ULTRAFILTRATION DYSFUNCTION IN PERITONEAL DIALYSIS: THE ROLE OF INFLAMMATION, FIBROSIS, AND ANGIOGENESIS.

By

PETER J MARGETTS

A Thesis

Submitted to the School of Graduate Studies

In Partial Fulfillment of the Requirements

for the Degree

Doctor of Philosophy

McMaster University

DOCTOR OF PHILOSOPHY (2002) McMaster University

(Medical Sciences) Hamilton, Ontario

TITLE: Ultrafiltration dysfunction in peritoneal dialysis: The role of inflammation, fibrosis, and angiogenesis.

AUTHOR: Peter J Margetts, BSc, MD, FRCPC

SUPERVISOR: Jack Gauldie, PhD

ABSTRACT

Background: Peritoneal dialysis (PD) is a valuable therapy for end-stage renal disease. Changes in the peritoneal transport properties, including increased solute transport and ultrafiltration (UF) dysfunction are main clinical limitations of PD. The capillary wall of peritoneal blood vessels plays a key role as a barrier to solute transport. Therefore, neovascularization of the peritoneal tissues is a main determinant in the functional changes in the peritoneal membrane. **I hypothesize that ultrafiltration dysfunction is caused by increased peritoneal vascularization with a concomitant increase in glucose transport from the peritoneal cavity and rapid loss of the ultrafiltration gradient. Further, I hypothesize that this increased vascular surface area is induced by profibrotic and inflammatory cytokines such as transforming growth factor (TGF) β, interleukin (IL) 1β, and tumour necrosis factor (TNF) α through upregulation of angiogenic cytokines such as vascular endothelial growth factor (VEGF).**

Methods: I used adenovirus mediated gene transfer of cytokines such as TGFβ1, IL-1β, and TNFα to the peritoneal of rats and studied the effects on histology, angiogenesis, gene regulation, solute transport, and UF. I also used an animal model of daily peritoneal exposure to dialysate solution. The angiogenic and fibrogenic response of the peritoneum to this model was detailed, along with the effect of overexpression of angiostatin and decorin by adenovirus mediated gene transfer.

Results: Adenovirus mediated gene transfer of TGFβ1 led to increased peritoneal fibrosis and angiogenesis which persisted to 28 days. These histologic changes were associated with increased solute transport of glucose and decreased UF. In vitro and in

vivo, TGFβ1 appeared to up regulate expression of VEGF. Inflammatory cytokines such as TNFα and IL-1β both induced fibrosis, angiogenesis, and peritoneal membrane dysfunction. The kinetics of the response was quite different. TNFα induced a very transient response with a complete resolution of changes by 21 days after adenovirus infection. The response induced by IL-1β was more sustained. I hypothesize that the strong expression of tissue inhibitor of metalloproteinase after IL-1β treatment may explain the prolonged fibrogenic response after IL-1β. Daily exposure to dialysate also induced a strong fibrogenic and angiogenic response in the peritoneum. The anti-angiogenic agent angiostatin, when overexpressed using adenovirus mediated gene transfer, reduced the vascularization and improved the ultrafiltration dysfunction. Decorin, a proteoglycan that binds and inactivates TGFβ, reduced peritoneal fibrosis, but did not alter the angiogenic response, nor did it improve peritoneal membrane function.

Discussion: These experiments have clarified the role of angiogenesis in UF dysfunction. The correlation between blood vessel density and ultrafiltration, along with the improvement in UF after treatment with angiostatin, is compelling evidence that vascularization of the peritoneal membrane causes UF dysfunction. There is a close association between fibrosis and angiogenesis in the peritoneum. Fibrosis appears to be necessary for a prolonged angiogenic response. Further work is required to identify the factors leading to the interaction between fibrosis and angiogenesis. The role of the interstitium and lymphatics in solute transport and UF dysfunction needs to be better defined. Finally, anti-angiogenic therapies need to be studied in these models of peritoneal membrane dysfunction and eventually applied to patients on PD to improve the quality and duration of this therapy.

ACKNOWLEDGEMENT

I would like to thank Dr. Jack Gauldie for the opportunity to pursue this work. The supervisory committee, Dr. Zhou Xing, Dr. Larry Arsenault, and Dr. David Ludwin, provided their time and advice for this work. Dr. Cathy Hoff and Baxter Healthcare provided tremendous support both financial and scientific for these projects. I have been supported by the Canadian Institutes of Health Research and the Kidney Foundation of Canada. I would also like to recognize my colleagues Dr. Martin Kolb, Lisa Yu, Tom Galt, Dr. Margaret Kelly, Jane Ann Schroeder, Mary Jo Smith, and Dr. Philippe Bonniaud for their assistance. Finally, my family deserves the most recognition for being endlessly patient with me.

LIST OF ABBREVIATIONS

AGE - advanced glycation endproducts
ALK - activin receptor-like kinase
ANG - angiopoietin
CTGF - connective tissue growth factor
EPS - encapsulating sclerosing peritonitis
ESRD - end stage renal disease
FGF - fibroblast growth factor
GDP - glucose degradation products
HIF - hypoxia inducible factor
IL - interleukin
LPS - lipopolysaccharide
MMP - matrix metalloproteinase
NOS - nitric oxide synthase
PAI - plasminogen activator inhibitor
PD - peritoneal dialysis
PDGF - platelet derived growth factor
RRD - residual renal function
TGFβ - transforming growth factor β
TGFβR- transforming growth factor β receptor
TIMP - tissue inhibitor of metalloproteinase
TNF - tumor necrosis factor
UF - ultrafiltration
VEGF - vascular endothelial growth factor
VSMC - vascular smooth muscle cell

TABLE OF CONTENTS

TITLE PAGE ---------- ii
ABSTRACT ---------- iii
ACKNOWLEDGEMENTS ---------- v
LIST OF ABBREVIATIONS ---------- vi
TABLE OF CONTENTS ---------- vii

CHAPTER 1: INTRODUCTION
Epidemiology ---------- 1
Outcomes ---------- 2
Encapsulating Peritoneal Sclerosis ---------- 5
Pathophysiology ---------- 5
Increased Vascular Surface Area ---------- 6
Aquaporin Dysfunction ---------- 7
Lymphatic and Tissue Reabsorption ---------- 8
Etiology ---------- 8
Bioincompatible Fluids ---------- 8
Peritonitis ---------- 10
Uremia and Systemic Inflammation ---------- 13
Treatment of Ultrafiltration Dysfunction ---------- 14
Icodextrin ---------- 14
Biocompatible Solutions ---------- 15
Alteration of the Interstitial Compartment ---------- 16
Anti-angiogenic Therapy ---------- 16
Peritoneal Fibrosis and the Role of TGFβ ---------- 18
Angiogenesis in Fibrotic Peritoneal Tissue ---------- 20
Animal Models of PD ---------- 24
Summary ---------- 26

CHAPTER 2: GENE TRANSFER OF TRANSFORMING GROWTH FACTOR β TO THE RAT PERITONEUM: EFFECTS ON MEMBRANE FUNCTION ---------- 28

CHAPTER 3: INFLAMMATORY CYTOKINES, ANGIOGENESIS, AND FIBROSIS IN THE RAT PERITONEUM ---------- 41

CHAPTER 4: A CHRONIC INFLAMMATORY INFUSION MODEL OF PERITONEAL DIALYSIS IN RATS ---------- 53

CHAPTER 5: ANTIANGIOGENIC AND ANTIFIBROTIC GENE THERAPY IN A CHRONIC INFUSION MODEL OF PERITONEAL DIALYSIS IN RATS ---------- 60

CHAPTER 6: DISCUSSION ---------- 69

REFERENCES ---------- 76

CHAPTER 1: INTRODUCTION

Peritoneal dialysis (PD) is a valuable therapy for end-stage renal disease (ESRD). It is used by over 100,000 patients world wide which accounts for approximately 15% of the dialysis population [1]. A lack of randomized clinical trials comparing dialysis modalities has meant that the relative value of PD is still uncertain and contested. The clinical experience with PD suggests that it is a useful early therapy in ESRD and is a necessary third modality to complement hemodialysis and transplantation.

Along with peritonitis and inadequate removal of uremic toxins, ultrafiltration (UF) dysfunction is a main clinical limitation of PD. Ultrafiltration refers to the net fluid removal across the peritoneal membrane. Over time, PD patients develop an increased transport of small molecular weight solutes across the peritoneal membrane with a concomitant decrease in ultrafiltration [2]. Solutes, such as urea, creatinine, phosphate, sodium, and potassium, travel from the blood to the peritoneal dialysis fluid. On the other hand, glucose travels from the dialysate solution to the blood. The capillary wall of peritoneal blood vessels plays a key role as a barrier to solute transport [3]. Therefore, neovascularization of the peritoneal tissues is a main determinant in the functional changes in the peritoneal membrane. This increased vascular surface area leads to an increase in glucose transport from the peritoneal cavity. Glucose is the main osmotic agent used in PD, and the rapid loss of glucose leads to a rapid loss of the UF gradient. The role of increased fluid absorption from the peritoneum by lymphatics and tissue [4], along with dysfunction of aquaporins in the peritoneal vessel walls [5], is less well defined.

Epidemiology

In various studies, the prevalence of UF dysfunction leading to technique failure has been reported between 1.7% [6] and 13.7% [7]. These data are confounded by the difficulty in accurately diagnosing the cause of technique failure. Using a clinical definition of UF dysfunction, prevalence rates have been reported between 15.3% [8] to 30.9% [9]. Ho-dac-Pannekeet and colleagues used the recommended 4 hour high osmolarity PET and found the prevalence of UF dysfunction to be 23% in a group of 68 stable PD patients [10].

Changes in the peritoneal membrane function over time have been documented in several studies and recently reviewed [11]. The majority of longitudinal studies of peritoneal membrane function find an increase in small molecular weight solute transport with time on dialysis and a coincident decrease in ultrafiltration [2;12]. The onset of UF dysfunction appears between 2 [13] and 4 [2] years after the initiation of PD. The inverse correlation between UF and solute transport has been clearly demonstrated in these studies. Lo and colleagues demonstrated a centripetal effect of membrane function over time [14]. Among 55 PD patients followed for a mean of 22 months, there was an increase in solute transport in patients with initial low transport peritoneal membranes, and a decrease in solute transport in high transporters over time. One possible explanation is a selective censoring by technique failure or death of high transport patients while the low transport group experience a slow increase in solute transport over time [11]. This may also explain to some extent the apparent stability of peritoneal membrane function over the first 2–4 years of PD.

Outcomes

Several features at the initiation of PD predict subsequent survival. These features include high transport status peritoneal membrane [15-18], low serum albumin [15;19], age [15;20], comorbid disease (including diabetes) [21;22], and residual renal function (RRF) [19;23]. High transport status at the initiation of dialysis has also been shown to be associated with decreased UF function and poor outcome [15]. Acquired UF dysfunction is associated with decreased survival in several studies. Wang and colleagues measured the peritoneal membrane function in a cross section of 46 established PD patients and found increased solute transport to be inversely associated with fluid removal and survival [17]. In a study of 125 incident PD patients, Ates and colleagues demonstrated the inpact of total fluid removal and sodium removal (including peritoneal and renal) on survival [23]. From a large longitudinal database, Davies and colleagues compared survivors and non-survivors and demonstrated that non-survivors had a progressive increase in small solute transport with time on dialysis [16].

UF dysfunction is also associated with other poor prognostic indicators such as clinical volume expansion [24] and diastolic hypertension [25]. Volume expansion in PD patients is also associated with hypoalbuminemia [26] and adjusting dialysis prescription to increase UF can reduce volume expansion and increase serum albumin [27].

In summary, there is evidence that both intrinsic and acquired UF dysfunction is associated with decreased survival in PD patients. UF dysfunction is associated with volume expansion and this may be associated, through hypertension, left ventricular hypertrophy, pre-existing co-morbid disease, systemic inflammation, and

hypoalbuminemia, with increased mortality and morbidity, primarily through cardiovascular outcomes (Figure 1).

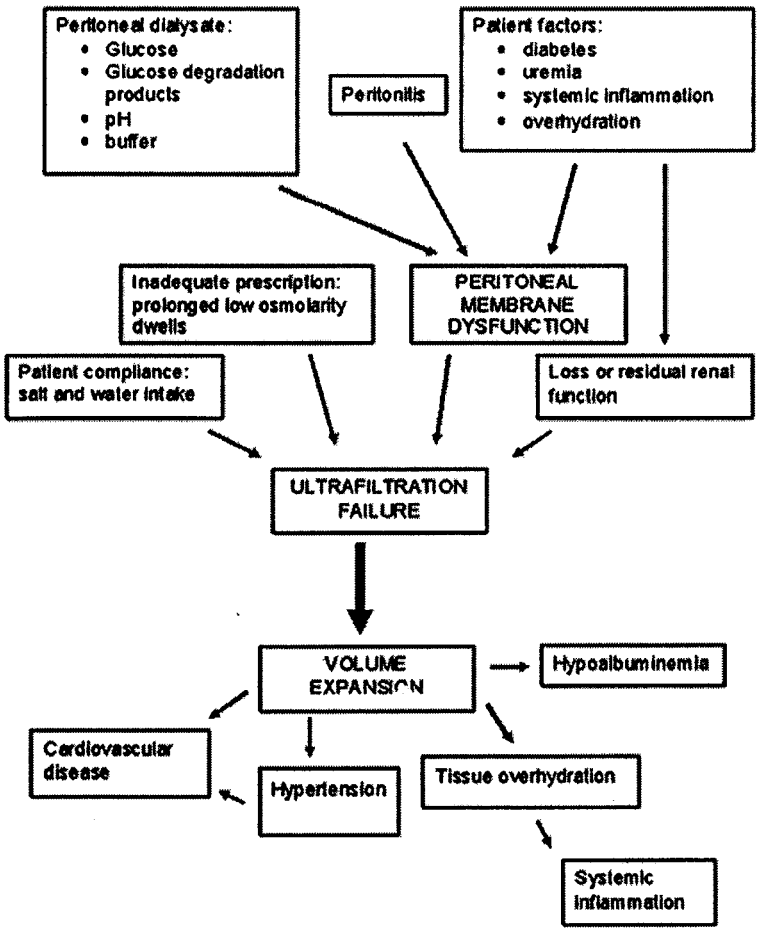

Figure 1. Components of peritoneal membrane dysfunction, ultrafiltration dysfunction, and outcomes of ultrafiltration failure

Encapsulating Peritoneal Sclerosis

In the peritoneum, progressive fibrosis alone can lead to a serious disorder known as encapsulating peritoneal sclerosis (EPS). This rare, poorly understood disorder occurs in less than 1% of PD patients [28]. Patients present with hemorrhagic peritoneal effluent, abdominal pain, and intermittent bowel obstruction [29]. Risk factors for this condition include severe unresolving peritonitis, chlorhexidine disinfectant, acetate buffered dialysis fluid, and certain medications [30]. The histologic changes observed in EPS includes a loss of mesothelial cells, a thick, acellular, collagen rind which encloses the loops of small bowel with severe intra-abdominal adhesions [30]. The involvement of inflammatory cells in this process has been controversial [31]. Treatment includes surgical debridement, cessation of PD, and immunosuppression with corticosteroids and other agents. Despite treatment, the outcome from this rare condition is generally very poor [29].

Pathophysiology

Ultrafiltration across the peritoneal membrane is the net effect of the osmotic force pulling fluid into the peritoneal cavity, and hydrostatic forces pushing fluid into the interstitium and into lymphatic reabsorption [32]. The 'membrane' across which the osmotic force acts has been described as a theoretical 3 pore model [33]. Ultrasmall pores correspond to aquaporin channels located on peritoneal venules and capillaries [5] and usually supply 40% of the net ultrafiltration [34]. Small sized pores are the major conduit for solute and a major source of fluid ultrafiltration and large pores are few in number and allow for macromolecular transport. The major determinant of the prevalence of the small pores is the vascular surface area. This explains why increased vascularization of

the peritoneal tissues will lead to rapid glucose transport, loss of osmotic gradient, and loss of ultrafiltration [3].

The peritoneal 'membrane' is comprised of an outer mesothelial cell layer covering a basement membrane which in turn overlays a thin interstitial layer comprised of connective tissue and blood vessels. Several biopsy studies have reported on the histologic changes induced by peritoneal dialysis. In the largest study reported to date, Williams and colleagues analyzed biopsy samples from 212 patients including normal, uremic, and peritoneal dialysis patients [35]. They identified an increase in the thickness of the submesothelial tissue with uremia and further increase with peritoneal dialysis. They also identified a vascular sclerosis which has been recognized in other biopsy studies [36;37] and correlated with time on peritoneal dialysis. When they analyzed the number of blood vessels in the submesothelial tissue, they did not find a correlation with time on dialysis, but did recognize a significant increase in vascularization in patients whose biopsy was performed at catheter removal due to 'membrane failure'. Several smaller studies have noted an increased vascularization of the submesothelial tissue in patients on peritoneal dialysis [37-39]. These studies have also noted a correlation between vascularization and interstitial fibrosis [38].

Increased vascular surface area

We hypothesize that increased vascularization of the peritoneal tissue is the primary mechanism of ultrafiltration failure. Between 50 and 75% of patients with UF dysfunction will have increased vascular area measured by increased small solute transport on a standard PET [9;10]. In animal models of peritoneal dialysate exposure, we

have seen a similar increase in submesothelial vascularization which correlates with glucose absorption and correlates inversely with net UF [40]. Furthermore, we were able to therapeutically reduce peritoneal vasculature using anti-angiogenic therapy, and this led to an improvement in UF function. Human biopsy studies confirm the association between submesothelial vascularization and UF dysfunction [35;37-39]. Also, careful studies of the peritoneal effluent have revealed an association between locally produced vascular endothelial growth factor (VEGF) and peritoneal solute transport [41].

Aquaporin dysfunction

Aquaporin dysfunction is a presumed mechanism leading to UF failure; however, the evidence is still somewhat contradictory. Monquil and colleagues identified patients with UF dysfunction but normal solute transport and low reabsorption rate. They concluded that impaired transcellular water transport (aquaporin dysfunction) was the mechanism for UF failure. Hyperosmolar PD solutions (3.86% glucose) did not improve ultrafiltration, and 5 of 6 patients required transfer to hemodialysis [42]. Aquaporin dysfunction as a cause of UF failure is also supported by animal studies of aquaporin 1 knock out mice [43]. These mice displayed significantly impaired water transport using a modified peritoneal function test.

The ability to accurately measure aquaporin function using a standard peritoneal function test using hyperosmolar solution (3.86% glucose) has been questioned based on computer simulation [34]. Despite the application of a correction for solute transport [44], low UF or high solute transport alone may blunt the effect of sodium dilution which is taken to be indicative of aquaporin dysfunction. Careful immunohistochemical analysis of

peritoneal tissue has led to the observation that aquaporin expression does not change in the face of acute peritonitis in an animal model [45]. Finally, normal aquaporin expression was found in a patient with presumed aquaporin dysfunction [46]. Alteration of the function of the aquaporin molecule could occur, but more work needs to be carried out in this area.

Lymphatic and tissue reabsorption

The importance of fluid reabsorption from the peritoneal cavity as a mechanism of ultrafiltration failure has been documented [9;47;48]. The interstitium of the peritoneal tissue consists mainly of collagen fibers and glycosaminoglycans such as hyaluronan. It is likely that alterations in the constitution of the interstitium will lead to alteration in hydrodynamic properties and macromolecular transport. Hydration will tend to expand the extracellular matrix and increase fluid conductance [48]. Hyaluronan may act to restrict fluid transport [48] and this may have therapeutic importance [49]. Risk factors for increased tissue and lymphatic reabsorption have not been identified, but recently, Fussholler and colleagues identified an association between increased fluid reabsorption and time on dialysis [50].

Etiology

Bioincompatible fluids

The observation that UF dysfunction increases with time on PD [12] has led to the conclusion that elements of the PD fluid are bioincompatible and damage the peritoneal membrane. Several studies have documented progressive UF dysfunction in patients in

the absence of peritonitis [2;51]. Animal models have confirmed the detrimental effect of peritoneal dialysate on the peritoneum [52;53]. Putative bioincompatible components of standard PD fluid include glucose, glucose degradation products (GDP), lactate buffer, or acidic pH. Observational [54] and animal studies [55] have attempted to better define suspect dialysis components.

Prolonged exposure to high concentrations of glucose in the peritoneal dialysate has long been suspected to damage the peritoneal membrane. Davies and colleagues have shown that increased exposure to hyperosmolar glucose dialysate predated by 2 years the onset of UF dysfunction [54]. In this case control study, patients with increasingly permeable peritoneal membranes also had an early loss of RRF which probably necessitated the increasing use of glucose to replace renal ultrafiltration. Selgas and colleagues identified a similar effect with increased glucose exposure and diabetes both predicting for early UF dysfunction [56]. In a mechanistic study, Zweers and colleagues noted a decrease in locally produced VEGF in patients switched to non-glucose PD solutions [57].

GDPs, such as methylglyoxal, glyoxal, and 3-deoxyglucasone, are reactive carbonyl compounds produced in peritoneal dialysate during the process of heat sterilization [58]. They have been shown to be more potent inducers of advanced glycation end-products (AGE) than glucose alone [59]. GDPs have been shown to alter mesothelial cell function and proliferation [60] and to induce VEGF production in vitro [61]. In animal models, conventional dialysate fluid induced vasodilation and capillary recruitment in the peritoneal tissues, whereas GDP-reduced PD fluid did not induce these changes [55;62].

AGEs are irreversibly altered proteins (eg. pentosidine, carboxymethyllysine) which have a wide range of vascular and non-vascular [63] activity in the peritoneal tissue, signaling through a specific AGE receptor [64]. Several studies have identified the appearance of AGE products such as pentosidine in the peritoneal effluent of patients on dialysis [65;66]. These are felt to be both transported from the blood and produced and cleared in the peritoneum. Effluent pentosidine levels also correlate with time on dialysis and the use of non-glucose dialysate appears to reduce effluent pentoside presumably through a 'washout' mechanism [67].

Biopsy studies have confirmed the deposition of AGE in the peritoneal tissues of patients on peritoneal dialysis [39;68-70]. AGEs are deposited in the interstitium and vessel walls and correlate with fibrosis [69], solute transport and ultrafiltration dysfunction [70], and co-localizes with VEGF in the peritoneal vasculature [39;61].

Conventional peritoneal dialysis fluid has a pH around 5 and uses lactate as a buffer. To date, the bioincompatible nature of these components is not clear, but appears to be less important than glucose or GDPs [55]. Previous clinical experience with acetate buffered PD fluid demonstrated increased rates of UF dysfunction [71].

