidase complex and DAB were used for the color reaction. PBS (0.01 M, pH 7.4) without primary antibody was used as a negative control (16).

Detection of Ang-2 mRNA in peritoneal tissues. Total RNA was isolated with a single-step method using TRIzol reagent, according to the manufacturer's instructions (17). An ultraviolet spectrophotometer XO-1101 (Kai-Di high-speed analytical instruments Co., Ltd., Nanjing, China) was used to measure the total RNA concentration. Complementary DNA (cDNA) was synthesized according to standard procedures (18). The primers were as follows: Ang-2 (100 bp product) sense, 5'-CGGCCACAGTCAACAACTCA-3' and antisense, 5'-GCTCTTATAGTCGGGCGATGA-3'; sTie2/Fc sense, 5'-CGGCCACAGTCAACAACTCA-3' and antisense, 5'-GCTCTTATAGTCGGGCGATGA-3'; and β-actin (222 bp product) sense, 5'-AGCCATGTACGTAGCCATCC-3' and antisense, 5'-GCTCTTATAGTCGGGCGATGA-3'.

Electrophoresis was performed and imaging system analysis software was used to capture images and determine the absorbance (A) value (Bio-Rad, Berkeley, CA, USA). Ang-2 mRNA was expressed as the ratio of its amplicon to β-actin amplicon (Amp). The omentum was removed from the sacrificed rats and fixed in a sufficient amount of 4% formaldehyde (Baxter, Deerfield, IL, USA). The specimens were dewaxed and rehydrated, treated with 3% H₂O₂ to block endogenous peroxidase and incubated in 5% goat antiserum for 30 min at 37°C. The sections were marked with Ang-2 antibody (1:80 dilution) and incubated overnight at 4°C. The sections were washed once with PBS (0.01 M, pH 7.4), then a biotin-labeled secondary antibody was added and incubated at 37°C for 35 min. Avidin biotin-peroxidase complex and DAB were used for the color reaction. PBS (0.01 M, pH 7.4) without primary antibody was used as a negative control (16).

Detection of Ang-2 mRNA in peritoneal tissues. Total RNA was isolated with a single-step method using TRIzol reagent, according to the manufacturer's instructions (17). An ultraviolet spectrophotometer XO-1101 (Kai-Di high-speed analytical instruments Co., Ltd., Nanjing, China) was used to measure the total RNA concentration. Complementary DNA (cDNA) was synthesized according to standard procedures (18). The primers were as follows: Ang-2 (100 bp product) sense, 5'-CGGCCACAGTCAACAACTCA-3' and antisense, 5'-GCTCTTATAGTCGGGCGATGA-3'; and β-actin (222 bp product) sense, 5'-AGCCATGTACGTAGCCATCC-3' and antisense, 5'-GCTCTTATAGTCGGGCGATGA-3'.

Detection of Ang-2 mRNA in peritoneal tissues. Total RNA was isolated with a single-step method using TRIzol reagent, according to the manufacturer's instructions (17). An ultraviolet spectrophotometer XO-1101 (Kai-Di high-speed analytical instruments Co., Ltd., Nanjing, China) was used to measure the total RNA concentration. Complementary DNA (cDNA) was synthesized according to standard procedures (18). The primers were as follows: Ang-2 (100 bp product) sense, 5'-CGGCCACAGTCAACAACTCA-3' and antisense, 5'-GCTCTTATAGTCGGGCGATGA-3'; and β-actin (222 bp product) sense, 5'-AGCCATGTACGTAGCCATCC-3' and antisense, 5'-GCTCTTATAGTCGGGCGATGA-3'.
cell, or a cluster of endothelial cells clearly separated from adjacent microvessels and other connective tissue elements were considered to be vessels.

Statistical analysis. Data were expressed as the mean ± SD. Multiple comparisons were initially subjected to one-way analysis of variance (ANOVA). Correlations were analyzed using the Pearson's correlation test. Statistical analysis was performed using SPSS13.0 software (SPSS Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Renal function of the uremic rat model. There were no statistically significant differences in the serum creatinine (Scr) levels between the control and sham surgery groups. However, serum creatinine levels were increased 2-3-fold in uremic rats (P<0.05) compared with the control group (Table I).
Effects of recombinant rat sTie2/Fc on the expression of Ang-2 in peritoneal tissue. Ang-2 mRNA and protein expression was observed in each group. There were no significant differences in Ang-2 mRNA and protein expression between the control and sham surgery groups. RT-PCR demonstrated that there was a significant difference in Ang-2 mRNA expression between the control, uremic and dialyze groups (0.196±0.036 vs. 0.334±0.041 vs. 0.796±0.019, respectively; P<0.05). Furthermore, Ang-2 mRNA expression was significantly different between the dialyze, dialyze + 0.25Tie2 and dialyze + 0.50Tie2 groups (0.796±0.019 vs. 0.607±0.033 vs. 0.349±0.021, respectively; P<0.05; Fig. 1). Ang-2 protein expression was observed in peritoneal capillary endothelial cells and in the cytoplasm of the peritoneal mesothelial cells using immunohistochemistry. Of all the groups, staining in the dialyze group was the strongest, whilst it was weakest in the dialyze + 0.50 Tie2 group (Fig. 2).