Peritonitis

Aside from the bioincompatible nature of conventional dialysate solutions, peritonitis is a major cause of peritoneal membrane dysfunction. There is an acute effect of peritonitis following the infectious event that clinically appears as decreased UF with increased solute transport [72]. The effect of a single episode of peritonitis on long term peritoneal membrane function is less certain. Ates and colleagues have suggested a subtle

longer term UF dysfunction after a single episode of peritonitis [72]. Others suggest that one episode of peritonitis has little long term effect [2;50;73]. Features of peritonitis which predict long term UF dysfunction include later infective episodes (>36 months after initiation of PD) [74], multiple events or increased inflammatory response [2;75], severe infections [73], or gram negative infections [76].

The response to acute infection involves resident peritoneal mesothelial cells and macrophages which work in a coordinated fashion to recruit other inflammatory cells including lymphocytes and neutrophils [77]. Heat killed bacterial solutions can induce peritoneal mesothelial cells to produce the inflammatory mediators interleukin (IL) -6 and prostaglandins, along with chemokines IL-8, RANTES, and monocyte chemotactic protein - 1 [78]. IL-1 and tumor necrosis factor (TNF) α are induced by bacterial products and endotoxins from peritoneal macrophages and impact on mesothelial cells and endothelium with the up-regulation of vascular adhesion molecules and increased vascular permeability.

The mechanism of long term changes after peritonitis episodes is not clear. There is an acute inflammatory event with upregulation of inflammatory cytokines [79]. Profibrotic cytokines such as transforming growth factor β (TGFβ) and fibroblast growth factor (FGF) -2 [79;80] have also been shown to be up-regulated after peritonitis episodes. In an animal model of peritonitis, Combet and colleagues demonstrated increased expression of endothelial nitric oxide synthase (NOS) in the peritoneal tissues [45]. This was associated with increased vascularization, increased protein loss, and UF failure. nitric oxide has been identified as an important and necessary cofactor in peritoneal angiogenesis [39]. Using adenovirus mediated overexpression of inflammatory cytokines

IL-1β and TNFα, we have shown that longer term effects on vascularization and fibrosis can be induced in the peritoneum after transient cytokine overexpression and IL-1β appears to be more potent in inducing these changes [81]. This finding is supported by other research in peritoneal inflammation. In vitro experiments suggest that IL-1 can lead to the upregulation of collagen, fibronectin [82], and PAI-1 [83] gene expression in peritoneal mesothelial cells. Peritoneal effluent from patients with peritonitis leads to increased mesothelial cell expression of the anti-inflammatory glycosaminoglycan hyaluronan and this effect was blocked with antibodies to IL-1 [84]. Several studies have noted that IL-1 production from peritoneal mesothelial cells is dependent on the species of infectious agent. S. aureus supernatant induces higher IL-1 expression than S. epidermidis, which is higher than E.coli [83;85]. Finally, there has been a demonstrated direct effect of IL-1 on the induction of VEGF and angiogenesis [86-88].

Therefore, there are pathways from acute inflammation to longer term fibrosis and angiogenesis in the peritoneum which may explain the association between peritonitis and UF dysfunction seen in several observational studies. The increased fibrogenic and angiogenic nature of IL-1, combined with the observation that variability in inflammatory response depends on bacterial species, suggests a mechanism whereby different infectious episodes could have different long term effects on peritoneal membrane function. This also suggests that early identification and treatment with antibiotics, combined with an IL-1 inhibiting agent [89], may be the optimal therapy for PD related peritonitis.

Uremia and systemic inflammation

The uremic environment has been associated with an increased expression of AGE proteins [90]. In rats made uremic with subtotal nephrectomy, significant changes in the peritoneal tissues occurred including increased AGE deposition, increased vascularization with increased expression of NOS, VEGF, and FGF-2 [91]. The effect of uremia on the peritoneal tissues have been confirmed in two human peritoneal biopsy studies [35;38] that demonstrated increased submesothelial thickening in pre-dialysis patients.

Systemic inflammation has been a controversial topic in chronic renal failure and in peritoneal dialysis. The observed association between markers of malnutrition, inflammation, and atherosclerosis suggests systemic inflammation is a common underlying condition in patients with renal failure [92]. In PD patients, the association between peritoneal membrane transport properties and markers of systemic inflammation has been contradictory. Wang and colleagues did not find any association in numerous markers of inflammation assessed [93]. In subsequent work, patients with signs of inflammation, malnutrition, and atherosclerosis had higher peritoneal transport at the initiation of dialysis [94]. In a second study, high C-reactive protein and declining RRF were both associated with increasing peritoneal membrane transport status from initiation to 1 year on dialysis [95]. In a retrospective study, we found patients with a high transport peritoneal membrane had low serum albumin before the initiation of PD and suggested this was the result of systemic factors such as inflammation or overhydration [96].

Overhydration is likely a result of a high transport status peritoneal membrane with concomitant UF dysfunction, but it may also impact on the peritoneal membrane

function [97]. Theoretic considerations suggest that peritoneal tissue hydration leads to dilution of extracellular matrix components which increases fluid conductivity and may thus lead to increased tissue and lymphatic reabsorption [48]. An older intervention study suggests that a decrease in extracellular fluid volume in PD patients, either through aggressive diuresis or hemofiltration, improved the UF properties of the peritoneal membrane [98].

Finally, two studies have demonstrated an association between diabetes and high transport status early in peritoneal dialysis [15;56]. The assumption is that high glucose levels on either side of the peritoneal membrane will lead to histologic changes with associated functional changes.

Treatment of Ultrafiltration Dysfunction

The treatment of UF dysfunction in PD patients involves three steps. First, the problem of UF dysfunction must be recognized through regular clinical volume assessment and routine measurement of peritoneal membrane function including net UF [99]. Prevention, with protection of the peritoneal membrane from injury due to peritonitis and bioincompatible dialysis solutions, is an important second step. Finally, treatment strategies for established UF dysfunction are limited at present, but should expand with further research in this area.

Icodextrin

Icodextrin is a promising dialysate solution for improving UF function. Icodextrin is a colloid and appears to promote UF through small pores in the peritoneal membrane

and is therefore effective in PD patients with increased peritoneal vascular area or in the setting of acute peritonitis [100]. In large randomized studies in which icodextrin was used in the night time dwell in continuous ambulatory PD patients [101] or the daytime dwell in automated PD patients [102], a similar UF rate was observed to that obtained with a hyperosmolar glucose solution (3.86% glucose). Two studies have assessed the value of icodextrin in patients with UF failure. The first prospective study took 17 patients with impending technique failure due to UF dysfunction and prescribed icodextrin [103]. Net daily UF increased by 570 ml, blood pressure improved, and technique survival was prolonged by one year. Patients who benefited the most from icodextrin were those who initially had very low UF.

Biocompatible solutions

Biocompatible solutions are produced in two chambered bags so that glucose is separated from the electrolytes at a very low pH. This prevents the formation of GDPs during heat sterilization [59] and allows for adjustment of final pH and buffer.

Conventional PD solutions have detrimental effects on cellular proliferation, function and inflammation which are reversed by more physiological solutions [60;104]. Animal studies using more physiological solutions have demonstrated reduced AGE deposition and preserved UF [105].

In a six month trial of 106 PD patients randomized to conventional or physiological solution, Tranaeus and colleagues found an increase in UF (150 ml/day) with stable peritoneal membrane characteristics [106]. In a voluntary 6 month extension of this study, significantly lower peritonitis rates were reported in the physiological solution

group. A common finding among clinical trials of physiological solutions is an increase in effluent CA125 suggesting improved mesothelial cell viability, and a decrease in effluent hyaluronan concentration, which is interpreted as a decrease in peritoneal inflammation [107;108]

Alteration of the interstitial compartment

Hyaluronan is a glycosaminoglycan which is present in the interstitium of the peritoneum. In animal models, hyaluronan has been shown to increase net ultrafiltration and decrease markers of inflammation [109;110]. These animal studies have also shown a decrease in protein transport into the peritoneal fluid. Aside from demonstrated anti-inflammatory properties, hyaluronan is felt to effect the interstitium and fluid absorption from the peritoneum [49].

Small observational studies in humans have been carried out to study the surface active phospholipid phosphatidylcholine and have demonstrated an increase net UF when delivered orally [111] or intraperitoneal [112].

Anti-angiogenic therapy

Our results demonstrate that anti-angiogenic therapy using gene transfer of angiostatin improves net UF in an animal model of peritoneal dialysis [40]. There are several advantages and disadvantages to using adenoviral mediated gene transfer in the peritoneum in human disease. The type 5 adenovirus used in our experiments is readily taken up by mesothelial cells in the naïve peritoneum [113], and in peritoneal tissue exposed to chronic peritoneal dialysis fluid [52]. The adenovirus is readily purified to high titre [114].

The negative aspects of peritoneal adenovirus gene therapy is the acute inflammatory reaction, the possibility that the adenovirus will be transported to the liver or systemically, and the transient nature of the expression. In our experiments, we found the null adenovirus had a minimal impact on the peritoneum. There was a transient inflammatory response which resolved by 48 hours after infection [81]. At very high doses, we did see expression of the virus in liver parenchyma, which suggested the adenovirus did transport out of the peritoneum, probably via lymphatic or hematologic routes.

The transient nature of gene expression with adenovirus gene transfer is likely the most limiting problem. The fibrosis and angiogenesis seen in patients on PD occur over a 2 to 4 year time period. Gene therapy for this problem would likely have to persist for considerably more time than the 10 to 14 days we saw with adenovirus mediated gene transfer [113]. On the other hand, we have investigated strategies for prolonging adenovirus gene transfer effects using repeated application with immunosuppression to prevent the host acute immune response [115]. In conjunction with biocompatible solutions, one could imagine a therapy whereby adenovirus mediated gene transfer is used to acutely decrease the vascularization of the peritoneal tissues, and biocompatible solutions are used to prevent recurrent of vascularization.

Ex-vivo gene transfer has been used in several animal models. In this technique, mesothelial cells are cultured and stably transfected with a gene of interest, the peritoneum is denuded using a gelfilm application, and the transfected mesothelial cells are re-inserted [116]. This leads to a more prolonged transgene expression, however, natural turn-over of mesothelial cells leads to eventual removal of the transfected cells.

The observation that decreasing the vasculature leads to improvement in ultrafiltration function suggests that non-gene therapy approaches should also be effective. Several anti-angiogenic agents, included thalidomide [117], TNP-470 [118], and VEGF receptor tyrosine kinase inhibitors [119] are available and have been used in animal and human studies and may be suitable for IP or oral administration for patients on PD with UF dysfunction.

Peritoneal fibrosis and the role of TGFβ

The response of the peritoneal tissues to systemic and local toxic and inflammatory events is similar to the process of wound healing and tissue repair in other organs. This 'response to injury' process is a ubiquitous physiological event which occurs with slight variations in the majority of living organ systems [120]. Normal wound healing returns the damaged tissue close to the pre-injury state. With severe or prolonged injury, residual scarring and some loss of function are probable. In aberrant wound healing, the process becomes dysregulated and collagen deposition occurs with minimal ongoing inflammation. This collagen deposition leads to progressive fibrosis and loss of organ function. Fibrosis is the end result of multiple injurious and inflammatory diseases and affects virtually every organ system. There is a high cost, mortality, and morbidity associated with progressive organ fibrosis [121-123].

The fibrogenic response involves a coordinated interaction between cellular and cytokine components. The initial insult leads to an upregulation of fibrogenic cytokines released from platelets during clot formation in wounded tissue [120]. Profibrotic cytokines can also be directly stimulated by high glucose, dialysis fluid, or inflammation, in the

peritoneum [80;124-126]. The initial tissue insult may also lead to an influx of inflammatory cells that are an alternate source for profibrotic cytokines [120]. Along with TGFβ1, other cytokines such as TGFβ2, TGFβ3, platelet derived growth factor (PDGF), FGFs, and connective tissue growth factor (CTGF) [127], are involved in the initiation of the fibrogenic process. Plasminogen activator inhibitor (PAI) -1 is likely involved at an early stage in peritoneal fibrosis with the inhibition of fibrinolytic activity on the surface of the peritoneal tissue and the subsequent accumulation of fibrin tissue which forms the initial scaffold for subsequent peritoneal fibrosis [128].

Fibroblasts enter the site of tissue damage drawn by chemotactic factors such as PDGF. They transform into myofibroblasts under the influence of mechanical stress and TGFβ [129]. The hallmark of early wound healing is the elaboration of collagen, new blood vessels, and nerves, in a temporary healing structure called granulation tissue [120]. The progression and maintenance of subsequent fibrosis is likely the result of a balance between collagenolytic enzymes (matrix metalloproteinases (MMP)) and their inhibitors (tissue inhibitor of metalloproteinase (TIMP)). The resolution of the fibrogenic process also involves apoptosis of myofibroblasts [129].

TGFβ1 is a multifunctional cytokine involved with many wound healing processes; fibroblast activation [123], collagen deposition [130], inhibition of fibrinolysis through PAI-1 [128], and angiogenesis [113;130-133] . TGFβ1 is also involved in cell cycle regulation through cyclin dependent kinase inhibitor p21 [134] inhibition and has immune modulating activity. Specifically, TGFβ inhibits T-helper cell differentiation and regulates lymphocyte proliferation [135]. TGFβ also prevents T-cell activation in the setting of apoptosis and is therefore potentially crucial in the maintenance of self-tolerance [136].

TGFβ is a member of a family of cytokines including activins and bone morphogenic proteins, which bind to the receptor TGFβRII that then recruits and activates TGFβRI. TGFβRI initiates a cellular signaling cascade through its serine/threonine kinase activity [134]. Non-signaling membrane associated proteins (TGFβRIII (biglycan) and endoglin [137]) modify the interaction between TGFβ and its receptor complex. TGFβ and related cytokines signal predominantly through Smad signal transduction proteins [138]. Non-Smad signaling after TGFβRI receptor ligation has also been demonstrated.

TGFβ1 exists in tissues in an inactive form bound to latency associated protein. It is activated through cleavage by integrin αVβ6, thrombospondin, plasmin, or cathepsin. The activity of unbound TGFβ1 is also modulated by matrix associated proteoglycans such as decorin and biglycan [139]. CTGF, a TGFβ1 induced protein, also binds to and increases the activity of TGFβ1 [140].

Angiogenesis in fibrotic peritoneal tissue

We have demonstrated that overexpression of TGFβ1 in the peritoneum using an adenovirus vector leads to fibrosis, angiogenesis, and similar functional changes as is seen in patients on long term peritoneal dialysis [113]. Human peritoneal biopsy studies have demonstrated a progression of fibrosis [35] and angiogenesis [39] with time on dialysis, and also support a direct correlation between peritoneal fibrosis and angiogenesis [141]. Taken together, these data suggests that angiogenesis is a direct result of the fibrotic process and occurs in response to profibrotic cytokines. However, certain components of dialysis fluid, such as GDPs, can directly upregulate VEGF [61], and AGE deposition

occurs in vascular walls and co-localizes with VEGF in the peritoneal vasculature [39;61]. These data suggests that there are fibrosis independent pathways to angiogenesis in the peritoneum and may explain the observation that peritoneal fibrosis is ubiquitous, but UF dysfunction due to increased peritoneal vascularization occurs in a smaller proportion of PD patients.

Angiogenesis is a complex and regulated process [142]. It has been studied in detail because of its role in cancer and ischemic heart disease. The process of angiogenesis in the setting of wound healing and tissue repair is likely similar in general to angiogenesis in malignancy, however certain features are probably unique [143]. In general, three distinct processes are involved in new blood vessel growth. Vasculogenesis is the appearance of new vessels from endothelial stem cells. Angiogenesis describes the sprouting of new vessels from existing vessels. Arteriogenesis is the maturation process whereby new blood vessels become less permeable and recruit vascular smooth muscle cells (VSMC) [144].

In pathologic situations such as malignancy and wound healing, angiogenesis is the primary mechanism of new blood vessel growth. Existing vascular structures undergo a series of changes at the initiation of angiogenesis. Vessels demonstrate increased permeability caused by VEGF, angiopoeitin (ANG) -2 [145], and placental growth factor [146]. Other factors involved in early vascular permeability include inflammatory markers such as TNFα [81], nitric oxide [147], and prostaglandins [148]. Next is the detachment of endothelial cells and the elaboration of basement membrane degradation enzymes such as MMP and PAI-1, which allow the migration of endothelial cells into interstitial tissue [144]. Integrins such as αvβ3 are integral in endothelial cell migration and survival [149].

Endothelial cells proliferate and form primitive tubules. These structures recruit vascular smooth muscle cells (VSMC) through cytokines such as PDGF [150]. Endothelial cells mature and become resistant to apoptosis through the vascular stabilization factor ANG-1 [151].

VEGF is the key angiogenic and vasculogenic cytokine and induces endothelial cell proliferation, migration [152], and proteolytic activity [153]. VEGF exists as a series of isoforms with differing angiogenic and heparin binding properties [153]. There are several VEGF related proteins including placental growth factor, VEGF-B, VEGF-C, and VEGF-D, which have differential angiogenic and lymphangiogenic activities [152]. VEGF acts through its receptors VEGFR-1 (Flt-1), VEGFR-2 (Flk-1/KDR), and VEGFR-3 [153]. VEGFR-2 is likely the dominant receptor which induces signaling pathways. VEGFR-1 may have some signaling properties and may also act as a decoy molecule. VEGFR-3 binds preferentially to VEGF-C and has a role in lymphangiogenesis. A fourth receptor, neuropilin-1, has been recently described and increases the activity of VEGFR-2 [152]. VEGF was initially identified as vascular permeability factor and has a potent effect in increasing macromolecular transport across the vessel wall [152]. VEGF is up-regulated in the setting of hypoxia through a cofactor, hypoxia inducible factor (HIF) -1 [144]. Recently, it has been identified that VEGF is modified in vitro by CTGF which binds to and inhibits the protein activity [154].

PDGF and FGF-2 are other growth factors independently associated with new blood vessel growth [143]. Not only can these two growth factors induce endothelial cell mitogenesis, but they also appear to induce the recruitment of VSMCs and thus complete

the blood vessel maturation process [150]. VEGF and FGF-2 have synergistic effects on blood vessel growth [155].

Other families of cytokines, the angiopoietins and ephrins, are involved in the modification of established or newly formed blood vessels [156;157]. ANG-1 and ANG-2 both interact with the Tie-2 receptor on endothelial cells with ANG-1 likely promoting receptor activation and ANG-2 acting to block this kinase activity. ANG-1 is secreted by perivascular cells [158] and is possibly upregulated in the fibrotic setting [159;160]. ANG-2 is secreted by endothelial cells and its production is induced by VEGF, FGF, and hypoxia [161]. ANG-1 induces a more mature phenotype in endothelial cells with decreased intracellular macromolecular transport [145] and resistance to apoptosis [162].

Fibrosis and angiogenesis are integrated processes [143]. Fibrosis could be viewed as a benign tumor, and the cells forming the fibrotic tissue require metabolic support through angiogenesis [163]. With this view, interruption of angiogenesis may impair the fibrotic process and several experiments have been carried out to demonstrate this [164]. Agents involved in the fibrotic process also appear to induce angiogenesis. FGF-2 and PDGF are involved in both fibrogenesis and angiogenesis. Nitric oxide is a necessary cofactor with VEGF in angiogenesis [165] and nitric oxide synthase is upregulated in the peritoneal tissues in patients on PD [39]. The injured or fibrotic tissue may suffer from hypoxia due to inadequate vascularization [163], and this may induce angiogenesis through HIF-1α. The inflammatory cytokine IL-1 is both strongly pro-fibrotic [166] and induces a sustained angiogenesis in peritoneal tissues [81;167].

The role of TGFβ in angiogenesis has been somewhat controversial. In cell culture, TGFβ has a biphasic effect on the growth of endothelial cells; facilitating at low

dose but inhibitory at high dose [133]. TGFβ synergistically acts with HIF-1α [131;168] and high glucose [130] in upregulating VEGF. TGFβ also directly upregulates VEGF in vitro and in vivo [113;169]. TGFβ appears to induce expression ANG-1 and may lead to the stabilization of blood vessels in fibrogenesis [160;170]. Subclasses of TGFβRI, activin receptor-like kinase (ALK) -1, may be involved in signaling involved in vascular maturation [171]. Mutations of endoglin, a TGFβ receptor complex related molecule, leads to the human disease hereditary hemorrhagic telangectasia [137;172]. TGFβ also has a role in the maturation of VSMCs after their recruitment by PDGF [173]. Finally, TGFβ knock out mice have lethal defects in blood vessel maturation and hematopoiesis [174]. Therefore, TGFβ1 has putative angiogenic roles both in the initiation phase through VEGF, and in the maturation phase.

Angiogenesis appears in the setting of fibrosis and is related to pro-fibrotic factors. There is preliminary evidence that angiogenic factors may have a direct role in collagen expression and fibrosis. Hypoxia upregulates VEGF and collagen 1 gene expression in rat model of liver fibrosis [175]. In a transgenic mouse model, combined overexpression of VEGF and ANG-2 lead to significant cardiac fibrosis [176].

Animal Models of PD

Animal models are required in PD research in order to study changes in the complex interaction between solute transport, fibrosis, and angiogenesis. Rats have been used most commonly as they are large enough to manipulate for accurate intraperitoneal injection and indwelling catheter placement but are small enough to be reasonably economical. Mice, rabbits, and dogs have been used in the past [177].

Two main models are utilized; the first is an acute model where fluid is placed by intraperitoneal injection. Some form of functional peritoneal test is performed before tissue is harvested for analysis. We have used a simplified PET where net UF, glucose, creatinine, and albumin transport is measured after a 4 hour dwell with 2.5% glucose dialysis solution [113]. Others have used a more extensive evaluation with frequent intraperitoneal samples taken and a volume marker such as ^{131}I labeled albumin used as a volume marker [178]. This allows the measurement of UF, and solute transport, and allows the estimation of the relative contribution of aquaporin transport, increased vascular surface area, and interstitial / lymphatic uptake to net fluid flux across the peritoneal membrane.

We have used this acute model to study the effect of overexpression of cytokines using adenovirus mediated gene transfer [81;113], which are effectively transferred to the peritoneal tissues. The effect of pharmacologic agents such as hyaluronan [109] or nitric oxide inhibitor [179] has been studied with these acute models. The effect of infectious episodes on peritoneal structure and function has also been studied with this acute model [45]. Combined with intravital microscopy, the effect of different dialysis solutions on vascular recruitment and leukocyte adhesion can be evaluated [55].

Chronic models involve the surgical placement of an indwelling catheter with daily infusion of dialysis solution. This model has been used extensively to study the effect of standard PD fluid on peritoneal tissue [52;180], to study biocompatible PD solutions [181], and to study the effects of novel therapies on peritoneal function [40]. These models have also been used to study the relative roles of systemic factors such as uremia, and local effects of PD solutions, on the peritoneum [91].

Summary

The main finding in peritoneal biopsies of patients on long term peritonitis is fibrosis, submesothelial thickening, and loss of the mesothelial cell layer [35]. This observation was difficult to reconcile with the hypothesized cause of loss of ultrafiltration function which appeared to be mainly due to increased vascularization of the peritoneal tissues [3]. Subsequent biopsy studies have confirmed an association between vascularization of the peritoneum, and ultrafiltration dysfunction [35;38].

I hypothesize that ultrafiltration dysfunction is caused by increased peritoneal vascularization with a concomitant increase in glucose transport from the peritoneal cavity and rapid loss of the ultrafiltration gradient. Further, I hypothesize that this increased vascular surface area is induced by profibrotic and inflammatory cytokines such as TGF β, IL-1β, and TNFα through upregulation of angiogenic cytokines such as vascular endothelial growth factor (VEGF).

Our work in this area has confirmed the association between angiogenesis and UF failure [52] and demonstrated a causality by experimentally reducing the vasculature using adenovirus mediated gene transfer of angiostatin with subsequent improvement in ultrafiltration [40]. We also further explored the causes of angiogenesis in the fibrotic environment and identified that TGFβ [113] and IL-1β [81] are both strongly pro-fibrotic and angiogenic in the peritoneum. These observations have several implications. The goal of improving the peritoneal membrane for patients on peritoneal dialysis should now focus on anti-angiogenic therapy, along with methods to prevent injury to the peritoneal membrane. We have demonstrated that IL-1 has a prominent role in inducing peritoneal damage. It is possible that therapy aimed at IL-1 may prevent progressive peritoneal

membrane damage in the setting of acute peritonitis. Finally, we have created a new model to study angiogenesis in the setting of fibrosis. This will allow further evaluation of interventions, and further experiments to understand the role of such agents as the angiopoietins, CTGF, TGFβ3, matrix metalloproteinases, and their inhibitors in the process of angiogenesis and fibrogenesis.

CHAPTER 2: GENE TRANSFER OF TRANSFORMING GROWTH FACTOR β TO THE RAT PERITONEUM: EFFECTS ON MEMBRANE FUNCTION

The following paper is published in the Journal of American Society of Nephrology 2001; 12: 2029-2039. The goal of this work was to investigate the effect of transient overexpression of TGFβ1 in the peritoneum. The adenovirus construct, AdTGFβ1 was created by Dr. Patricia Sime. It was made with standard co-transfection techniques using the porcine TGFβ1 gene plasmid (supplied by Dr. Anita Roberts). This gene sequence is mutated so that a serine replaces a cysteine at the 223 and 225 locations. This mutation impairs the ability of TGFβ1 to bind to its latency associated protein, and it is therefore secreted in the active form.

We demonstrate expression of the adenovirus vector transgene between 4 and 14 days after infection. Using an adenovirus construct expressing the reporter gene β-galactosidase, we demonstrated virtually 100% adenovirus uptake by mesothelial cells. We also identified a lack of significant inflammatory reaction to our control adenovirus AdDL70. AdTGFβ1 had a dramatic effect on the structure and function of the peritoneum. There was submesothelial thickening with persisting angiogenesis. There was a significant ultrafiltration dysfunction that persisted until 28 days after adenoviral infection. This was associated with increased transport of glucose out of the peritoneal fluid, and albumin into the peritoneal fluid.

We also identified an increased presence of VEGF in the peritoneal effluent of rats exposed to AdTGFβ1. We explored this further in cell culture experiments, and

demonstrated the production of VEGF by primary mesothelial cells exposed to recombinant TGFβ1.

These experiments, therefore, suggested that TGFβ1 up-regulation may explain the changes seen in patients on long term peritoneal dialysis.

The concept for this paper was developed by Dr. Gauldie, Dr. Cathy Hoff, and me. I designed the experiments with suggestions on the animal model from Dr. Hoff and Dr. Gauldie. I developed the concept for, and the experiments to measure, angiogenesis and VEGF. The animal experiments were carried out by Dr. Kolb, Tom Galt, and me. I carried out all benchwork including ELISA, hydroxyproline, and cell culture. Histology support was carried out by Mary Jo Smith; I performed the immunohistochemistry. I analyzed the data and wrote the paper. The manuscript was reviewed by Dr. Gauldie, Dr. Hoff, and Dr. Shockley.

J Am Soc Nephrol 12: 2029–2039, 2001

Gene Transfer of Transforming Growth Factor-β1 to the Rat Peritoneum: Effects on Membrane Function

PETER J. MARGETTS,*† MARTIN KOLB,† TOM GALT,† CATHERINE M. HOFF,‡ TY R. SHOCKLEY,‡ and JACK GAULDIE†

**Division of Nephrology and †Centre for Gene Therapeutics, McMaster University, Hamilton, Canada; and ‡Renal Division Scientific Affairs, Baxter Healthcare Corp., McGaw Park, Illinois.*

Abstract. Long-term peritoneal dialysis is limited by physiologic changes in the peritoneum that lead to ultrafiltration failure. To determine the role of profibrotic cytokines in the alteration of peritoneal transport, a rodent model of transforming growth factor-β (TGF-β)-mediated peritoneal fibrosis was established. An adenoviral vector driving the active form of TGF-β1 (AdTGFβ1) was administered intraperitoneally, and peritoneal structure and function were evaluated for 28 d after infection. Seven days after AdTGFβ1 infection, thickening of the peritoneum, with cellular proliferation and increased vascularization, was noted. By day 28, there was persistent thickening and extensive collagen deposition. The mesenteric collagen content was significantly elevated, compared with control adenovirus-treated animals, 21 d after infection (2.9 *versus* 1.8 mg hydroxyproline/g tissue, $P = 0.006$). Blood vessel density, as measured using factor VIII immunohistochemical analyses, was significantly increased from day 4 to day 21 but decreased by day 28. Animals infected with AdTGFβ1 demonstrated increased transport of solutes and decreased net ultrafiltration, which was maximal on day 7 and returned to baseline levels by day 28. It was demonstrated *in vitro* and *in vivo* that TGF-β1 induced production of vascular endothelial growth factor. Overexpression of TGF-β1 after adenovirus-mediated gene transfer causes peritoneal fibrosis, neoangiogenesis, and increased peritoneal membrane solute transport. This model should allow further delineation of the relative contributions of profibrotic and angiogenic cytokines to changes in peritoneal function and may lead to potential new interventions for peritoneal membrane failure.

Peritoneal dialysis is an attractive option for the treatment of patients with end-stage renal disease. However, recurrent peritonitis and alterations in the peritoneal membrane transport of solutes complicate long-term peritoneal dialysis (1). The most common functional change in the peritoneal membrane with long-term peritoneal dialysis is ultrafiltration failure, which is the cause of technique failure for 9 to 27% of patients undergoing peritoneal dialysis (2). The relationship between the duration of peritoneal dialysis and ultrafiltration failure has been confirmed (1,3–5). In population-based studies, ultrafiltration was inversely correlated with increased peritoneal membrane transport of solutes (1,5,6).

The pathophysiologic features of the changes in peritoneal membrane transport with time are not well understood. The main barrier to peritoneal transport of solutes seems to be the vascular wall (7,8). The observed changes in peritoneal membrane function with time are therefore attributable to increases in the permeability of individual blood vessels or increases in the total vascular surface of the peritoneum (9,10). Recent studies of human peritoneal biopsy samples demonstrated increases in the density of the peritoneal microvasculature with dialysis time (11,12). Human (13,14) and animal (15) biopsy samples revealed a generalized thickening of the submesothelial collagenous zone of the peritoneum, with connective tissue deposition and microvascular sclerosis, corresponding to the duration of dialysis. α-Smooth muscle actin (α-SMA)-positive cells, suggesting myofibroblast differentiation induced by fibrogenic cytokines (16), were identified in the peritoneum and were correlated with the degree of fibrosis and the density of blood vessels (13).

The role of increased peritoneal vascular area in ultrafiltration failure suggests that angiogenic cytokines such as vascular endothelial growth factor (VEGF) are upregulated in the process of peritoneal dialysis. VEGF has been identified, by both immunohistochemical and immunoblot analyses, in biopsy samples from patients undergoing long-term dialysis (11). VEGF concentrations in dialysate fluid have been correlated with measures of solute transport among patients undergoing chronic peritoneal dialysis who are in stable condition (17).

VEGF may be induced directly in the peritoneum of patients undergoing peritoneal dialysis. Glucose degradation products, formed by heat-sterilization of dialysate, have been demonstrated to be toxic to mesothelial cells (18) but also enhance the production of VEGF by those cells (19). Rats with streptozocin-induced diabetes mellitus exhibit changes in peritoneal membrane transport similar to those of patients undergoing

Received November 6, 2000. Accepted March 29, 2001.
Correspondence to Dr. Jack Gauldie, Department of Pathology and Molecular Medicine, McMaster University, 1200 Main Street W., Room 2N16, Hamilton, Ontario, Canada L8N 3Z5. Phone: 905-521-2100, ext. 76332; Fax: 905-577-0198; E-mail: gauldie@mcmaster.ca

1046-6673/1210-2029
Journal of the American Society of Nephrology

long-term dialysis, and this effect can be reversed with the intraperitoneal administration of anti-VEGF antibodies (20).

Renal failure and dialysis expose the peritoneum to inflammatory mediators (21), and this exposure may induce the expression of profibrotic cytokines such as transforming growth factor-β (TGF-β). Peritoneal mesothelial cells may play a role in this process, because TGF-β can be induced in mesothelial cells by high-concentration glucose solutions and spent dialysate (22). Two studies noted increased dialysate TGF-β concentrations with increased duration of dialysis (23,24), and dialysate TGF-β concentrations were correlated with peritoneal membrane solute transport in a population of peritoneal dialysis patients in stable condition (25). In addition to local factors, membrane transport at the initiation of peritoneal dialysis is variable and predictive of outcome (26), which suggests that systemic processes, such as diabetes mellitus, atherosclerosis, systemic inflammation, and malnutrition, may also play a role in membrane dysfunction (27).

TGF-β is a multifunctional cytokine that is central in the process of fibrogenesis (28). By inducing fibroblasts to increase production of collagen I and III and increasing expression of the tissue inhibitor of metalloproteinase (29), TGF-β promotes the formation of extracellular matrix. As part of the fibroproliferative response, TGF-β has been demonstrated to enhance the expression of VEGF in several cell lines (30–32). In turn, VEGF is a mitogen for endothelial cells and induces angiogenesis (33). VEGF is also a vascular permeability factor and may directly induce endothelial cells to increase cellular fenestrations (34).

To study the relationship between TGF-β and changes in the structure and function of the peritoneum, we used adenovirus-mediated gene transfer of active TGF-β1 to the peritoneum in a rat model. This adenoviral construct (AdTGFβ1) was previously used to transfer this gene to the rat lung, resulting in transient overexpression of TGF-β1, with subsequent progressive pulmonary fibrosis (35).

We demonstrated that transient overexpression of TGF-β1 after intraperitoneal administration leads to histologic changes consistent with peritoneal fibrosis, including thickening of the submesothelial zone and increased interstitial collagen deposition. There was a TGF-β1-mediated increase in dialysate VEGF concentrations and increased vascularization. We observed changes in solute transport induced by TGF-β that mimicked those observed in patients undergoing long-term peritoneal dialysis but were transient and resolved with the resolution of the increased peritoneal vascularity.

Materials and Methods

Adenoviral Construct

A replication-deficient recombinant adenovirus expressing the biologically active form of porcine TGF-β1 was used in these studies. The construction of AdTGFβ1 was previously described (35). In this construct, the TGF-β1 gene is mutated at residues 223 and 225, and the resulting protein does not bind to its latency-associated protein, allowing production of active TGF-β1. An adenovirus construct coding for β-galactosidase (AdLacZ) (36) and an empty (null) virus (AdDL70) (36) were also used. Virus preparations were expanded, purified, and plaque-titered as described previously (37).

Animal Studies

All animal studies were performed according to the Canadian Council on Animal Care guidelines (38). Food and water were provided *ad libitum*. Animals were anesthetized with isoflurane (MTC Pharmaceuticals, Cambridge, Canada). Sprague-Dawley rats (225 to 275 g; Harlan, Indianapolis, IN) were given intraperitoneal injections of AdTGFβ1, AdDL70, or phosphate-buffered saline (PBS) (control). To ensure intraperitoneal delivery, an intravenous catheter over a 25-gauge needle (Becton-Dickson, Franklin Lakes, NJ) was introduced through the abdominal wall of anesthetized animals. The needle was removed, the catheter was fully advanced into the peritoneal cavity, and the substance was administered. The animals received 2×10^9 plaque-forming units of adenovirus, diluted in 50 μl of PBS, or PBS alone on day 0. Three to six animals per group were euthanized on days 4, 7, 21, and 28. The day 21 and 28 groups were repeated. Until euthanasia, a survival peritoneal equilibration test (PET) was performed in each animal on days 4, 7, 14, and 21. This test involved the intraperitoneal administration of a 0.09 ml/g dose of 2.5% Baxter Dianeal (Baxter Healthcare, Round Lake, IL). Four hours later, a tail blood sample and a peritoneal fluid sample were obtained. A PET was performed before euthanasia, and an accurate ultrafiltration measurement was made. One group was euthanized on day 0 for baseline assessments of histologic features, ultrafiltration, and membrane transport. Two animals were administered AdLacZ and euthanized on day 4 for β-galactosidase analysis.

Solute Transport

Whole blood was centrifuged at 5000 rpm for 10 min, and the serum was removed. Peritoneal fluid samples obtained at the time of PET were centrifuged at 1500 rpm for 5 min, and the supernatant was removed for analysis. Samples were analyzed with an Hitachi 917 automated chemistry analyzer (Roche Diagnostics, Laval, Canada). Creatinine levels were measured using the picric acid-modified Jaffé method, and peritoneal samples were corrected for glucose levels [corrected creatinine concentration = measured creatinine concentration − (0.137 × measured glucose concentration)]. Albumin levels were measured by using the bromcresol green method, and glucose levels were measured by using a standard enzymatic test. The following measures of peritoneal transport were calculated: creatinine and albumin dialysate/plasma concentration ratios after a 4-h peritoneal dwell time and the glucose 4-h/initial dialysate concentration ratio. Solute clearance or mass transfer was also measured at time points when the animals were euthanized and an accurate ultrafiltrate volume could be assessed. Because the initial solute concentration for creatinine and albumin was 0, the 4-h clearance was calculated as follows: final Dianeal solute concentration × final volume/plasma concentration. Mass transfer of glucose out of the peritoneum was calculated as follows: (initial dialysate glucose concentration × initial volume) − (final dialysate glucose concentration × final volume). These values were corrected for animal weights at the time of euthanasia.

Cell Counts and Differential Determinations

The cell pellets remaining after centrifugation of the peritoneal fluid samples were resuspended in PBS. The numbers of cells were counted by using a hemocytometer. The cells were centrifuged onto a glass slide (Shandon, Pittsburgh, PA) and stained (Biochemical Science, Swedesboro, NJ). Cell differentials were determined with at least 300 cells.

Cell Culture

Rat peritoneal mesothelial cells were collected from a whole section of anterior abdominal wall placed in an isolation device (39). The parietal peritoneal surface was washed once with PBS and once with 0.25% trypsin (Life Technologies, Burlington, Canada) for 5 min and was exposed to fresh trypsin for 30 min at 37°C. The surface was gently scraped, and the cells were recovered. After centrifugation at 1000 rpm at 4°C for 5 min, the cell pellet was resuspended in Dulbecco's modified Eagle's medium/F-12 medium with 10% fetal bovine serum (Sigma Chemical Co., St. Louis, MO), 1% penicillin/streptomycin (Life Technologies), and 0.08% Fungizone (Life Technologies), and cells were plated in 40-cm^2 flasks (Corning, Cambridge, MA). Mesothelial cells in culture exhibited a cobblestone appearance at confluence and were vimentin-positive, with the eccentric nuclei characteristic of mesothelial cells (data not shown). Cells were used between passage 4 and passage 8.

Cells were plated on 100-mm plates (Corning) and grown to confluence. Plated cells were rested in 1% fetal bovine serum-containing medium for 24 h and were then exposed to recombinant human active TGF-β1 (R&D Systems, Minneapolis, MN), at concentrations of 1 or 10 ng/ml, for 12 h. The cells were then washed three times with PBS, and fresh medium with 1% fetal bovine serum was added. Supernatant samples were obtained at 12, 24, 48, and 72 h after exposure to recombinant TGF-β1.

Histologic Assessments

Cytochemical staining for β-galactosidase was performed on samples obtained from animals 4 d after intraperitoneal infection with AdLacZ. Fresh tissue samples were fixed in 2% formaldehyde/0.2% glutaraldehyde for 3 h. Fixation was followed by staining for 6 h in a solution containing potassium ferrous cyanide, potassium ferric cyanide, and magnesium chloride (Sigma), with Triton X-100 and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Boehringer Mannheim, Indianapolis, IN). The samples were stored in 70% ethanol and then paraffin-processed and -embedded, and 5-μm sections were cut. These sections were counterstained with nuclear fast red.

Tissue samples were obtained from the anterior abdominal wall, liver, and mesentery of AdTGFβ1- and control virus-treated animals on days 4, 7, 21, and 28 and were fixed in a sufficient amount of 4% phosphate-buffered formaldehyde for 24 h. The tissue samples were then paraffin-processed and -embedded, and 3-μm sections were cut. Cut sections were stained with hematoxylin and eosin and with Masson's trichrome stain.

Immunohistochemical Analyses

Using the same 4-, 7-, 21-, and 28-d samples, 3-μm sections were cut on aptex-coated slides and immunohistochemical analyses were performed with antibodies to α-SMA, vimentin, and von Willebrand factor/factor VIII-related antigen (Dako Corp., Carpenteria, CA). Negative control sections were assayed in parallel, with nonimmune mouse or rabbit Ig diluted to the same concentrations as the primary antibodies. All sections were deparaffinized in xylene, followed by 100% ethanol, and were then placed in a freshly prepared methanol/H_2O_2 solution for 30 min, for blockade of endogenous peroxidase activity. After hydration to water with graded alcohols, the sections were placed in 0.05 M Tris-buffered saline (TBS) (pH 7.6). The sections for α-SMA assays were blocked for 20 min with 1% normal swine serum (NSS) in TBS, followed by overnight incubation with mouse anti-human α-SMA (1:100) in 1% NSS. Sections were then incubated with a biotinylated rabbit anti-mouse Ig (1:300; Dako) for 1 h, followed by a 45-min incubation with a streptavidin-peroxidase conjugate (1:600; Dako). Sections for vimentin and factor VIII were pretreated before primary incubations. For vimentin assays, microwave antigen retrieval was performed with hot 1 mM ethylenediaminetetraacetate for 10 min, and sections were allowed to reach room temperature before subsequent procedures were performed. Sections were then blocked with 1% NSS for 20 min, followed by overnight incubation with mouse anti-human vimentin (1:300; Dako) in antibody-diluting fluid. Sections were incubated for 1 h with biotinylated rabbit anti-mouse Ig (1:300) in antibody-diluting fluid, followed by a 45-min incubation with a streptavidin-peroxidase conjugate (1:600; Dako) in peroxidase-stabilizing buffer. For factor VIII, the sections were digested for 17 min with 0.05% Pronase (Sigma) in TBS with calcium chloride, at room temperature. Sections were blocked with 5% normal goat serum, followed by a 1-h incubation with rabbit anti-human factor VIII (1:500) in 1% normal goat serum. Sections were then incubated with a prediluted, biotinylated, goat anti-rabbit Ig preparation, followed by a streptavidin-peroxidase conjugate (Zymed Laboratories, San Francisco, CA), according to the instructions provided by the manufacturer. All immunohistochemical incubations were performed at room temperature, and all sections were washed with 0.05 M TBS (pH 7.6) for 3 × 5 min between incubations, except before the addition of primary antibodies. All sections were rinsed with 0.05 M acetate buffer (pH 5.0) before development with an aminoethylcarbazole chromogen substrate for 15 min. All sections were counterstained with Mayer's hematoxylin for 2 min before being mounted with glycerin gelatin.

Blood Vessel Analysis

Sections of the anterior abdominal wall that had been immunostained for factor VIII were studied in a blinded manner, by using a standardized microscope grid to count peritoneum-associated blood vessels and to measure the thickness of the peritoneum. The area examined included all tissue from the abdominal wall muscle to the peritoneal cavity. Slides contained one to four separate tissue pieces, and each slide was examined in 10 to 12 randomly selected, high-power views. All measurements were performed at the same magnification. The number of blood vessels counted per grid was standardized to a count per 1-mm length of peritoneum.

Cytokine Analyses

Peritoneal fluid and cell culture supernatants were collected and centrifuged at 1500 rpm for 5 min. The samples were analyzed using a human TGF-β1 enzyme-linked immunosorbent assay (ELISA) kit, a mouse VEGF ELISA kit, or a rat tumor necrosis factor-α (TNF-α) ELISA kit (R&D Systems). The sensitivities of these assays were 7.0, 3.0, and 5.0 pg/ml, respectively. Samples were assayed for active TGF-β1 concentrations without treatment; samples were also treated with HCl, pH-adjusted with NaOH, and then assayed for total (active and latent) TGF-β1.

Hydroxyproline Assay

A portion of mesentery was frozen for an hydroxyproline assay, which was modified from the method described by Woessner (40). Tissues were weighed, homogenized in water, and centrifuged at 1000 rpm for 5 min, and the superficial fatty material was removed by vacuum suction. Solid material was precipitated with TCA, with centrifugation at 1500 rpm for 15 min at 4°C. Samples were hydrolyzed overnight in 6 N HCl at 110°C. Hydroxyproline contents were quantified with Erlich's reagent (Sigma) and were assayed by measurement of the OD at 557 nm. Standard hydroxyproline samples (Sigma) were used to create a standard curve.

Statistical Analyses

Data are presented as mean ± SEM unless otherwise noted. Comparisons between groups were performed by *t* test. We used linear regression analyses to compare Dianeal cytokine levels and solute transport. ANOVA was used to examine the mean clearances of creatinine and albumin and the mass transfer of glucose in the treated and control animals during the course of the experiments.

Results

Adenoviral Gene Transfer to Peritoneal Mesothelial Cells

Adenovirus is highly infective for mesothelial cells, as demonstrated by widespread expression of β-galactosidase in the peritoneal cavity 4 d after AdLacZ administration (Figure 1). At the dose used, adenoviral transgene product was not observed in the liver, spleen, skeletal muscle tissue, or submesothelial interstitium. We searched for evidence of inflammation attributable to the adenovirus (Table 1). Specifically, we did not observe a significant difference between AdTGFβ1- and AdDL70-treated animals with respect to inflammatory cells in the peritoneal fluid. The day 4 TNF-α concentrations in the dialysate were low and were not different between groups. This finding suggests that the inflammatory response to the adenovirus was limited and was similar for control and active virus. Previous work identified a mild early inflammatory response to adenovirus (as indicated by neutrophilia and increased TNF-α concentrations), which resolved by 48 h (41).