Effects of recombinant rat sTie2/Fc on MVD. Few new microvessels were observed in the control and sham surgery groups. However, there was a significant difference in the MVD between control, uremic and dialyze groups (1.78±0.29 vs. 4.16±0.31 vs. 9.53±0.33, respectively; P<0.05). Moreover, there was a significant difference in the MVD between the dialyze, dialyze + 0.25Tie2 and dialyze + 0.50Tie2 groups (9.53±0.33 vs. 7.27±0.27 vs. 5.21±0.25, respectively; P<0.05; Fig. 3).

Spearman results. A significant positive correlation was observed between Ang-2 mRNA expression and MVD (r=0.9790; P=0.003; Fig. 4).

Discussion

UFF is one of the main reasons that long-term PD patients choose to discontinue their treatment (20). Peritoneal angiogenesis is considered to be the most probable mechanism leading to UFF (1). Increases in peritoneal angiogenesis lead to an increase in peritoneal vascular surface area, which accelerates the transport of small soluble molecules; this causes rapid disappearance of the osmotic gradient, thus inducing the loss of the peritoneal UF capacity. These events may predict not only technical failure, but also patient mortality (21,22). Studies have revealed that anti-angiogenic treatments, such as angiostatin, slow the process of peritoneal angiogenesis, which is a more effective method of protecting the peritoneal membrane function than directly inhibiting peritoneal fibrosis (23). As with any disease, early intervention, diagnosis and treatment may improve the outcome for PD patients.

In 1996, Hanahan and Folkman (24) proposed a hypothesis on the balance of the angiogenesis switch, where angiogenesis is regulated by an equilibrium between inducers and inhibitors. Once the balance is broken, the endothelial cells are activated and promote neoangiogenesis. Other studies have demonstrated that in a uremic and high-glucose environment, the levels of certain inducers (including the fibroblast growth factor and VEGF) were raised in the peritoneum and positively correlated with angiogenesis (24-27). Inhibitors (including thrombospodin-1, 16 kD prolactin, interferon α/β, platelet factor-4 and angiostatin) are able to inhibit peritoneal neoangiogenesis by downregulating the expression of inducer factors in the peritoneum (24,27).

It has been demonstrated that the Ang/Tie pathway is an inducer factor in vasculogenesis and angiogenesis, but little is known about its involvement in peritoneal angiogenesis. One study confirmed increased angiogenesis and fibrosis in the uremic rat model under PD, and suggested that this increase was due to increased Ang-2 levels associated with decreased Tie2, indicating that this pathway may be targeted in order to preserve the peritoneum (9). The present study revealed that in a uremic and high-glucose environment, Ang-2 mRNA and protein expression increased in peritoneal tissues, indicating that upregulation of Ang-2 was the likely cause of peritoneal angiogenesis. Furthermore, we observed a positive correlation between Ang-2 and peritoneal angiogenesis.

A previous study (27) reported that soluble receptors for angiogenic growth factors, which were used as endogenous ligand antagonists, may be useful in inhibiting signal transduction, thus influencing the biological action of ligands. Studies showed that the Ang/Tie2 system was involved in cancer tissue and retinal neovascularization, and that the use of a sTie-2/Fc effectively inhibited angiogenesis (28,29). Results of the present study are consistent with a previous study (9) demonstrating that the Ang/Tie2 system is upregulated in a uremic rat model treated with PD. Results suggest that inhibiting this pathway may be an effective method of preventing peritoneal angiogenesis, allowing PD patients to continue with their treatment and improving survival rates. This study demonstrated that sTie-2/Fc may be used to effectively inhibit peritoneal angiogenesis. VEGF and VEGF receptor inhibitors have been approved for angiogenesis inhibition in a number of studies, but their use alone is unable to completely prevent angiogenesis (28,30). As VEGF is also upregulated in uremic patients under PD (31), the combined use of a VEGF inhibitor and sTie-2/Fc may be an effective method to reduce angiogenesis in PD patients (28).

In conclusion, results from this study suggest that targeting Ang-2 using a soluble Tie-2 receptor may be an effective method for preventing peritoneal angiogenesis in uremic patients undergoing PD. This study also provides an experimental basis for the development of a future clinical treatment using sTie-2/Fc.
References