The animals that received AdTGFβ1 demonstrated significant elevations in active TGF-β1 levels in the dialysate from day 4 to day 14 (Figure 2). Levels peaked on day 4 (1920 *versus* 87 pg/ml for AdDL70, $P < 0.001$). There was a significant secondary increase in total TGF-β1 concentrations that was maximal on day 7 (16.4 *versus* 0.3 ng/ml, $P < 0.001$). Biologically active TGF-β1 is the form produced after infection with AdTGFβ1, and the total (active plus latent) amount likely represents autoinduction of latent TGF-β1 by resident peritoneal cells (42). The time course of transient expression of gene product after AdTGFβ1 infection was similar to that demonstrated in the rat lung model (35).

Figure 1. Adenovirus-mediated gene transfer to the peritoneal mesothelium. Four days after delivery of AdLacZ, the peritoneum was fixed and stained for β-galactosidase activity. (A) Whole section of the anterior abdominal wall, demonstrating extensive uniform expression (blue staining) of adenoviral gene product. (B) Section of the anterior abdominal wall, demonstrating that β-galactosidase staining was limited to the mesothelial cell monolayer. The section was counterstained with nuclear fast red. Magnification, ×100.

Effects of TGF-β1 on the Structure of the Peritoneum

Animals were euthanized on days 4, 7, 21, and 28, and parietal and visceral peritoneal surfaces were evaluated by microscopic and immunohistochemical techniques. By day 4, there were significant changes in the peritoneum of AdTGFβ1-treated animals. There was thickening of the peritoneum, with increased cellularity and vascularization (Figure 3). By day 7, these effects were even more prominent. On day 7, increased collagen amounts were present in the peritoneum. The more superficial cells in the thickened peritoneum were predominantly vimentin-positive (data not shown). Immunohistochemical analyses demonstrated the accumulation of α-SMA-positive interstitial cells (Figure 4). Vascular proliferation was prominent in factor VIII-stained sections (Figure 4). By day 21, the proliferative response had diminished but there was still generalized thickening of the submesothelial collagenous zone, with increased vascularity. By day 28, the submesothelial zone remained thickened but the degree of vascularity had decreased.

We noted similar changes in visceral peritoneal sections from animals exposed to TGF-β1. Specifically, mesenteric tissue demonstrated increased collagen deposition and vascularity. Also, in the day 7 samples from animals exposed to TGF-β1, there seemed to be increased blood vessel density extending into the superficial muscle layer of the abdominal peritoneal wall. These changes were not quantified. No histologic changes were detected in the control adenovirus-treated groups, compared with PBS-treated animals (Figure 3).

Mesenteric Collagen Content

Hydroxyproline is a component of collagen, and its concentration is directly correlated with the collagen content. The hydroxyproline contents of mesenteric samples from AdTGFβ1-treated animals were unchanged, compared with control adenovirus-treated animals, on day 4 but were elevated

Table 1. Inflammatory changes after adenoviral administration, measured as cellular responses and TNF-α concentrations in peritoneal fluid[a]

Parameter	PBS		AdTGFβ1		AdDL70	
	Day 4	Day 7	Day 4	Day 7	Day 4	Day 7
Total cells[b]	8.3 ± 5.3	19.2 ± 9	5.3 ± 2.1	10.9 ± 3.5	13.0 ± 2.9	20.8 ± 10.2
Lymphocytes (%)[c]	30.7 ± 2	20.6 ± 7.7	32.4 ± 12.3	22.7 ± 11.4	17.6 ± 8.8	29.4 ± 20.6
Macrophages (%)[c]	56.0 ± 8.5	44.4 ± 10.6	61.1 ± 10.1	73.4 ± 11.3	69.8 ± 6.1	43.7 ± 29.1
Neutrophils (%)[c]	0.1 ± 0.2	7.6 ± 2.5	2.6 ± 2.4	3.2 ± 2.9	6.1 ± 7.5	0.4 ± 0.5
TNF-α (pg/ml)	ND	ND	32.2 ± 17.1	42.4 ± 22.5	25.8 ± 3.8	35.9 ± 13.3

PBS, phosphate-buffered saline; TNF-α, tumor necrosis factor-α; ND, not determined.
$P > 0.05$ for comparisons between AdTGFβ1 and AdDL70 groups at each time point.
[a] All values are mean ± SD.
[b] Total cell count (total cells in peritoneal fluid × 10^6).
[c] Differential counts (percentage of total cells).

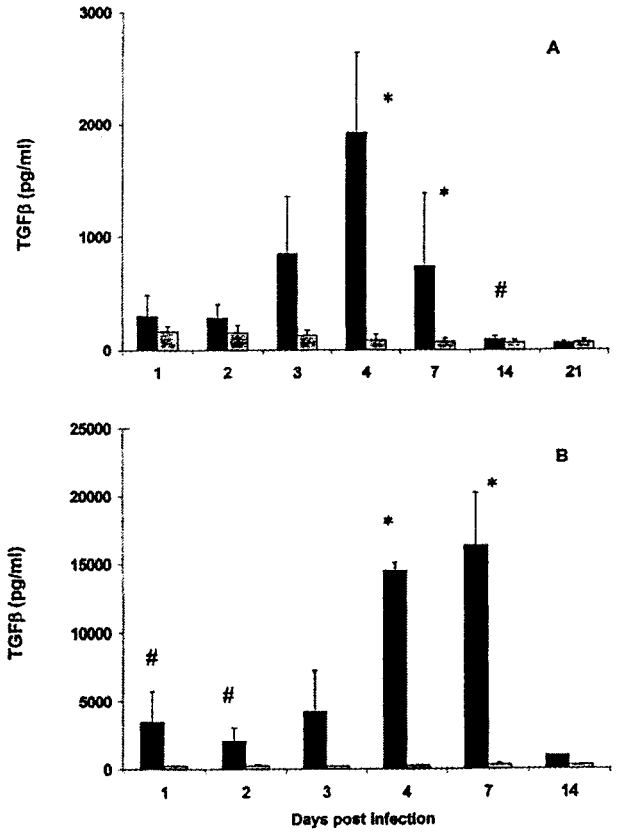

Figure 2. Transforming growth factor-β (TGF-β) concentrations in peritoneal dialysate after adenoviral infection. (A) Active TGF-β1 concentrations in AdTGFβ1-treated animals (■) were significantly elevated, compared with AdDL70-treated animals (▨), from day 4 to day 14. (B) Total TGF-β dialysate concentrations were elevated in AdTGFβ1-treated animals on days 1 to 2 and days 4 and 7. *$P <$ 0.001, #$P < 0.05$. Data are mean ± SD (n = 3 or 4 for days 1 to 3, n = 5 to 22 for days 4 to 14).

on days 7, 21, and 28 (data not shown). The difference reached statistical significance on day 21 (2.9 ± 0.7 *versus* 1.8 ± 0.3 mg/g tissue for control AdDL70, $P = 0.006$).

Quantitative Morphologic Assessments

Quantitative morphologic assessments performed using a standardized microscope grid and factor VIII-stained sections demonstrated significantly greater average thicknesses of the peritoneum for AdTGFβ1-treated animals on days 4, 7, and 21 (Figure 5). With resolution of the fibroproliferative phase on day 7, the thickness of the peritoneum decreased to 67 μm by day 21. This change persisted to day 28 (64 μm), but the difference was no longer significant. The number of vessels per 1-mm length of peritoneum was maximal on day 7 (11.4 vessels/mm) and then progressively decreased from day 21 (8.9 vessels/mm) to day 28 (4.4 vessels/mm). No differences were noted between control virus-treated animals and day 0 PBS-treated animals with respect to thickness (29.8 *versus* 32.2 μm for day 21 control virus, $P = 0.43$) or number of blood vessels (1.2 *versus* 1.2 vessels/mm for day 21 control virus, $P = 0.99$) (data not shown).

Effects of TGF-β1 on the Function of the Peritoneal Membrane

The transport characteristics of the peritoneal membrane in AdTGFβ1-, AdDL70-, or PBS-treated animals were evaluated using a modified PET (Table 2). Animals treated with AdTGFβ1 exhibited significant reductions in ultrafiltration from day 4 to day 21 ($P < 0.05$). The ultrafiltration impairment resolved by day 28 (Figure 6).

We used both the standard dialysate/plasma solute ratio and clearance (for creatinine and albumin) or mass transfer (for glucose) to analyze the alterations in transport properties after transient exposure to TGF-β1. Because of the significant differences in net ultrafiltration identified, solute clearance or mass transfer better estimates the diffusive transport nature of the peritoneum (10). Using these measures, we observed no significant change in the clearance of creatinine (Table 2). We did, however, note a significant increase in the clearance of albumin ($P = 0.006$, compared with AdDL70, by ANOVA) and in the mass transfer of glucose ($P < 0.001$) during the course of the experiment. These differences were most apparent on days 4 and 7, and values returned to baseline levels by day 28.

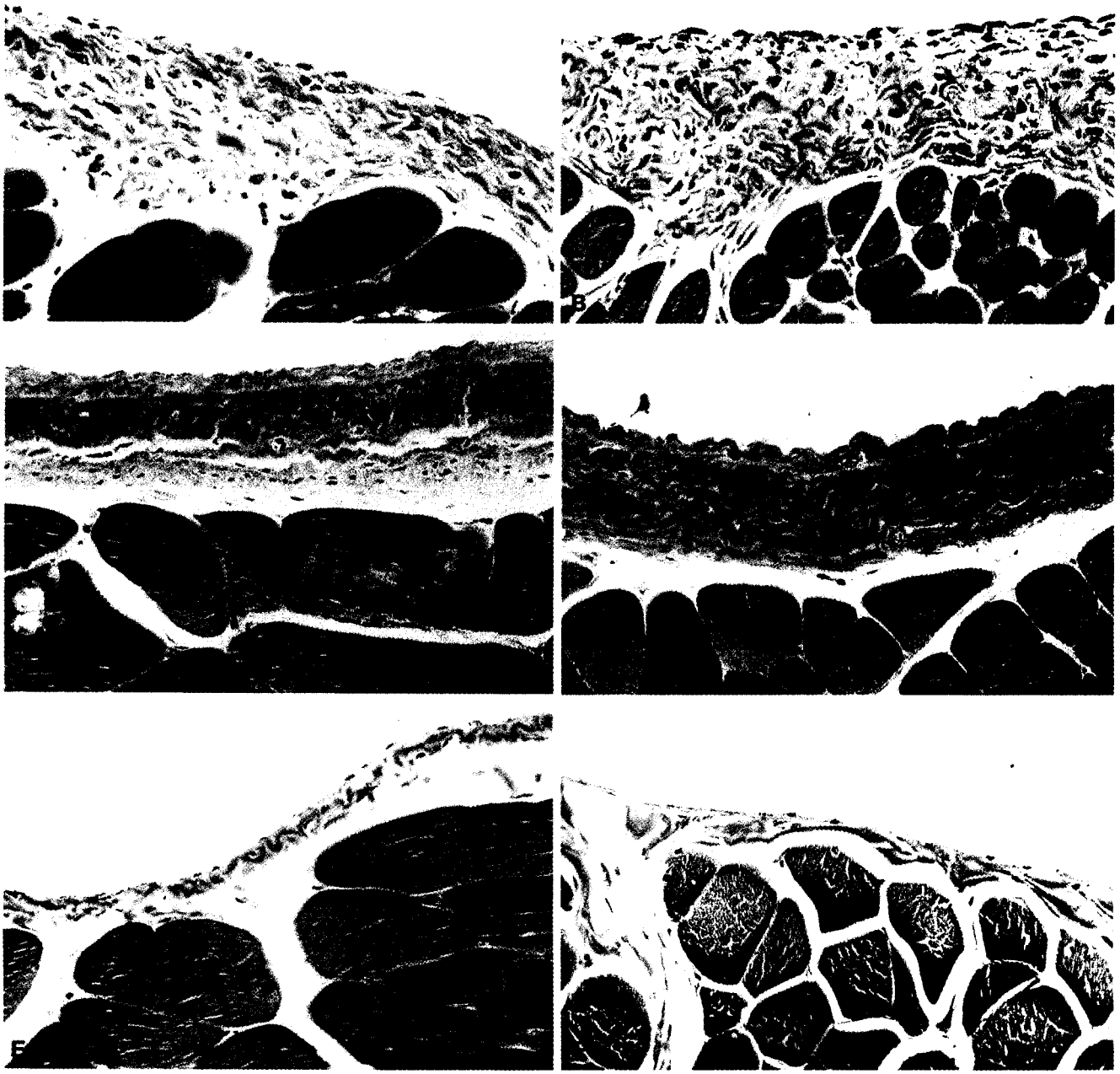

Figure 3. Histologic features of the anterior abdominal wall after exposure to AdTGFβ1. (A) On day 4 after infection, there is a fibroproliferative response, with increased thickness, cellularity, and vascularization. (B) On day 7, these changes are exaggerated, with some increase in collagen deposition. (C) By day 21, the cellularity of the peritoneal membrane has decreased but there is ongoing vascularization and extensive collagen deposition. (D) Twenty-eight days after infection, the collagen deposition remains but vascularity is decreased. (E) Four days after control virus (AdDL70) infection, the peritoneum exhibits no acute virus-related changes. (F) Normal rat peritoneum on day 21 after phosphate-buffered saline (PBS) treatment is also shown. All sections, Masson's trichrome stain. Magnification, ×200.

Role of VEGF and Increased Peritoneal Membrane Transport

Primary mesothelial cells in culture produce VEGF in response to exposure to recombinant TGF-β1 (Figure 7). Forty-eight hours after exposure to 10 ng/ml TGF-β1, mesothelial cells produced 2.4 times the amount of VEGF measured in the supernatant by ELISA ($P = 0.001$).

The dialysate concentration of VEGF was significantly increased for AdTGFβ1-treated rats, compared with control rats, on days 4 and 7 (1512 *versus* 92 pg/ml for AdDL70-treated animals on day 7; $P < 0.001$) (Figure 7). The VEGF dialysate concentration decreased by day 14 but remained significantly elevated, compared with dialysate from AdDL70-treated animals, until day 21 (118 *versus* 64 pg/ml, $P = 0.04$).

There was a correlation between TGF-β1 exposure and VEGF expression. On day 4, the dialysate TGF-β1 concentra-

Figure 4. Immunohistochemical analyses of the anterior abdominal wall. (A) α-Smooth muscle actin (α-SMA) staining 7 d after AdTGFβ1 infection demonstrates hypercellularity of the peritoneal membrane, with staining of myofibroblasts and smooth muscle of small arterioles. (B) α-SMA staining 7 d after AdDL70 control virus infection demonstrates a normal thin peritoneal membrane, with staining of smooth muscle cells in occasional deep arterioles. (C) Factor VIII staining 7 d after exposure to AdTGFβ1 demonstrates a thickened membrane with markedly increased vascularity, which is not evident in the control virus-treated animal (D). (E) Factor VIII staining on day 21 demonstrates the persistence of increased vascularity, which is not evident in AdDL70-treated animals on day 21 (F). Magnification (all sections), ×200.

tion was correlated with the VEGF concentration ($r^2 = 0.240$, $P = 0.03$) (Figure 7).

Discussion

In these experiments, we demonstrated that adenovirus-mediated gene transfer to the peritoneum is a useful technique for studying the effects of cytokines on peritoneal membrane structure and function. Using a β-galactosidase adenoviral vector in mouse peritoneum, Osada *et al.* (43) demonstrated the effectiveness of adenoviral gene transfer to mesothelial cells and the lack of acute virus-induced changes. Similarly, we observed efficient infection of rat mesothelial cells by replica-

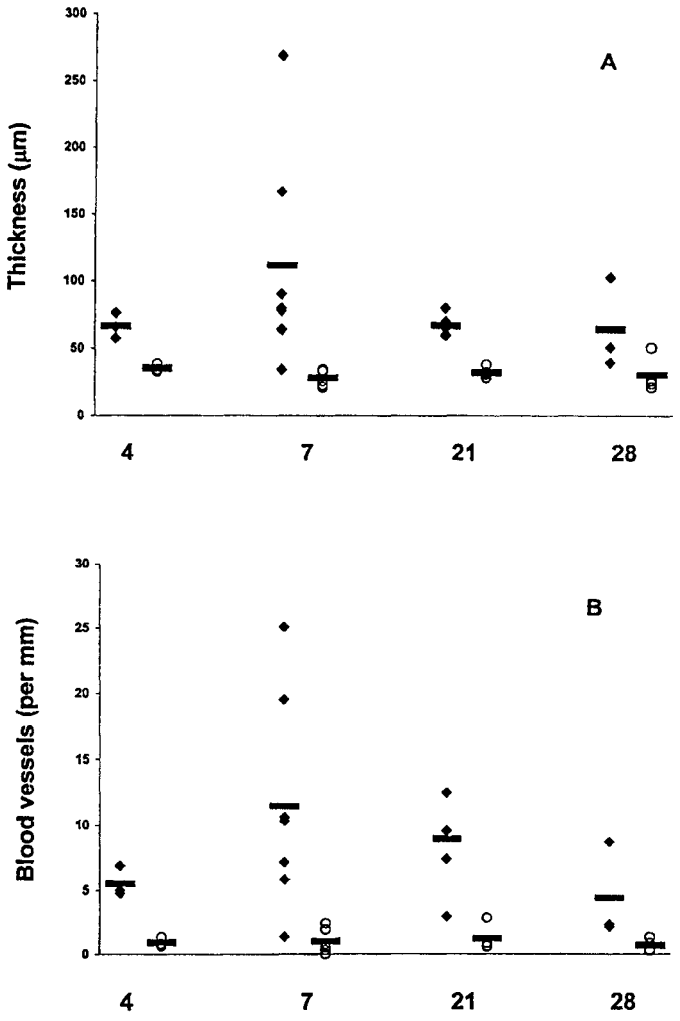

Figure 5. AdTGFβ1-mediated changes in membrane thickness and vascularity, as assessed by quantitative immunohistochemical analysis of factor VIII-stained sections of parietal peritoneum. All data points are presented with the black bars representing mean values. (A) Thickness of the peritoneal membrane in AdTGFβ1-treated animals (◆) and control (AdDL70-treated) animals (○). (B) Number of blood vessels counted per millimeter length of peritoneal membrane in AdTGFβ1-treated animals (◆) and control animals (○). Ten to 12 fields were counted for each section. $P < 0.05$ for comparison between AdTGFβ1- and AdDL70-treated animals (days 4 to 21) for both thickness and vessels per millimeter.

tion-deficient adenoviral vectors. In comparisons of control virus- and PBS-treated animals, we observed no demonstrable, acute, virus-induced effects on morphologic features or peritoneal membrane transport at the vector dose used. In comparisons of the AdDL70 control and AdTGFβ1 virus, we observed no difference in markers of acute inflammation after 4 d.

There are similarities between our animal model of AdTGFβ1-induced peritoneal fibrosis and the peritoneum of patients undergoing long-term dialysis. As has been observed in biopsy samples from patients undergoing long-term peritoneal dialysis (11,12,14), we noted increased thickness of the submesothelial collagenous zone, with increased α-SMA staining and increased vascularity. The solute transport properties of the AdTGFβ1-treated peritoneum also mimic the changes observed for long-term dialysis patients, with increased transport of glucose and albumin and decreased ultrafiltration capacity. Some of the changes we observed in our model were transitory. The early fibrocellular proliferation and increased vascularization declined after 7 and 21 d, respectively. The changes in membrane transport and ultrafiltration also normalized with the decline in vascularization.

Variability was noted in the histologic changes observed in the peritoneum of animals treated with AdTGFβ1. These changes were fairly uniform among sections from individual animals, suggesting that inadequate sampling did not affect these results. It is more likely that there was variability in the level of TGF-β1 expressed in each animal, despite delivery of similar adenoviral doses, and this range of exposure explains the variable histologic presentation.

Previous studies in the lung with AdTGFβ1 demonstrated a progressive fibrogenic response (35). In the peritoneum, there seems to be sustained expansion of the extracellular matrix, as indicated by both a sustained increase in the thickness of the submesothelial collagenous zone and increased hydroxyproline contents in the mesenteric tissue. Our findings are therefore in agreement with a maintained "fibrotic" response to transient overexpression of TGF-β1. Of importance is the identified dissociation between "fibrosis," as indicated by extracellular matrix deposition, and the vascular component.

There is a narrow concentration range in which TGF-β1 produces effects in the peritoneum. To identify a suitable dose of AdTGFβ1 to use in this study, we initially performed a dose-response study. We observed that doses of AdTGFβ1 of $<1 \times 10^9$ plaque-forming units led to transient TGF-β1 levels of <1000 pg/ml, with no histologic or functional effects. At higher doses, excessive morbidity resulted from a striking early fibroproliferative response, which led to stricture and bowel obstruction. This resistance to the effects of TGF-β1 could be explained by the upregulation of natural inhibitors of TGF-β1, such as the proteoglycan decorin, or by inhibitors of the fi-

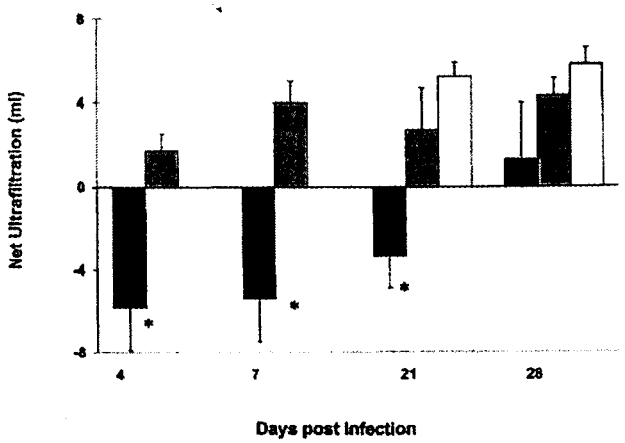

Figure 6. Net ultrafiltration after adenovirus infection. AdTGFβ1 treatment led to temporary ultrafiltration failure. AdTGFβ1-treated animals (■) were compared with AdDL70-treated (▨) and PBS-treated (□) animals. *$P < 0.05$.

Table 2. Transport properties of the peritoneal membrane after adenovirus-mediated gene transfer of TGF-β1[a]

Day	Group	Creatinine Clearance (ml/kg)	Glucose Transport (mmol/g)	Albumin Clearance (ml/kg)	Creatinine (d/p)	Glucose (d/d_0)	Albumin (d/p) (10^{-3})
4	AdTGFβ1	32 ± 11	9.8 ± 0.2	4.7 ± 2.0	0.59 ± 0.05[b]	0.08 ± 0.01[c]	65.5 ± 59
	AdDL70	42 ± 2	7.4 ± 0.7	1.1 ± 1.3	0.43 ± 0.11	0.29 ± 0.03	21.4 ± 26.0
	PBS	ND	ND	ND	0.22 ± 0.08	0.29 ± 0.02	0
7	AdTGFβ1	46 ± 13	8.9 ± 1.8	10 ± 7	0.68 ± 0.17[c]	0.07 ± 0.01[c]	245 ± 117[c]
	AdDL70	41 ± 4	6.2 ± 0.3	2.1 ± 0.2	0.41 ± 0.09	0.25 ± 0.10	13.7 ± 24.6
	PBS	ND	ND	ND	0.44 ± 0.20	0.27 ± 0.03	0
14	AdTGFβ1	ND	ND	ND	0.42 ± 0.06	0.12 ± 0.06[b]	57.6 ± 44.4[b]
	AdDL70	ND	ND	ND	0.47 ± 0.10	0.25 ± 0.09	12.8 ± 28.6
	PBS	ND	ND	ND	0.43 ± 0.04	0.30 ± 0.03	0
21	AdTGFβ1	38 ± 5	8.3 ± 0.7	2.6 ± 2.3	0.46 ± 0.09	0.20 ± 0.06[b]	43.7 ± 58.0
	AdDL70	46 ± 10	7.4 ± 1.1	2.3 ± 1.8	0.38 ± 0.09	0.26 ± 0.06	17.9 ± 17.2
	PBS	49 ± 2	6.4 ± 0.8	0	0.42 ± 0.07	0.24 ± 0.08	0
28	AdTGFβ1	48 ± 15	7.3 ± 0.7	0.6 ± 1.3	0.40 ± 0.09	0.27 ± 0.02	9.1 ± 20.3
	AdDL70	49 ± 7	6.8 ± 0.4	1.0 ± 1.3	0.39 ± 0.04	0.28 ± 0.03	7.2 ± 11.2
	PBS	52 ± 9	6.6 ± 0.4	0	0.40 ± 0.03	0.27 ± 0.02	0

[a] All data are mean ± SD. d/p, dialysate/plasma concentration ratio; d/d_0, 4-h/initial concentration ratio; TGF-β1, transforming growth factor-β1; ND, not determined.
[b] $P < 0.02$ by t test for comparison between AdTGFβ1 and AdDL70 groups.
[c] $P < 0.001$.

brotic response, such as the metalloproteinases. The resolution of the vascular component of the fibrotic response suggests that there may be upregulation of natural inhibitors to angiogenesis in response to the transient high levels of TGF-β or a natural decrease in vascularity with the withdrawal of VEGF. If the peritoneum can recover from a transient insult, then perhaps prolonged or repeated exposure to profibrotic cytokines (via recurrent infection or daily dialysis) may induce more lasting changes in the structure and function of the peritoneal membrane. Chronic rat models of peritoneal dialysis have indeed confirmed this hypothesis (44,45).

The time course of neovascularization coincides with the changes in peritoneal membrane transport. This suggests that alteration of blood vessels, through either increased vascular surface area (represented by the number of vessels per unit length of peritoneal membrane in cross-sections) or increased vascular permeability, is responsible for the changes in peritoneal membrane transport we observed. The increased albumin transport observed on day 4, at a time when the dialysate concentration of VEGF was very high and vascularization was only moderately increased, suggests a direct permeability response of existing blood vessels to VEGF at earlier time points. The ongoing ultrafiltration defect at day 21, *i.e.*, a time when VEGF levels had returned to baseline values and blood vessel counts were still above control levels, supports a role for increased vascularization in the demonstrated changes in peritoneal membrane function.

On the basis of our limited *in vitro* observations and recently reported observations that exposure of rat primary mesothelial cells to TGF-β led to an increase in VEGF protein and mRNA production (32), we suggest that mesothelial cells may be responsible for *in vivo* angiogenic cytokine production after TGF-β1 exposure. Other resident peritoneal cell populations, such as macrophages, fibroblasts, and endothelial cells, may also participate in this response.

We have established a model of TGF-β1-mediated peritoneal fibrosis in rats. This model can be used to elucidate the molecular mechanisms and pathobiologic features of peritoneal fibrosis and the consequent effects on solute transport. Our model suggests that TGF-β1 induces structural alterations in the peritoneum, which lead to increased solute permeability and decreased ultrafiltration. We demonstrate that VEGF expression is dramatically increased in peritoneal membranes exposed to TGF-β1 and mesothelial cells likely have a role in this process. Changes in peritoneal membrane function seem to be correlated with exposure to VEGF, and the changes observed resolve as the neovascularization declines. Our observation that angiogenic factors play an important role may lead to potential therapeutic interventions for the prevention or treatment of peritoneal membrane failure among patients undergoing long-term peritoneal dialysis. Effective adenoviral gene therapy using antiangiogenic genes such as angiostatin has been demonstrated in tumor models (46). Because of their potential use in cancer therapy, new antiangiogenic compounds are being developed and tested (47,48), and these compounds may be useful in protecting the peritoneal membrane during chronic peritoneal dialysis.

Acknowledgments

This research was funded by Baxter Healthcare USA and the Medical Research Council of Canada. Dr. Margetts holds a joint Medical Research Council/Kidney Foundation of Canada fellowship. We thank Mary Jo Smith and Brian Hewlett for their expertise in histologic and immunohistochemical analyses.

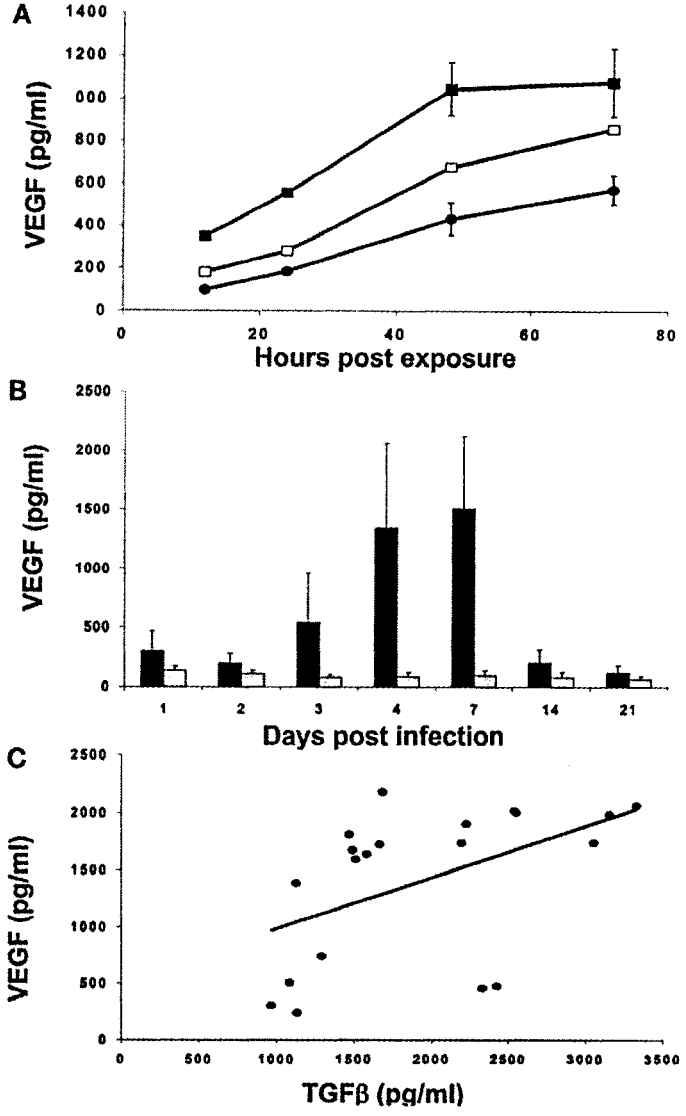

Figure 7. Relationship between TGF-β1 and vascular endothelial growth factor (VEGF). (A) *In vitro* expression of VEGF in mesothelial cell culture supernatants after exposure for 12 h to 10 ng/ml recombinant TGF-β1 (■), 1 ng/ml TGF-β (□), or PBS (●). Significant differences ($P < 0.01$) were observed at 48 and 72 h (10 ng/ml *versus* control). (B) Peritoneal dialysate VEGF concentrations after exposure to AdTGFβ1 (■) or AdDL70 (control) (▨). Significant differences ($P < 0.001$) were observed for days 4, 7 ($P < 0.001$), and 14 ($P = 0.03$). Values shown are mean ± SD ($n = 3$ to 5 for days 1 to 3 and $n = 10$ to 21 for days 4 to 14). (C) Correlation between day 4 TGF-β peritoneal dialysate concentrations and day 4 VEGF concentrations ($r^2 = 0.240$, $P = 0.03$).

References

1. Davies SJ, Bryan J, Phillips L, Russell GI: Longitudinal changes in peritoneal kinetics: The effects of peritoneal dialysis and peritonitis. *Nephrol Dial Transplant* 11: 498–506, 1996
2. Davies SJ, Phillips L, Griffiths AM, Russell LH, Naish PF, Russell GI: What really happens to people on long-term peritoneal dialysis? *Kidney Int* 54: 2207–2217, 1998
3. Slingeneyer A, Canaud B, Mion C: Permanent loss of ultrafiltration capacity of the peritoneum in long-term peritoneal dialysis: An epidemiological study. *Nephron* 33: 133–138, 1983
4. Heimbürger O, Waniewski J, Werynski A, Tranæus A, Lindholm B: Peritoneal transport in CAPD patients with permanent loss of ultrafiltration capacity. *Kidney Int* 38: 495–506, 1990
5. Selgas R, Fernandez-Reyes M, Bosque E, Bajo M, Borrego F, Jimenez C, Del Peso G, De Alvaro F: Functional longevity of the human peritoneum: How long is continuous peritoneal dialysis possible? Results of a prospective medium long-term study. *Am J Kidney Dis* 23: 64–73, 1994
6. Davies SJ, Brown B, Bryan J, Russell GI: Clinical evaluation of the peritoneal equilibration test: A population based study. *Nephrol Dial Transplant* 8: 64–70, 1993
7. Krediet RT: The peritoneal membrane in chronic peritoneal dialysis. *Kidney Int* 55: 341–356, 1999
8. Flessner MF: Osmotic barrier of the parietal peritoneum. *Am J Physiol* 267: F861–F870, 1994
9. Struijk DG, Krediet RT, Koomen GCM, Boeschoten EW, Hoek FJ, Arisz L: A prospective study of peritoneal transport in CAPD patients. *Kidney Int* 45: 1739–1744, 1994
10. Krediet RT: Evaluation of peritoneal membrane integrity. *J Nephrol* 10: 238–244, 1997
11. Combet S, Miyata T, Moulin P, Pouthier D, Goffin E, Devuyst O: Vascular proliferation and enhanced expression of endothelial nitric oxide synthase in human peritoneum exposed to long term peritoneal dialysis. *J Am Soc Nephrol* 11: 717–728, 2000
12. Mateijsen MAM, van der Wal AC, Hendriks PMEM, Zweers MM, Mulder J, Struijk DG, Krediet RT: Vascular and interstitial changes in the peritoneum of CAPD patients with peritoneal sclerosis. *Perit Dial Int* 19: 517–525, 1999
13. Honda K, Nitta K, Horita S, Yumura W, Nihei H, Nagai R, Ikeda K, Horiuchi S: Accumulation of advanced glycosylation end products in the peritoneal vasculature of continuous ambulatory peritoneal dialysis patients with low ultra-filtration. *Nephrol Dial Transplant* 14: 1541–1549, 1999
14. Topley N, Craig KJ, Fallon M, MacKenzie R, Von Ruhland C, Newman GR, Williams GT, Mujais S, Williams JD: Morphological changes in the peritoneal membrane of patients on peritoneal dialysis are related to time on treatment [Abstract]. *J Am Soc Nephrol* 10: 324A, 1999
15. Nakamoto M: Pathogenesis of peritoneal fibrosis and peritoneal small vessel changes. *Perit Dial Int* 16[Suppl 1]: S39–S41, 1996
16. Gauldie J, Sime PJ, Xing Z, Marr B, Tremblay GM: Transforming growth factor β gene transfer to the lung induces myofibroblast presence and pulmonary fibrosis. *Curr Topics Pathol* 93: 35–45, 1999
17. Zweers MM, deWaart DR, Smit W, Struijk DG, Krediet RT: Growth factors VEGF and TGF β1 in peritoneal dialysis. *J Lab Clin Med* 134: 124–132, 1999
18. Witowski J, Korybalska K, Wisniewska J, Breborowicz A, Gahl GM, Frei U, Passlick-Deetjen J, Jörres A: Effect of glucose degradation products on human peritoneal mesothelial cell function. *J Am Soc Nephrol* 11: 729–739, 2000
19. Inagi R, Toshio M, Yamamoto T, Suzuki D, Urakami K, Saito A, van Ypersele de Strihou C, Kurokawa K: Glucose degradation product methylglyoxal enhances the production of vascular endothelial growth factor in peritoneal cells: Role in the functional and morphological alterations of peritoneal membranes in peritoneal dialysis. *FEBS Lett* 463: 260–264, 1999
20. DeVriese AS, Tilton RG, Stephan CC, Lameire NH: Vascular endothelial growth factor is essential for hyperglycemia-induced structural and functional alterations of the peritoneal membrane. *J Am Soc Nephrol* 12: 1734–1741, 2001

21. Breborowicz A, Oreopoulos G: Evidence for the presence of chronic inflammation during peritoneal dialysis: Therapeutic implications. *Perit Dial Int* 17[Suppl 2]: S37–S41, 1997
22. Kang K, Hong Y, Lim HJ, Choi J, Han K, Yoon K: High glucose solution and spent dialysate stimulate the synthesis of transforming growth factor β1 of human peritoneal mesothelial cells: Effect of cytokine costimulation. *Perit Dial Int* 19: 221–230, 1999
23. Mlambo NC, Hylander B, Brauner A: Increased levels of transforming growth factor β1 and basic fibroblast growth factor in patients on CAPD: A study during non-infected steady state and peritonitis. *Inflammation* 23: 131–139, 1999
24. Wang T, Ghen YG, Ye RG, Mai WY, Zhen ZH, Li HQ: Enhanced expression of TGFβ1 by peritoneal macrophages in CAPD patients. *Adv Perit Dial* 11: 11–14, 1995
25. Lai KN, Lai KB, Szeto CC, Lam CWK, Leung JCK: Growth factors in continuous ambulatory peritoneal dialysis effluent. *Am J Nephrol* 19: 416–422, 1999
26. Churchill DN, Thorpe KE, Nolph KD, Keshaviah PR, Oreopoulos DG, Pagé D: Increased peritoneal membrane transport is associated with decreased patient and technique survival for continuous peritoneal dialysis patients. *J Am Soc Nephrol* 9: 1285–1292, 1998
27. Margetts PJ, McMullin JP, Rabbat C, Churchill DC: Peritoneal membrane transport and hypoalbuminia: Cause or effect? *Perit Dial Int* 20: 14–18, 2000
28. Border WA, Noble NA: Transforming growth factor β in tissue fibrosis. *New Engl J Med* 331: 1286–1292, 1994
29. Mozes MM, Böttinger EP, Jacot TA, Kopp JB: Renal expression of fibrotic matrix proteins and of transforming growth factor β isoforms in TGFβ transgenic mice. *J Am Soc Nephrol* 10: 271–280, 1999
30. Pertovaara L, Kipainen A, Mustonen T, Orpanan A, Ferrara N, Saksela O, Alitalo K: Vascular endothelial growth factor is induced in response to transforming growth factor β in fibroblastic and epithelial cells. *J Biol Chem* 269: 6271–6274, 1994
31. Saadeh PB, Mehrara BJ, Steinbrech DS, Dudziak ME, Greenwald JA, Luchs JS, Spector JA, Ueno H, Gittes GK, Longaker MT: Transforming growth factor β1 modulates the expression of vascular endothelial growth factor by osteoblasts. *Am J Physiol* 277: C628–C637, 1999
32. Hoff CM, Inman KL, Piscopo D, Shockley TR: TGF-β mediated production of VEGF by rat primary mesothelial cells [Abstract]. *J Am Soc Nephrol* 11: 310A, 2000
33. Ferrara N: Role of vascular endothelial growth factor in the regulation of angiogenesis. *Kidney Int* 56: 794–814, 1999
34. Hippenstiel S, Krüll M, Ikemann A, Risau W, Clauss M, Suttorp N: VEGF induces hyperpermeability by a direct action on endothelial cells. *Am J Physiol* 274: L678–L684, 1998
35. Sime PJ, Xing Z, Graham FL, Csaky KG, Gauldie J: Adenovector-mediated gene transfer of active transforming growth factor β1 induces prolonged severe fibrosis in rat lung. *J Clin Invest* 100: 768–776, 1997
36. Bett AJ, Haddara W, Prevac L, Graham FL: An efficient and flexible system for construction of adenovirus vectors with insertions or deletions in early regions 1 and 2. *Proc Natl Acad Sci USA* 91: 8802–8806, 1994
37. Xing Z, Ohkawara Y, Jordana M, Graham FL, Gauldie J: Transfer of granulocyte-macrophage colony-stimulating factor gene to rat lung induces eosinophilia, monocytosis, and fibrotic reactions. *J Clin Invest* 97: 1102–1110, 1996
38. Canadian Council on Animal Care: *Guide to the Care and Use of Experimental Animals*, 2nd Ed., Ottawa, Canada, Canadian Council on Animal Care, 1993
39. Hjelle JT, Golinska BT, Waters DC, Steidley KR, McCarroll DR, Dobbie JW: Isolation and propagation *in vitro* of peritoneal mesothelial cells. *Perit Dial Int* 9: 341–347, 1989
40. Woessner JF Jr: The determination of hydroxyproline in tissue and protein samples containing small proportion of this amino acid. *Arch Biochem Biophys* 93: 440–444, 1961
41. Hoff CM: *Ex vivo* and *in vivo* gene transfer to the peritoneal cavity. *Nephrol Dial Transplant* 16[Suppl 1]: 1–4, 2001
42. Kim SJ, Angel P, Lafyatis R, Hattori K, Kim KY, Sporn MB, Karin M, Roberts AB: Autoinduction of transforming growth factor β1 is mediated by the AP-1 complex. *Mol Cell Biol* 10: 1492–1497, 1990
43. Osada S, Ebihara I, Setoguchi Y, Takahashi H, Tomino Y, Koide H: Gene therapy for renal anemia in mice with polycystic kidney using an adenovirus vector encoding the human erythropoietin gene. *Kidney Int* 55: 1234–1240, 1999
44. Kredeit RT, Zweers MM, Ho-dac-Pannekeet MM, van der Wal AC, Smit W, Douma CE, Mateijsen MAM, Struijk D: The effect of various dialysis solutions on peritoneal membrane viability. *Perit Dial Int* 19[Suppl 2]: S257–S263, 1999
45. Kim Y, Kim SH, Kim JH, Kim SJ, Kim CD, Cho DK, Kim YJ, Moberly JM: Effects of peritoneal rest on peritoneal transport and peritoneal membrane thickening in continuous ambulatory peritoneal dialysis rats. *Perit Dial Int* 19[Suppl 2]: S384 –S387, 1999
46. Gyorffy SF, Podor TJ, Palmer K, Hitt M, Gauldie J: A novel role for combined anti-angiogenesis therapy and immunotherapy: Treatment of a murine breast cancer model using adenoviral gene therapy vectors expressing angiostatin and interleukin-12. *Blood* 94[Suppl 1]: 620a, 1999
47. Ferrara N, Alitalo K: Clinical applications of angiogenic growth factors and their inhibitors. *Nat Med* 5: 1359–1364, 1999
48. Verheul HM, Hoekman K, Jorna AS, Smit EF, Pinedo HM: Targeting vascular endothelial growth factor blockade: Ascites and pleural effusion formation. *Oncologist* 5[Suppl 1]: 45–50, 2000

CHAPTER 3: INFLAMMATORY CYTOKINES, ANGIOGENESIS, AND FIBROSIS IN THE RAT PERITONEUM

This paper is published in the American Journal of Pathology 2002; 160: 2285-2294. We used similar techniques as was used in the AdTGFβ1 model described above. This time, we looked specifically at two inflammatory cytokines - TNFα and IL-1β. We had some suggestions from other work that IL-1β may be a more fibrogenic cytokine than TNFα [166]. It was therefore of some interest to see the effect on the peritoneal function and angiogenesis.

IL-1β treated animals had a more potent inflammatory reaction with prolonged weight loss and increased peritoneal effluent markers of acute inflammation. One interesting finding was that adenovirus mediated gene transfer of TNFα led to an up-regulation of IL-1β, but IL-1β did not induce any expression of TNFα. This confirms that TNFα is an upstream, early inflammatory mediator.

TNFα induced a potent, but very transient angiogenesis with ultrafiltration dysfunction. There was also a transient increase in peritoneal fibrosis measured by hydroxyproline concentration in peritoneal tissues. IL-1β, in contrast, induced a very prolonged angiogenesis, ultrafiltration dysfunction, and peritoneal fibrosis. In trying to understand this, we identified a prolonged significant increased expression of TIMP-1 and PAI-1 mRNA expression in the peritoneal tissues of animals exposed to IL-1β. We felt that this inhibition of metalloproteinases and collagenolysis may explain the persisting fibrosis. Of note was the observation that TGFβ1 mRNA levels were very similar between TNFα and IL-1β treated animals.

This study identified the potential for angiogenesis, even very transient angiogenesis, to be a mechanism of acute ultrafiltration failure seen in PD patients who develop peritonitis. It also provided a potential link, through IL-1β, between peritonitis and longer term changes in fibrosis and ultrafiltration dysfunction.

The AdIL-1β was created by Dr. Daniel Anthony from the University of Southampton. The AdTNFα was created by Dr. Robert Marr who was previously in Dr. Frank Graham's lab.

I developed the concept for this paper. I designed the experiments with some input from Dr. Gauldie and Dr. Hoff. I designed the experiments. All work was carried out by me, with some technical support from Martin Kolb and Lisa Yu. I analyzed the data and wrote the paper. The manuscript was reviewed by Drs. Gauldie, Hoff, and Cliff Holmes.

American Journal of Pathology, Vol. 160, No. 6, June 2002

Animal Model

Inflammatory Cytokines, Angiogenesis, and Fibrosis in the Rat Peritoneum

Peter J. Margetts,*† Martin Kolb,* Lisa Yu,* Catherine M. Hoff,‡ Clifford J. Holmes,‡ Daniel C. Anthony,§ and Jack Gauldie*

From the Department of Pathology and Molecular Medicine and Division of Nephrology,† Centre for Gene Therapeutics, McMaster University, Hamilton, Ontario, Canada; Renal Division Scientific Affairs,‡ Baxter Healthcare Corporation, McGaw Park, Illinois; and the CNS Inflammation Group, the University of Southampton,§ Southhampton, United Kingdom*

Peritonitis, a common complication of peritoneal dialysis, is followed by acute changes in the function of the peritoneum. The role of inflammatory cytokines in these processes is not clearly identified. We used adenoviral-mediated gene transfer to transiently overexpress interleukin (IL)-1β (AdIL-1β) or tumor necrosis factor (TNF)-α (AdTNF-α) in the rat peritoneum then used a modified equilibrium test to study the histological and functional changes. Overexpression of IL-1β or TNF-α led to an acute inflammatory response. Both inflammatory cytokines induced an early expression of the angiogenic cytokine, vascular endothelial growth factor, along with increased expression of the profibrotic cytokine, transforming growth factor-β1, along with fibronectin expression and collagen deposition in peritoneal tissues. Both inflammatory cytokines induced angiogenesis, increased solute permeability, and ultrafiltration dysfunction at earlier time points. Changes in structure and function seen in AdTNF-α-treated animals returned to normal by 21 days after infection, whereas AdIL-1β-treated animals had persistently increased vasculature with submesothelial thickening and fibrosis. This was associated with up-regulation TIMP-1. TNF-α or IL-1β both induce acute changes in the peritoneum that mimic those seen in peritoneal dialysis patients who experience an episode of peritonitis. These functional changes were associated with early angiogenesis that resolved rapidly after exposure to TNF-α. IL-1β exposure, however, led to a different response with sustained vascularization and fibrosis. IL-1β inhibition may be a therapeutic goal in acute peritonitis to prevent peritoneal damage. (Am J Pathol 2002, 160:2285–2294)

Peritonitis is a common complication of peritoneal dialysis.[1] The response of the peritoneum to infective organisms involves the inflammatory cytokines and the interaction between resident cell populations: macrophages, mesothelial cells, and fibroblasts.[2,3] The earliest response involves tumor necrosis factor (TNF)-α and interleukin (IL)−1[3] derived from macrophages in response to bacterial products.[4] What follows is a complex interaction between inflammatory cytokines, such as IL-6, chemokines and subsequent leukocyte transmigration, prostaglandins, nitric oxide, and adhesion molecules. There is also an anti-inflammatory response consisting of soluble receptors to TNF-α,[5] IL-1 receptor antagonist,[6] and repair molecules such as hyaluronan.[7]

In the first month after peritonitis, patients develop an acute dysfunction of the peritoneum as a dialysis membrane.[8] This is characteristically associated with increased transport of small molecular weight solutes and ultrafiltration failure. The mechanism for this is not well established, but vascular effects of nitric oxide[9] or prostaglandins[10] have been hypothesized to play a role.

Recurrent peritonitis has been shown, in some studies, to be associated with long-term peritoneal membrane changes.[11,12] As in the acute changes after peritonitis, long-term changes are associated with increased solute transport and decreased ultrafiltration. In this setting, human biopsy studies[13–15] and animal experiments[16] have identified an increase in the peritoneal-associated vasculature, which seems to be the primary cause of increased solute transport. A fibrogenic response and

Supported by Baxter Health Care, the Canadian Institutes of Health Research, and the Kidney Foundation of Canada.

This paper was presented in abstract form at the IX Congress of the International Society for Peritoneal Dialysis, Montreal, PQ, Canada, June 2001.

Accepted for publication March 5, 2002.

Address reprint requests to Dr. J. Gauldie, Chair, Department of Pathology and Molecular Medicine, McMaster University, 1200 Main St. W, Room 2N16, Hamilton, Ontario, Canada, L8N 3Z5. E-mail: gauldie@mcmaster.ca.

associated vasculopathy have also been identified in patients on long-term peritoneal dialysis.[17]

The association between peritoneal inflammation, angiogenesis, and fibrosis is not clear. IL-1β has been identified *in vivo* as having strong fibrogenic properties through up-regulation of transforming growth factor (TGF)-β.[18] This has been confirmed in *in vitro* mesothelial cell culture work.[19] Both IL-1β and TNF-α have angiogenic properties as has been demonstrated in studies using rat mesenteric window assays[20,21] and TNF-α has been associated with up-regulation of the angiogenic cytokine vascular endothelial growth factor (VEGF) in cell culture.[22]

We have previously described the use of adenovirus-mediated gene transfer of cytokines and growth factors as an effective tool in elucidating functional and morphological changes in the peritoneum.[23] In the following experiments, we compared the effects of transient overexpression of TNF-α or IL-1β on the peritoneum of rats using adenovirus-mediated gene transfer (AdTNF-α or AdIL-1β) and controlled with a null adenovirus (AdDL70). Four days after infection with either AdTNF-α or AdIL-1β we saw increased vascularity of the peritoneum, increased solute transport, and decreased ultrafiltration. By day 21, the animals treated with AdTNF-α returned to normal peritoneal morphology and function, but the AdIL-1β-treated animals remained quite abnormal with peritoneal fibrosis and persistent vascularization.

Materials and Methods

Adenovirus

The construction of the adenovirus vectors AdIL-1β,[18] AdTNF-α,[24] and AdDL70[25] have been previously described. Adenovirus preparations were purified by CsCl gradient centrifugation and PD-10 Sephadex chromatography (Amersham Pharmacia, Baie d'Urfe, Canada) and plaque-titered on 293 cells as previously described.[26]

Animals

All animal studies were performed according to the Canadian Council on Animal Care Guidelines. Three groups of female Sprague-Dawley rats (Harlan, Indianapolis, IN), 200 to 250 g (corresponding to 6 to 8 weeks of age[27]) were studied. The first group (n = 27) received an intraperitoneal injection of AdIL-1β. The second group (n = 20) received AdTNF-α and the third group (n = 28) received a null control virus (AdDL70). All were administered adenovirus at a dose of 1.5 to 2.0 $\times$ 10^9 plaque-forming units/ml diluted to 100 μl in phosphate-buffered saline on day 0. Animals did not receive anti-inflammatory agents during the experimental protocol. Animals from each group were sacrificed on days 4, 7, 21, and 28 after adenovirus administration. Before sacrifice, 0.09 ml/g of 2.5% Dianeal (Baxter Health Care, McGaw Park, IL) was administered intraperitoneally. Four hours later, the peritoneum was opened and the entire fluid content removed for accurate ultrafiltration measurement. Net ultrafiltration was the volume of fluid removed after 4 hours minus the volume of fluid administered. Blood samples were drawn and the entire anterior abdominal wall was removed after skin and subcutaneous tissue was removed. The lower portion of this tissue was stored in formalin and the upper portion taken for RNA extraction. Mesenteric tissue was taken and frozen in liquid nitrogen.

Whole blood was centrifuged at 5000 rpm for 10 minutes and the serum removed. Peritoneal fluid samples were centrifuged at 1500 rpm for 5 minutes. Samples were analyzed on a Hitachi 917 automated chemistry analyzer (Roche Diagnostics, Laval, Canada) for glucose and albumin. Mass transport of glucose out of the peritoneum was calculated as (initial dialysate glucose $\times$ initial volume) $-$ (final dialysate glucose $\times$ final volume). Albumin clearance was calculated as mass transport divided by the serum solute concentration. All values were corrected for animal weight at sacrifice.

Cell number in the peritoneal fluid was counted with a hemocytometer. The fluid was plated to a glass slide by cytospin (Shandon Inc., Pittsburgh, PA) and stained (Biochemical Science Inc., Swedesboro, NJ). Cell differential was counted on at least 300 cells.

Histology

Tissue samples at sacrifice from the lower anterior abdominal wall or omentum were taken and fixed in a sufficient amount of 4% phosphate-buffered formaldehyde for 24 hours. The tissue samples were then paraffin-processed, embedded, and 5-μm sections cut. Cut sections were then stained for Masson's trichrome and immunohistochemistry was performed with antibodies to Factor VIII-von Willebrand factor (vWF) antigen (DAKO Corp., Carpinteria CA). Negative control sections were run in parallel with nonimmune mouse or rabbit serum. All sections were deparaffinized in xylene followed by 100% ethanol and then placed in a methanol H_2O_2 solution for 30 minutes to block endogenous peroxidase activity. After hydration to water with graded alcohols, the sections were placed in 0.05 mol/L Tris-buffered saline, pH 7.6. The sections were digested with 0.05% Pronase (Sigma Chemical Co., St. Louis, MO) in Tris-buffered saline with calcium chloride for 17 minutes at room temperature then blocked in 5% normal goat serum followed by a 1-hour incubation in the 1:500 rabbit anti-human factor VIII in 1% normal goat serum. Sections were then incubated in a prediluted kit of a biotinylated goat anti-rabbit followed by a streptavidin/peroxidase conjugate (Zymed Labs, San Francisco, CA) as per the manufacturer's instructions. Immunohistochemistry incubations were performed at room temperature and sections were washed in between incubations 3 $\times$ 5 minutes with 0.05 mol/L Tris-buffered saline, pH 7.6, except before the addition of primary antibody. Sections were rinsed in 0.05 mol/L acetate buffer, pH 5.0, before development in an 3-amino-9-ethylcarbazole (AEC) chromogen substrate for 15 minutes. Sections were counterstained in Mayer's hematoxylin for 2 minutes before mounting with glycerin gelatin.

Quantitative Immunohistochemistry

Low-power fields from factor VIII-immunostained sections of the anterior abdominal wall were digitized using a Leica DMR microscope (Leica Microsystems, Wetzlar, Germany). All sections were analyzed by the same image-processing algorithm using Leica Qwin Image Processing Software (Leica Imaging Systems, Cambridge, England). Results are reported as number of vessels/mm^2 of peritoneal tissue. We were able to estimate the total vessel cross-section area in each digitized image and we could therefore calculate an average cross-sectional area per vessel for each slide analyzed.

Hydroxyproline Assay

A portion of mesentery was taken and frozen for a hydroxyproline assay, modified from Woessner's method.[28] Tissues were weighed, homogenized in water, centrifuged at 1000 rpm for 5 minutes, and the superficial fatty material removed by vacuum suction. Solid material was precipitated with trichloroacetic acid with centrifugation at 1500 rpm for 15 minutes at 4°C. Samples were hydrolyzed overnight in 6 N HCl at 110°C. Hydroxyproline content is quantified by Erlich's reagent (Sigma) and assayed by measuring the optical density at 557 nm. A hydroxyproline standard sample (Sigma) was used to create a standard curve.

Cytokine Analysis

Peritoneal fluid taken at sacrifice was analyzed for the following cytokines using enzyme-linked immunosorbent assay (ELISA) (all R&D Systems, Minneapolis, MN) as directed by the manufacturer: human TGF-β (cross-reactive with rat), murine VEGF (cross-reactive with rat), rat IL-6, rat TNF-α (cross-reactive with murine), human IL-1β (not cross-reactive with rat), and murine TNF-soluble receptor II (TNFsrII, cross-reactive with rat). To measure total TGF-β, samples were first activated using 1 mol/L HCl for 10 minutes then normalized with 1 N NaOH to dissociate TGF-β from its latency-associated binding protein.

Frozen omental tissue was taken and homogenized in Trizol reagent (Life Technologies, Burlington, Ontario, Canada). Protein was extracted from the phenol layer after centrifugation according to the manufacturer's protocol. Total protein in this extract was assayed using a standard protein assay method (BioRad DC Protein Assay; BioRad Laboratories, Mississauga, Canada). Equal quantities of protein were then assayed using murine VEGF ELISA (R&D Systems).

RNase Protection Assay

The peritoneal surfaces of the anterior abdominal wall sections were immersed for 15 minutes in Trizol reagent. The parietal peritoneum was gently scraped and the Trizol collected and processed according to the manufacturer's instruction for isolation of RNA. The concentration of RNA resuspended in RNase-free water was measured by optical density at 260 nm. RNA (8 μg) was then hybridized overnight with a custom probe set (Pharmingen, Mississauga, ON) labeled with α-^{32}P-UTP (New England Nuclear, Boston, MA). The custom probe set contains different length probes for fibronectin, TGF-β1, tissue inhibitor of metalloproteinase-1 (TIMP-1), and housekeeping genes GAPDH and L32 for loading control. The hybridized samples were extracted using phenol/chloroform and acetate precipitation and then washed with ethanol. The extracted bound RNA was then run on a 5% polyacrylamide gel, transferred to blotting paper, dried, and exposed for 4 days to film (Eastman Kodak, Rochester, NY).

The images obtained were digitized and analyzed for band density using Scion Image software (Scion Corp, Frederick, MD). Densities were standardized to L32.

Statistics

Data are presented ± SEM unless otherwise noted. Comparison between groups was made by *t*-test. We used regression analysis to compare the cytokine concentration on day 4 in the peritoneal fluid and subsequent vascularization of the peritoneal membrane and combined all three treatment groups (AdIL-1β, AdTNF-α, AdDL70) in these results.

Results

Adenoviral Response

In previous work, we have identified an early (48 hour) inflammatory response to AdDL70 in similar dosage used in these experiments.[29] There were no adenoviral effects identified by 4 days,[23] the earliest time point used in our experiments. We therefore did not include a nonadenoviral control group in these experiments.

We have previously shown that adenovirus is effectively taken up, and the transgene product expressed, by mesothelial cells in the peritoneum.[23] The peak of expression is 4 to 7 days after infection, and the total duration of expression is 10 to 14 days. In these experiments, we confirmed the high levels of expression of the transgene product by analysis of the peritoneal dialysis fluid with the appropriate ELISA. Four days after infection peritoneal fluid was taken after a 4-hour dwell and analyzed by ELISA. Animals treated with AdIL-1β had 326 ± 89 pg/ml of human IL-1β and animals treated with AdTNF-α had 2076 ± 1131 pg/ml of rodent TNF-α measured in the peritoneal fluid (Table 1).

Inflammatory Response

The inflammatory response after intraperitoneal administration of AdTNF-α, AdIL-1β, or control adenovirus was measured from various markers in the peritoneal fluid and the results are shown in Table 2. The cellular response to overexpression of IL-1β and TNF-α was dramatic with a significant increase in the total number of cells and a

Table 1. Cytokine Concentration in the Peritoneal Fluid and Tissue 4, 7, and 21 Days after Infection with Control Adenovirus, AdTNF-α, or AdIL-1β

	Day 4			Day 7			Day 21		
	AdDL70	AdTNF-α	AdIL-1β	AdDL70	AdTNF-α	AdIL-1β	AdDL70	AdTNF-α	AdIL-1β
Human IL-1β (pg/ml)	<10	<10	326 ± 89*†	<10	<10	77 ± 105	<10	<10	<10
Rat IL1β (pg/ml)	32 ± 10	302 ± 255*	310 ± 186*	31 ± 10	114 ± 92	638 ± 800	46 ± 21	28 ± 2	251 ± 301
TNF-α (pg/ml)	69 ± 8	2076 ± 1131*	69 ± 7†	58 ± 14	365 ± 482*	83 ± 12†	nd	66 ± 11	69 ± 9
Total TGF-β (pg/ml)	131 ± 80	186 ± 82	756 ± 441*†	88 ± 50	306 ± 137*	1056 ± 610*†	63 ± 2	64 ± 18	166 ± 101
VEGF (pg/ml)	75 ± 44	172 ± 130*	210 ± 173*	85 ± 49	205 ± 133*	262 ± 157*	63 ± 24	30 ± 4	47 ± 17
Tissue VEGF (pg/ml)	43 ± 10	72 ± 40	76 ± 16	47 ± 14	45 ± 21	78 ± 8*	nd	nd	nd

*$P < 0.05$ compared with AdDL70 control.
†$P < 0.05$ compared with AdTNF-α.
Data shown ± SD.
nd, No data.

disproportionate increase in neutrophils measured in the peritoneal fluid. There was also an associated weight loss that was more dramatic in the AdIL-1β-treated animals compared to control adenovirus-treated animals. Rodent IL-6 was measured by ELISA in peritoneal fluid and was elevated after exposure to IL-1β or TNF-α (Table 1). Finally, we measured rodent TNFsrII by ELISA in peritoneal fluid and this was significantly elevated 4 and 7 days after infection with AdIL-1β and AdTNF-α (Table 1).

We also noted a quantitative difference between the inflammatory response to AdIL-1β or AdTNF-α. Specifically the response to transient overexpression of IL-1β was more intense, as measured by total cell count and percentage of neutrophils, especially 7 days after infection. Also, two animals in the AdIL-1β group became very ill in the first week after treatment and required euthanasia. The response to IL-1β was more prolonged, with levels of TNFsrII remaining significantly elevated in AdIL-1β-treated animals 21 days after infection.

Cytokine Response after Transient Overexpression of TNF-α or IL-1β

We analyzed the peritoneal fluid after a 4-hour dwell for rat IL-1β, TNF-α, TGF-β, and VEGF using ELISA (Table 1). Both AdTNF-α and AdIL-1β treatment induced the production of endogenous IL-1β measured using a rodent-specific assay. Interestingly, AdIL-1β-treated animals did not show an increase in peritoneal concentration of TNF-α. Overexpression of both TNF-α and IL-1β induced a significant increase in the peritoneal fluid concentration of total TGF-β1 and VEGF compared to AdDL70-treated animals 7 days after infection. We identified a threefold increase in peritoneal concentration of TGF-β1 7 days after AdIL-1β treatment compared to AdTNF-α. Increases in peritoneal VEGF concentrations were similar after exposure to IL-1β and TNF-α.

To confirm that VEGF was present in the tissues after adenovirus infection, we isolated protein from omental homogenates and assayed for VEGF. We noted an increase in tissue VEGF that was significantly elevated 7 days after treatment with AdIL-1β (Table 1).

We isolated RNA from parietal peritoneal tissue of animals 4, 7, and 21 days after receiving adenovirus and analyzed it using an RNase protection assay (Figure 1). Quantitative density analysis indexed to L32 and referenced to AdDL70-treated animals revealed that at 4 and 7 days after infection, both TNF-α and IL-1β led to an increase in mRNA signal for fibronectin and TGF-β1. These changes declined to baseline by day 21 except for a small persistent elevation in fibronectin mRNA in AdIL-1β-treated animals at day 21. Most striking was the progressive increase in TIMP-1 mRNA expression seen in AdIL-1β-treated animals at day 21 (Figure 1).

Changes in Peritoneal Membrane Morphology

Transient overexpression of both IL-1β and TNF-α led to early, significant histological changes in the parietal peritoneum compared to animals treated with the control

Table 2. Inflammatory Changes Measured 4 and 7 Days after Infection with Control Adenovirus, AdTNF-α, AdIL-1β

	Day 4			Day 7		
	AdDL70	AdTNF-α	AdIL-1β	AdDL70	AdTNF-α	AdIL-1β
White blood cells (10^4/ml)	77 ± 69	158 ± 81*	952 ± 275*†	132 ± 164	262 ± 148	763 ± 541*†
Monocyte, %	19.4	15.3	8.0*	28.9	20.3	13.2
Macrophage, %	46.2	41.4	30.7	50.5	49.4	27.1
Neutrophil, %	4.2	31.7*	48.3*	3.5	25.9*	58.3*†
Eosinophil, %	27.4	9.8*	11.3*	19.4	3.8	1.1
Change in weight from day 0 (g)	1.3 ± 5.3	−2.1 ± 2.8	−5.6 ± 4.1	2.8 ± 6.1	2.2 ± 3.9	−5.9 ± 4.2
IL-6 (pg/ml)	59.1 ± 0.3	988 ± 857	1451 ± 497	66 ± 2	74 ± 8	1788 ± 746
TNFsrII (pg/ml)	34 ± 16	604 ± 420*	751 ± 364*	63 ± 11	1156 ± 275*	917 ± 388*

*$P < 0.05$ compared with AdDL70 control.
†$P < 0.05$ compared with AdTNF-α.
Data shown ± SD.

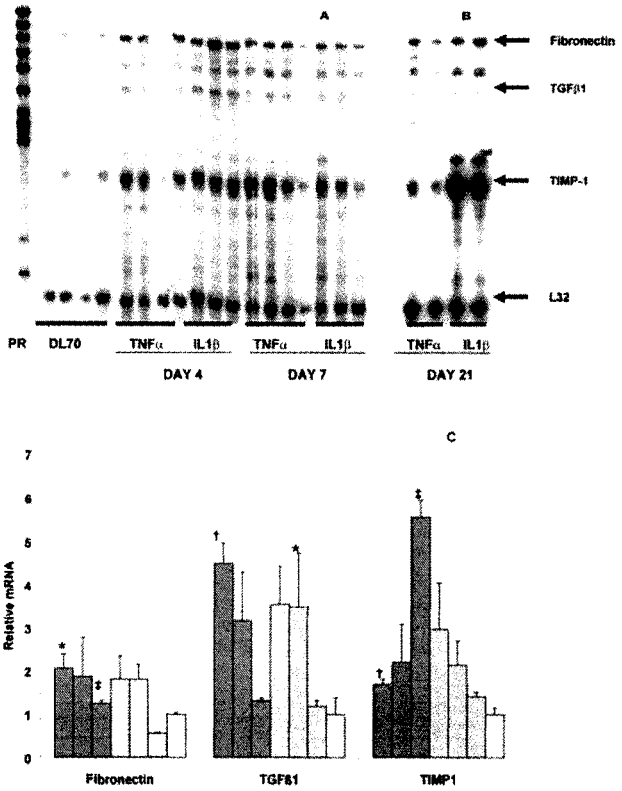

Figure 1. RNase protection assay. RNA was extracted from parietal peritoneal tissue and hybridized with a radiolabeled probe set. Samples were then RNase-digested, purified, and separated by polyacrylamide gel electrophoresis. **A:** PR is unprotected probe. Samples are from animals treated with AdDL70, AdTNF-α, or AdIL-1β 4 and 7 days after infection. **B:** Day 21 samples run on a separate gel. **Arrows** indicate signal from hybridized mRNA for fibronectin, TGF-β1, TIMP-1, and housekeeping gene L32 for loading control. **C:** Quantification of band density. Images were digitized and band density analyzed. Density was corrected for loading by L32 and then normalized to AdDL70-treated animals. **Dark gray bars**, AdIL-1β-treated animals; **light gray bars**, AdTNF-α-treated animals; **open bars**, AdDL70-treated animals. The three bars for each treatment group represent days 4, 7, and 21 after adenovirus administration. AdDL70 animals are day 4 after adenoviral infection. There was a similar up-regulation of fibronectin and TGF-β1 in AdIL-1β- and AdTNF-α-treated animals at days 4 and 7. TIMP-1 was strongly expressed at day 21 in AdIL-1β but not AdTNF-α animals. Data represents three to four animals at each data point. *, $P < 0.01$ compared with AdDL70 control; †, $P < 0.05$ compared with AdDL70; ‡, $P < 0.01$ for AdIL-1β compared with AdTNF-α-treated animals.

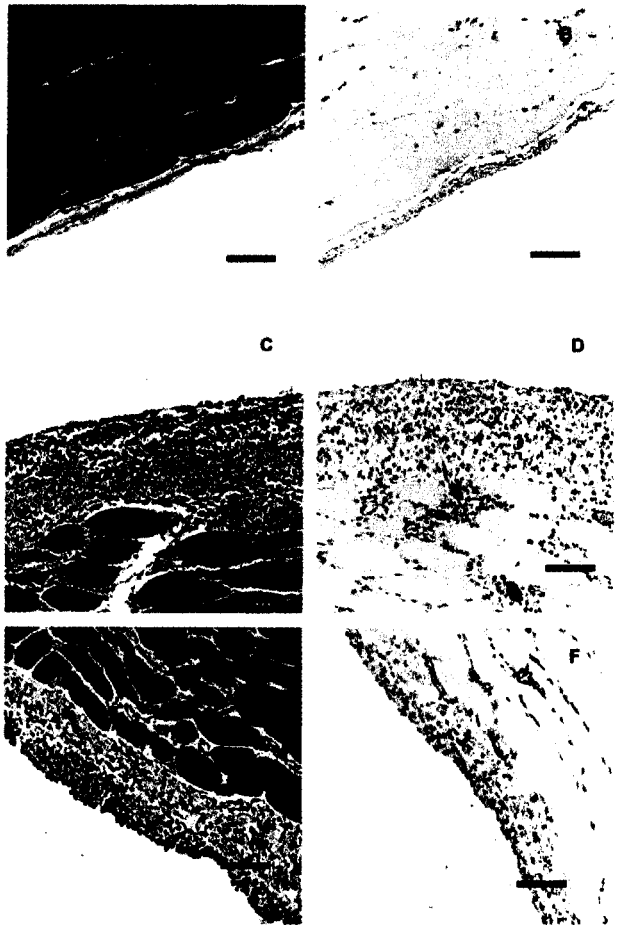

Figure 2. Histology of the anterior abdominal wall 7 days after infection with AdDL70 (**A** and **B**), AdIL-1β (**C** and **D**), or AdTNF-α (**E** and **F**). After control adenovirus infection, the parietal peritoneum has normal histology (**A**) with thin submesothelial zone with few cells, intact mesothelial cell layer, and few blood vessels (**B**). Seven days after infection with AdIL-1β or AdTNF-α, similar changes are observed. There is an increase in the submesothelial thickness, collagen deposition, and markedly increased cellularity (**C** and **E**). There is an associated increase in neovascularization (**D** and **F**). Scale bars, 100 μm. Masson's trichrome (**A**, **C**, and **E**) and Factor VIII-vWF immunohistochemistry (**B**, **D**, and **F**).

adenovirus, AdDL70 (Figure 2). The mesothelial cells, which normally are flattened against the basement membrane, became rounded up with enlarged nuclei. The submesothelial zone became substantially thickened with inflammatory cells and edematous changes. These changes were evident in both parietal and visceral peritoneal tissue as seen in omental tissue 7 days after infection (Figure 3).

Seven days after infection with AdIL-1β and AdTNF-α there was a dramatic angiogenesis that occurred in the peritoneum as evidenced by factor VIII-stained sections (Figure 2). Peritoneal-associated vessels were evaluated using digital image analysis from these sections (Figure 4). Overexpression of IL-1β or TNF-α led to significantly different kinetics of angiogenesis. Animals treated with AdIL-1β had increased peritoneal-associated blood vessels at each time point studied compared with AdDL70-treated animals. AdTNF-α-treated animals had an increase in peritoneal vasculature that was significantly elevated over AdDL70-treated animals 4 and 7 days after adenovirus infection, but by day 21, the peritoneal-associated vasculature in the AdTNF-α-treated animals had returned to baseline.

There was an alteration in morphology of blood vessels after exposure to TNF-α and IL-1β. We observed an increase in the average cross-sectional area of the blood vessels after exposure to inflammatory cytokines (IL-1β, 146 ± 18; TNF-α, 136 ± 17 *versus* AdDL70, 73 ± 23 μm^2 4 days after infection). This dilation of blood vessels persisted to 21 days after infection.

In both AdIL-1β- and AdTNF-α-treated animals there was an early accumulation of collagen evident by day 7 (Figures 2 and 3). We confirmed the histological evidence of collagen accumulation by analyzing mesenteric tissue for hydroxyproline content. As shown in Figure 5, AdIL-1β-treated animals had persistent elevation in hydroxyproline, and therefore, collagen content, of mesenteric tissue at each time point evaluated. AdTNF-α-

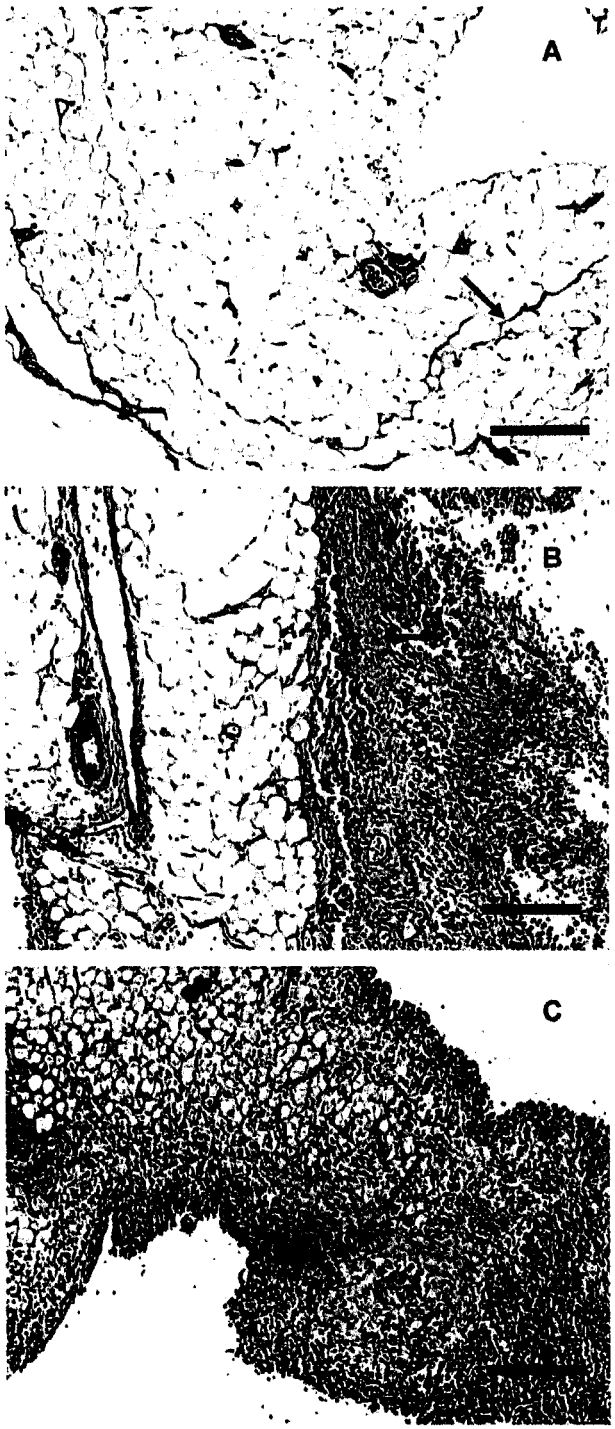

Figure 3. Histology of the visceral peritoneum (omentum) 7 days after infection with AdDL70 (**A**), AdIL-1β (**B**), or ADTNF-α (**C**). AdIL-1β and AdTNF-α-treated animals demonstrate increased cellular infiltration in omental tissue with collagen deposition (**arrows**). Scale bars, 200 μm. Masson's trichrome.

treated animals showed a significant early accumulation of collagen but by day 21, this had resolved.

The most significant histological difference after exposure to TNF-α or IL-1β can be seen in the day 21 samples (Figure 6). AdIL-1β-treated animals had submesothelial thickening, now with collagen deposition (Figure 6A). There was persistent angiogenesis seen in the factor

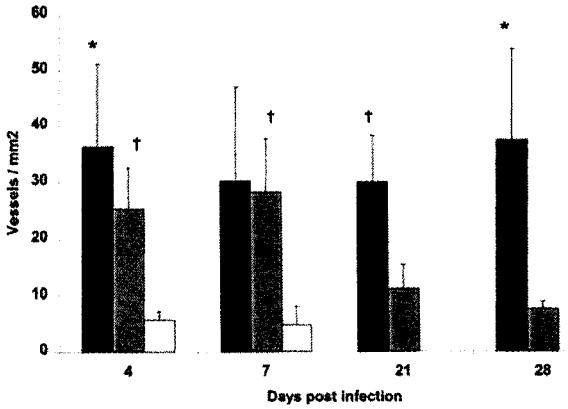

Figure 4. Vascular density in the submesothelial zone of the parietal peritoneum was measured using image analysis from digitized Factor VIII-vWF-stained sections taken from animals exposed to AdIL-1β (**black bars**), AdTNF-α (**gray bars**), or control adenovirus (AdDL70, **open bars**). Animals treated with AdIL-1β demonstrated increased vascular density in the submesothelial zone at all time points. In contrast, animals treated with AdTNF-α demonstrated a transient induction of angiogenesis that resolved by 21 days after infection. *, $P < 0.01$ compared with AdDL70-treated animals; †, $P < 0.05$ compared with AdDL70-treated animals. Data represents four to six animals for each data point.

VIII-stained section (Figure 6B). The AdTNF-α-treated animals had peritoneal histology indistinguishable from normal rat peritoneum 21 days after infection and demonstrated thin, compact submesothelial tissue and virtual absence of peritoneal-associated vessels (Figure 6, C and D).

Using regression analysis of all treatment groups combined, we could find a weak but significant correlation between dialysate VEGF concentration 4 days after adenovirus administration and subsequent vascularization (Figure 7A). Of note, there was a stronger correlation between TGF-β peritoneal fluid concentration on day 4 and subsequent vascularization (Figure 7B).

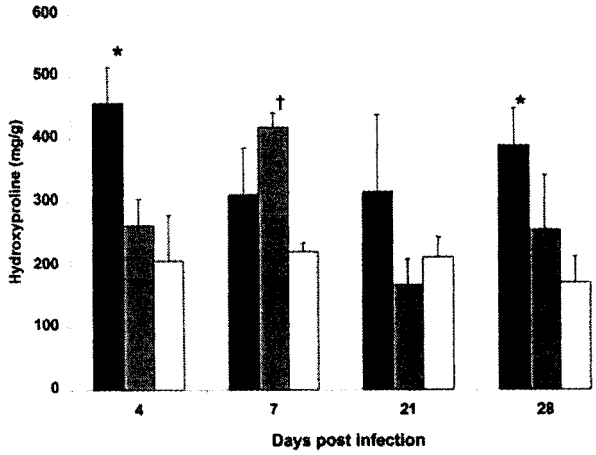

Figure 5. Hydroxyproline analysis of mesenteric tissue after infection with AdIL-1β (**black bars**), AdTNF-α (**gray bars**), or control adenovirus (AdDL70, **open bars**). AdIL-1β-treated animals demonstrated sustained fibrosis measured by hydroxyproline content of mesenteric tissue whereas TNF-α-treated animals demonstrated a transient accumulation of collagen by day 7 that disappeared by day 21. *, $P < 0.01$ compared with AdDL70; †, $P < 0.05$ compared with AdDL70. Data represents three to six animals at each time point.

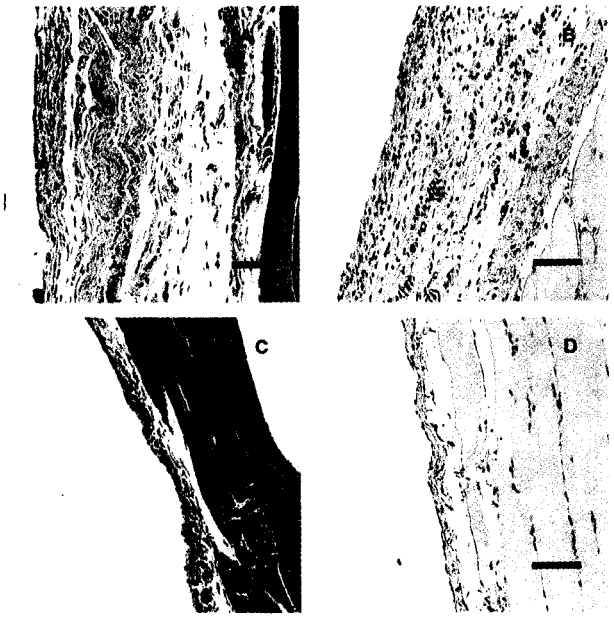

Figure 6. Histology of the anterior abdominal wall 21 days after infection with AdIL-1β (**A** and **B**) or AdTNF-α (**C** and **D**). Animals infected with AdIL-1β show persisting thickening of the submesothelial zone with collagen deposition (**A**) and persisting vascularization (**B**). After infection with AdTNF-α, the parietal peritoneum returned to normal histology (**C**) with absence of vascularization (**D**). Scale bars, 100 μm. Masson's trichrome (**A** and **C**); Factor VIII-vWF immunohistochemistry (**B** and **D**).

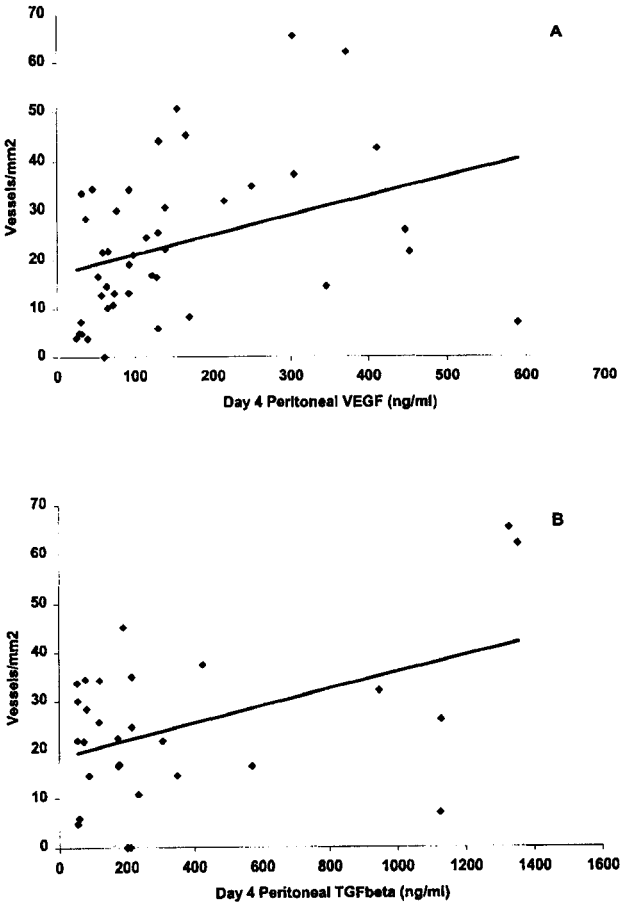

Figure 7. Correlation between peritoneal cytokine concentrations in all three treatment groups and vascularization measured by vessels/mm^2. **A:** Day 4 peritoneal VEGF concentration correlates weakly with vascularization (r = 0.35, P = 0.02). **B:** A stronger correlation exists between peritoneal TGF-β1 concentration and vascularization (r = 0.45, P = 0.01).

Peritoneal Membrane Function

We studied the function of the peritoneum as a dialysis membrane using net ultrafiltration, glucose mass transport, and albumin clearance, after a 4-hour dwell of 2.5% Dianeal. The net ultrafiltration (Figure 8A) reveals a significant impairment 4 days after infection with AdTNF-α and AdIL-1β compared with AdDL70-treated animals. The ultrafiltration function of the AdTNF-α-treated animals recovered by day 7, but remained impaired in IL-1β-treated animals until day 28. The glucose transport (Figure 8B) showed a similar trend with early increased glucose transport after overexpression of both inflammatory cytokines, but with increased glucose transport persisting until day 28 for AdIL-1β-treated animals.

Transient overexpression of TNF-α not only caused significant peritoneal vascularization by day 7, but albumin clearance suggests an increased permeability to macromolecules at this time point. The albumin clearance in AdTNF-α-treated animals, 7 days after adenovirus infection, was 30 ± 23 L/kg, which was significantly elevated over AdDL70-treated animals (3 ± 1 L/kg, P = 0.03), and elevated over AdIL-1β-treated animals (7 ± 1 L/kg, P = ns).

Discussion

Adenovirus-mediated gene transfer is a versatile tool to analyze the effects of transient overexpression of cytokines on peritoneal morphology and function.[23] Both inflammatory cytokines IL-1β and TNF-α induced an early acute inflammatory response not seen in control adenovirus-treated animals. This response consisted of increased peritoneal inflammatory cell infiltration, notably by neutrophils. There was a secondary up-regulation of IL-6 and TNFsrII, as has been seen in patients with peritonitis.[5]

IL-1β and TNF-α also induced functional changes similar to those seen in patients who develop peritonitis. Specifically, we saw an early ultrafiltration failure with increased transport of glucose and loss of albumin. These functional changes corresponded to increased expression of VEGF and TGF-β1 and increased peritoneal angiogenesis. We, and others, have demonstrated that angiogenesis is a key component in increased glucose transport and ultrafiltration dysfunction[16,30] and we suggest that, in this model, angiogenesis is an integral factor causing the acute peritoneal dysfunction. Others have observed a role for nitric oxide[9] or prostaglandins[10] in acute peritoneal dysfunction associated with peritonitis. We did not assay for these compounds in our experiments and they may play a role, but they are likely downstream components and the inflammatory cytokines can alone initiate peritoneal angiogenesis and acute ultrafiltration failure.

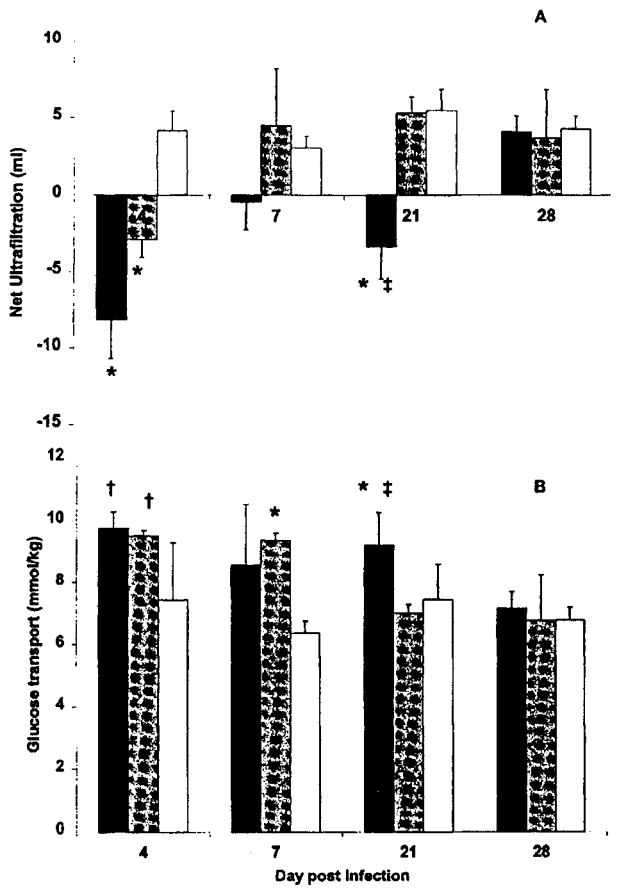

Figure 8. Peritoneal membrane function after adenovirus infection with AdIL-1β (**black bars**), AdTNF-α (**gray bars**), or control adenovirus (AdDL70, **open bars**) represented by net ultrafiltration (**A**) and mass transport of glucose transport across the peritoneal membrane (**B**). AdIL-1β-treated animals displayed impaired peritoneal function with decreased ultrafiltration and increased solute transport until day 28. AdTNF-α-treated animals demonstrated a more transient membrane dysfunction with resolution of ultrafiltration failure at day 7 and normalization of solute transport by day 21 after infection. *, $P < 0.01$ compared with AdDL70; †, $P < 0.05$ compared with AdDL70; ‡, $P < 0.05$ AdIL-1β compared with AdTNF-α. Data represents three to six animals for each data point.

The role of inflammatory cytokines in angiogenesis has been studied previously and the results are in agreement with our findings. Specifically, in a series of studies using the rat mesenteric window model, Norrby[20,21] has shown that both inflammatory cytokines induce angiogenesis, but the angiogenesis induced by IL-1β is more prolonged.

The most interesting findings from our experiments derive from the differences we observed between IL-1β and TNF-α. Specifically, TNF-α induced a transient, dramatic angiogenesis with collagen deposition and increased macromolecular permeability. These changes resolved completely by 21 days after adenovirus-mediated gene transfer. In contrast, IL-1β exposure induced a persisting vasculogenesis with associated up-regulation of profibrotic cytokines and induction of submesothelial fibrosis.

There was a quantitative difference in inflammatory response to IL-1β and TNF-α with the former leading to a more severe and prolonged inflammation measured by persisting neutrophilia and up-regulation of TNFsrII. We do not think this quantitative increased inflammatory response alone explains the qualitative fibrotic and angiogenic response seen by days 21 and 28. We saw a similar angiogenic and fibrogenic response between TNF-α and IL-1β at early time points. This was accompanied by an equal up-regulation of TGF-β and fibronectin mRNA and tissue VEGF. We hypothesize that the different fibrogenic response at later time points is related to the significant up-regulation of TIMP-1 as demonstrated by RNase protection assay (Figure 1). Increasing evidence would suggest that persisting fibrosis is related to imbalance between collagenase proteins and their inhibitors.[31] The profibrotic nature of IL-1β has been demonstrated previously in which overexpression in the lung led to progressive fibrosis,[18] however overexpression of TNF-α in the lung led to a patchy, mild fibrosis.[32]

One possible explanation for the differential angiogenic response to IL-1β and TNF-α is the recognized property of TNF-α to induce apoptosis through binding to its receptor p55.[33] IL-1β is not known to directly induce apoptosis. It is possible that the high peritoneal concentration of TNF-α seen during the first 7 days induces inflammation and angiogenesis and, as the concentration falls, there is a secondary induction of apoptosis and reversion of the membrane to normal histology by day 21.

Another possibility is that some element, or elements, of the profibrotic environment initiated by IL-1β is responsible for protecting the viability and stability of endothelial cells. Recent experiments have revealed that angiogenesis involves the growth of new immature blood vessels following a stabilization phase.[34] This stabilization involves different stimuli such as angiopoietin-1[35] along with the recruitment of perivascular cells and these factors may be enhanced in the fibrotic environment.

We cannot rule out a differential response to human IL-1β, the gene insert in the adenovirus used in these experiments. However, IL-1β has significant homology across mammalian species[36] and the receptor apparatus is known to be remarkably well preserved.[37] Only very minor differences in response to different species IL-1β has been documented previously.[38] Therefore, we do not feel that the differential effects between IL-1β and TNF-α seen in these experiments are because of the presence of human IL-1β.

We have not clearly identified the major angiogenic cytokine in this model. VEGF concentration was up-regulated in the peritoneal dialysate but this is not an accurate marker of local production.[39] Peritoneal cytokine concentration is effected by shifts of water into or out of the peritoneal cavity as well as transport of the cytokine into the peritoneum across permeable blood vessels. The tissue concentration of VEGF was elevated in animals exposed to TNF-α and IL-1β but the changes from AdDL70-treated animals were not striking (Table 2). It could be that higher tissue VEGF levels are present at earlier time points, as angiogenesis is already present by day 4. It is also possible that other fibrosis-related cytokines such as platelet-derived growth factor or basic fibroblast growth factor are important modulators of angiogenesis in this setting of acute inflammation.[40] This could explain the observation that peritoneal fluid TGF-β1

concentration correlates more closely with vascularization than VEGF concentration (Figure 7).

Some discrepancies exist between functional and morphological findings in the peritoneum. Seven days after infection with AdTNF-α, there is persisting angiogenesis and increased glucose transport, but the ultrafiltration has reverted to normal in AdTNF-treated animals (Figure 8). One possible confounding variable is the extremely high albumin transport measured in TNF-α-exposed animals at this time point. The increased osmotic component of the peritoneal protein may counteract the rapid loss of glucose gradient and preservation of ultrafiltration. Functional properties of IL-1β-treated animals improved at day 28 despite ongoing evidence of angiogenesis and fibrosis. We saw a similar pattern after adenoviral-mediated gene transfer of TGF-β1.[23] It is possible that there are progressive alterations in the interstitium of the peritoneum in fibrosis, such as increased deposition of hyaluronan, that may improve the peritoneal membrane function despite ongoing angiogenesis.[41] Alternatively, the peritoneum may regulate the blood supply to the peritoneum throughout time so that, despite the histological persistence of blood vessels, the overall volume of blood supply slowly returns to a basal level.

Using adenoviral-mediated gene transfer of the proinflammatory cytokines IL-1β and TNF-α, we have shown that these cytokines individually can induce peritoneal angiogenesis and functional changes similar to those found in peritoneal dialysis patients who suffer acute peritonitis. More importantly, IL-1β and TNF-α induce differing fibrogenic and angiogenic responses. These differential responses suggest that IL-1β may have a greater and more prolonged impact on the peritoneum through induction of sustained vasculogenesis and fibrosis. IL-1β may be the link between recurrent peritonitis and longer-term changes in the function of the peritoneum. This may explain why some patients have a poor outcome after one or several episodes of peritonitis. Targeted inhibition of IL-1β, perhaps through its receptor antagonist, may preserve the peritoneum after acute peritonitis. Finally, the further study of the angiogenic response in this inflammatory model may yield insights into the supporting role of fibrosis in angiogenesis and this may have implications beyond maintenance of the peritoneum as a dialysis membrane.

Acknowledgments

We thank Duncan Chong and Xueya Feng for technical support, Mary Jo Smith for help with histology, and Drs C. Richards and A. Ingram for their helpful review.

References

1. Bunke CM, Brier ME, Golper TA: Outcomes of single organism peritonitis in peritoneal dialysis: gram negatives versus gram positives in the Network 9 Peritonitis Study. Kidney Int 1997, 52:524–529
2. Topley N, Liberek T, Davenport A, Li FK, Fear H, Williams JD: Activation of inflammation and leukocyte recruitment into the peritoneal cavity. Kidney Int 1996, 56:S17–S21
3. Jorres A, Ludat K, Sander K, Dunkel K, Lorenz F, Keck H, Frei U, Gahl GM: The peritoneal fibroblast and the control of peritoneal inflammation. Kidney Int 1996, 56:S22–S27
4. Visser CE, Brouwer-Steenbergen JJ, Struijk G, Krediet RT, Beelen RH: Production of IL-1 beta and TNF-alpha by peritoneal macrophages depends on the bacterial species and the inoculum. Adv Perit Dial 1997, 13:201–204
5. Zemel D, Imholz AL, de Waart DR, Dinkla C, Struijk DG, Krediet RT: Appearance of tumor necrosis factor-alpha and soluble TNF-receptors I and II in peritoneal effluent of CAPD. Kidney Int 1994, 46:1422–1430
6. Brauner A, Hylander B, Wretlind B: Tumor necrosis factor-alpha, interleukin-1 beta, and interleukin-1 receptor antagonist in dialysate and serum from patients on continuous ambulatory peritoneal dialysis. Am J Kidney Dis 1996, 27:402–408
7. Yung S, Coles GA, Davies M: IL-1 beta, a major stimulator of hyaluronan synthesis in vitro of human peritoneal mesothelial cells: relevance to peritonitis in CAPD. Kidney Int 1996, 50:1337–1343
8. Ates K, Koc R, Nergizoglu G, Erturk S, Keven K, Sen A, Karatan O: The longitudinal effect of a single peritonitis episode on peritoneal membrane transport in CAPD patients. Perit Dial Int 2000, 20:220–226
9. Combet S, Van Landschoot M, Moulin P, Piech A, Verbavatz JM, Goffin E, Balligand JL, Lameire N, Devuyst O: Regulation of aquaporin-1 and nitric oxide synthase isoforms in a rat model of acute peritonitis. J Am Soc Nephrol 1999, 10:2185–2196
10. Steinhauer HB, Schollmeyer P: Prostaglandin-mediated loss of proteins during peritonitis in continuous ambulatory peritoneal dialysis. Kidney Int 1986, 29:584–590
11. Davies SJ, Bryan J, Phillips L, Russell GI: Longitudinal changes in peritoneal kinetics: the effects of peritoneal dialysis and peritonitis. Nephrol Dial Transplant 1996, 11:498–506
12. Lin CY, Chen WP, Yang LY, Chen A, Huang TP: Persistent transforming growth factor-beta 1 expression may predict peritoneal fibrosis in CAPD patients with frequent peritonitis occurrence. Am J Nephrol 1998, 18:513–519
13. Mateijsen MA, van der Wal AC, Hendriks PM, Zweers MM, Mulder J, Struijk DG, Krediet RT: Vascular and interstitial changes in the peritoneum of CAPD patients with peritoneal sclerosis. Perit Dial Int 1999, 19:517–525
14. Plum J, Hermann S, Fussholler A, Schoenicke G, Donner A, Rohrborn A, Grabensee B: Peritoneal sclerosis in peritoneal dialysis patients related to dialysis settings and peritoneal transport properties. Kidney Int 2001, 59(Suppl 78):S42–S47
15. Combet S, Miyata T, Moulin P, Pouthier D, Goffin E, Devuyst O: Vascular proliferation and enhanced expression of endothelial nitric oxide synthase in human peritoneum exposed to long-term peritoneal dialysis. J Am Soc Nephrol 2000, 11:717–728
16. Margetts PJ, Kolb M, Yu L, Hoff CM, Gauldie J: A chronic inflammatory infusion model of peritoneal dialysis in rats. Perit Dial Int 2001, 21:S319–S323
17. Honda K, Nitta K, Horita S, Yumura W, Nihei H: Morphological changes in the peritoneal vasculature of patients on CAPD with ultrafiltration failure. Nephron 1996, 72:171–176
18. Kolb M, Margetts PJ, Anthony DC, Pitossi F, Gauldie J: Transient expression of IL-1beta induces acute lung injury and chronic repair leading to pulmonary fibrosis. J Clin Invest 2001, 107:1529–1536
19. Yang WS, Kim BS, Lee SK, Park JS, Kim SB: Interleukin-1beta stimulates the production of extracellular matrix in cultured human peritoneal mesothelial cells. Perit Dial Int 1999, 19:211–220
20. Norrby K: Interleukin-1-alpha and de novo mammalian angiogenesis. Microvasc Res 1997, 54:58–64
21. Norrby K: TNF-alpha and de novo mammalian angiogenesis. Microvasc Res 1996, 52:79–83
22. Ryuto M, Ono M, Izumi H, Yoshida S, Weich HA, Kohno K, Kuwano M: Induction of vascular endothelial growth factor by tumor necrosis factor alpha in human glioma cells. Possible roles of SP-1. J Biol Chem 1996, 271:28220–28228
23. Margetts PJ, Kolb M, Hoff CM, Shockley TR, Gauldie J: Gene transfer of transforming growth factor beta 1 to the rat peritoneum: effects on membrane function. J Am Soc Nephrol 2001, 12:2029–2039
24. Marr RA, Addison CL, Snider D, Muller WJ, Gauldie J, Graham FL: Tumour immunotherapy using an adenoviral vector expressing a

membrane-bound mutant of murine TNF alpha. Gene Ther 1997, 4:1181–1188
25. Bett AJ, Haddara W, Prevec L, Graham FL: An efficient and flexible system for construction of adenovirus vectors with insertions or deletions in early regions 1 and 3. Proc Natl Acad Sci USA 1994, 91:8802–8806
26. Xing Z, Ohkawara Y, Jordana M, Graham F, Gauldie J: Transfer of granulocyte-macrophage colony-stimulating factor gene to rat lung induces eosinophilia, monocytosis, and fibrotic reactions. J Clin Invest 1996, 97:1102–1110
27. Aleman CL, Mas RM, Rodeiro I, Noa M, Hernandez C, Menendez R, Gamez R: Reference database of the main physiological parameters in Sprague-Dawley rats from 6 to 32 months. Lab Anim 1998, 32:457–466
28. Woessner Jr JF: The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. Arch Biochem Biophys 1961, 93:440–447
29. Hoff CM, Piscopo D, Inman KL, Shockley TR: Adenovirus-mediated gene transfer to the peritoneal cavity. Perit Dial Int 2000, 20:128
30. Krediet RT, Zweers MM, van der Wal AC, Struijk DG: Neoangiogenesis in the peritoneal membrane. Perit Dial Int 2000, 20(Suppl 2):S19–S25
31. Benyon RC, Arthur MJ: Extracellular matrix degradation and the role of hepatic stellate cells. Semin Liver Dis 2001, 21:373–384
32. Sime PJ, Marr RA, Gauldie D, Xing Z, Hewlett BR, Graham FL, Gauldie J: Transfer of tumor necrosis factor-alpha to rat lung induces severe pulmonary inflammation and patchy interstitial fibrogenesis with induction of transforming growth factor-beta1 and myofibroblasts. Am J Pathol 1998, 153:825–832
33. Dinarello CA: Proinflammatory cytokines. Chest 2000, 118:503–508
34. Yancopoulos GD, Davis S, Gale NW, Rudge JS, Wiegand SJ, Holash J: Vascular-specific growth factors and blood vessel formation. Nature 2000, 407:242–248
35. Thurston G, Rudge JS, Ioffe E, Zhou H, Ross L, Croll SD, Glazer N, Holash J, McDonald DM, Yancopoulos GD: Angiopoietin-1 protects the adult vasculature against plasma leakage. Nat Med 2000, 6:460–463
36. Howard RD, McIlwraith CW, Trotter GW, Nyborg JK: Cloning of equine interleukin 1 receptor antagonist and determination of its full-length cDNA sequence. Am J Vet Res 1998, 59:712–716
37. O'Neill LA, Greene C: Signal transduction pathways activated by the IL-1 receptor family: ancient signaling machinery in mammals, insects, and plants. J Leukoc Biol 1998, 63:650–657
38. Dejana E, Breviario F, Erroi A, Bussolino F, Mussoni L, Gramse M, Pintucci G, Casali B, Dinarello CA, Van Damme J, Mantovani A: Modulation of endothelial cell functions by different molecular species of interleukin 1. Blood 1987, 69:695–699
39. Zweers MM, de Waart DR, Smit W, Struijk DG, Krediet RT: Growth factors VEGF and TGF-beta1 in peritoneal dialysis. J Lab Clin Med 1999, 134:124–132
40. Kalluri R, Sukhatme VP: Fibrosis and angiogenesis. Curr Opin Nephrol Hypertens 2000, 9:413–418
41. Breborowicz A, Polubinska A, Moberly J, Ogle K, Martis L, Oreopoulos D: Hyaluronan modifies inflammatory response and peritoneal permeability during peritonitis in rats. Am J Kidney Dis 2001, 37:594–600

CHAPTER 4: A CHRONIC INFLAMMATORY INFUSION MODEL OF PERITONEAL DIALYSIS IN RATS

This short paper appeared in Peritoneal Dialysis International 2001; 21 (Suppl 3): S368 – S372. In this paper, we describe our initial experience with a chronic daily infusion model of peritoneal dialysis. In this model, we surgically placed an indwelling catheter attached to a subcutaneous port and daily injected the rats with various solutions. We used LPS in this model to induce an acute inflammatory reaction, but subsequent experience demonstrated that the LPS was not necessary.

We saw a small effect on peritoneal morphology of the catheter placement alone with no fluid infusion. Ringer's Lactate or buffered saline solution had a definite effect on peritoneal morphology. We saw an impact on UF, thickness of the submesothelium, and angiogenesis, with this 'physiological saline' solution. This was contrary to what others have observed in longer models [180]. I believe the catheter alone induces an inflammatory reaction, and the fluid instillation allows the catheter to move around the peritoneum and extend the affected area.

Dialysis solutions had a more dramatic effect on structure and function of the peritoneum compared with physiological saline infusion. This effect was likely due to the additional injurious effect of high glucose and GDPs. We did see an inverse association between peritoneal vascularization and UF. We also demonstrated the ability to express AdLacZ in the peritoneal tissues in this daily infusion model.

I developed the concept and designed these experiments. The work was carried out by myself, Dr. Kolb, and Lisa Yu. I wrote the paper which was reviewed by Drs Gauldie and Hoff.

Proceedings of the ISPD 2001 — The IXth Congress of the ISPD
June 26 - 29, 2001, Montréal, Canada
Peritoneal Dialysis International, Vol. 21 (2001), Supplement 3

0896-8608/01 $3.00 + .00

A CHRONIC INFLAMMATORY INFUSION MODEL OF PERITONEAL DIALYSIS IN RATS

Peter J. Margetts,[1,2] Martin Kolb,[1] Lisa Yu,[1] Catherine M. Hoff,[3] Jack Gauldie[1]

Centre for Gene Therapeutics,[1] Department of Pathology and Molecular Medicine, and Division of Nephrology,[2] McMaster University, Hamilton, Ontario, Canada; and Baxter Healthcare,[3] Renal Division Scientific Affairs, Baxter Healthcare Corporation, McGaw Park, Illinois, U.S.A.

♦ ***Objectives:*** **Peritoneal membrane changes are related to daily exposure to non physiologic dialysate and recurrent acute inflammation. We modified a daily infusion and inflammation model and evaluated it for fibrotic and angiogenic features. The feasibility of adenovirus-mediated gene transfer in the model was also assessed.**

♦ ***Methods:*** **Peritoneal catheters were implanted in rats. Over a period of 4 weeks, the animals received a daily infusion of Dianeal 4.25% (Baxter Healthcare Corporation, Deerfield, IL, U.S.A.) with an initial three doses of lipopolysaccharide (LPS) or physiologic saline. Peritoneal fluid was assayed for transforming growth factor beta (TGFβ) and vascular endothelial growth factor (VEGF). Animals were humanely killed at week 5. Net ultrafiltration was then measured, and tissue samples were immunostained for factor VIII. Mesenteric tissue was assayed for hydroxyproline content. Adenovirus-mediated gene transfer of β-galactosidase was assayed by intraperitoneal administration of the virus, 4 days before the end of the experiment.**

♦ ***Results:*** **Animals treated with either Dianeal or physiologic saline showed peritoneal membrane thickening and increased vascularity. Fibrosis was demonstrated by increased hydroxyproline concentration. Ultrafiltration was impaired. We found increased concentrations of VEGF and TGFβ in the peritoneal fluid of animals treated with LPS and daily infusion. Adenovirus-mediated gene transfer to the peritoneal membrane was demonstrated in the model.**

♦ ***Conclusions:*** **Exposure to LPS and daily Dianeal or physiologic saline leads to peritoneal fibrosis and neoangiogenesis. Vascularization and glucose transport correlate with ultrafiltration failure. The present animal model mimics changes seen in humans on peritoneal dialysis and may be valuable for evaluating short-term interventions to prevent membrane damage.**

Correspondence to: J. Gauldie, Department of Pathology and Molecular Medicine, McMaster University, 1200 Main Street West, Room 2N16, Hamilton, Ontario L8N 3Z5 Canada.

gauldie@mcmaster.ca

KEY WORDS: Fibrosis; angiogenesis; vascular endothelial growth factor; transforming growth factor; ultrafiltration; animal model.

Long-term peritoneal dialysis is characterized by alterations in the peritoneum leading to increased solute transport and decreased ultrafiltration (1). Those effects are likely due to exposure to non physiologic dialysate and recurrent inflammatory events. After long-term dialysis, the peritoneum shows signs of fibrosis with collagen deposition, thickening, and perivascular sclerosis (2). Several biopsy studies have identified increased vascularization in the peritoneum and correlated that finding with membrane dysfunction (3). Profibrotic cytokines such as transforming growth factor beta 1 (TGFβ1) (4) and angiogenic factors such as vascular endothelial growth factor (VEGF) (5) have been identified in peritoneal effluent and correlated with membrane function.

We previously developed an acute model that uses adenovirus-mediated gene transfer to study the effects on the peritoneum of transient exposure to a high concentration of a cytokine (TGFβ1) (6). In later experiments, we adopted a shorter-term chronic model (7) in which we could intervene with adenovirus to alter the environment of the peritoneum and attempt to modify the damaging effects of daily infusion. The original model, described by Kim and colleagues (7) used a combination of twice-daily exposure to dialysate with an initial administration of lipopolysaccharide (LPS). That model induced significant alterations in the peritoneum after 4 weeks. That model therefore seemed appropriate for the introduction of adenovirus-mediated gene transfer to the peritoneum, which has demonstrated transgene expression for a period of 10 – 14 days.

Here we describe our initial experience with the model. We show that significant histologic and functional changes occur in the parietal peritoneum after 4 weeks of daily infusion. The effects include increased

thickness of the peritoneum, neovascularization, increased glucose transport, and significantly impaired ultrafiltration. Those effects were seen both with physiologic saline solution and with high-concentration glucose dialysate. We also demonstrate that, in the model, adenovirus is effectively taken up and expressed by mesothelial cells.

METHODS

All animal studies were carried out according to the guidelines of the Canadian Council on Animal Care. Sprague–Dawley rats, 200 – 250 g (Harlan, Indianapolis, IN, U.S.A.) were anesthetized with ketamine. A 7-French silicone catheter (Access Systems, Newark, NJ, U.S.A.) was inserted into the peritoneum through a small incision in the abdominal wall. The catheter was tunneled subcutaneously to an implanted port (Access Systems). The animals were allowed to recover for 1 week before daily fluid infusion was initiated. The animals were treated with antibiotics.

Four groups of animals were studied, and all were humanely killed after 5 weeks. The first group ($n = 5$) had no catheter inserted. A second group ($n = 3$) had a catheter inserted, but no fluid instilled. A third group ($n = 8$) had catheters and daily infusion of phosphate-buffered saline or Ringer's lactate from week 1 to week 5. The last group ($n = 11$) had daily instillation of dialysate (Dianeal 4.25%: Baxter Healthcare Corporation, Deerfield, IL, U.S.A.) along with 75 μg LPS (Sigma–Genosys, Oakville, Canada) on days 8, 10, and 12. Animals having fluid infused received 15 mL for the first 3 days, then 20 mL for 3 days, and then 25 mL for the remainder of the duration of the experiment. At week 3, a peritoneal fluid sample was taken from all animals 4 hours after infusion of 20 mL Dianeal 2.5%. At the end of the experiment, 20 mL of Dianeal 2.5% was infused, and 4 hours later the animals were killed, an accurate ultrafiltration volume was measured, and blood and tissue samples were taken.

Peritoneal fluid samples were centrifuged at 1500 rpm for 5 minutes. Glucose was measured by a standard enzymatic test on a Hitachi 917 automated chemistry analyzer (Roche Diagnostics, Laval, Canada). Mass transfer of glucose out of the peritoneum was calculated using the formula:

(initial dialysate glucose × initial volume)
– (final dialysate glucose × final volume)

Values were corrected for animal weight at the time of death.

To demonstrate effective adenoviral gene transfer, a replication-deficient recombinant adenovirus expressing β-galactosidase (AdLacZ) was administered through the indwelling catheter in 2 animals. Adenovirus at a dose of 2×10^9 plaque-forming units diluted to 200 μL with PBS was administered 7 days after initiation of the daily dialysate infusion. The animals were killed 4 days later. Cytochemical staining for β-galactosidase was carried out on samples from animals treated with adenovirus. Fresh tissue samples were fixed in 2% formaldehyde, 0.2% glutaraldehyde for 3 hours. Fixation was followed by staining for 6 hours in a potassium ferrous cyanide, potassium ferric cyanide, magnesium chloride solution (Sigma), Triton X100, and X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside: Boehringer Mannheim, Indianapolis, IL, U.S.A.). Sections were counterstained with nuclear fast red.

Tissue samples were taken from the lower anterior abdominal wall distant from the site of catheter placement. Sections from both sides of the midline were taken and fixed in a sufficient amount of 4% phosphate-buffered formaldehyde for 24 hours. The tissue samples were then paraffin processed and embedded, and 3 μm sections were cut. Cut sections were then stained for Masson trichrome and immunohistochemistry was carried out with antibodies to von Willebrand factor–factor VIII–related antigen (Dako Corporation, Carpenteria, CA, U.S.A.).

Sections of the anterior abdominal wall, immunostained for factor VIII, were studied in a blinded fashion using a standardized microscope grid to count peritoneum-associated blood vessels and to measure the thickness of the peritoneum. Slides contained at least 4 separate tissue pieces, and each slide was examined in 10 – 12 random high-power views.

Peritoneal fluid samples were analyzed using a human TGFβ1 ELISA kit or mouse VEGF ELISA kit (R&D Systems, Minneapolis, MN, U.S.A.). Samples were treated with HCl, pH-adjusted with NaOH, and then assayed for total (active and latent) TGFβ1.

A portion of mesentery was taken and frozen for a hydroxyproline assay. Tissues were weighed and homogenized in water. Solid material was precipitated with trichloroacetic acid. Samples were hydrolyzed overnight in 6N HCl at 110°C. Hydroxyproline content was quantified by Erlich reagent (Sigma) and assayed by measuring the optical density at 557 nm.

Data are presented as mean ± standard deviation unless otherwise noted. Comparisons between groups were made by t-test. Linear regression was used to compare cytokine levels, quantitative immunohistochemistry, solute transport, and net ultrafiltration.

RESULTS

In general, we saw significant changes in the histologic and functional characteristics of the peritoneum in animals exposed to daily physiologic saline

or 4.25% Dianeal as compared to animals in the catheter-only or no-catheter control groups (Table 1). Net ultrafiltration after a 4-hour dwell with Dianeal 2.5% was significantly impaired in animals exposed to daily fluid, and the mass transfer of glucose was significantly increased. We also demonstrated a significant increase in the number of peritoneal vessels and in the thickness of the submesothelial tissue (Table 1, Figure 1). Animals exposed to daily fluid, both isotonic saline and Dianeal 4.25%, showed increased collagen deposition as measured by mesenteric hydroxyproline content. The thickness of the peritoneum in rats in the catheter-only group was increased, with a nonsignificant increase in vascularization. Those changes did not seem to affect functional characteristics such as glucose transport and net ultrafiltration.

The peritoneal effluent taken at week 3 showed a significant elevation in VEGF concentration in animals exposed to daily Dianeal 4.25% (135 pg/mL vs 70 pg/mL in no-catheter control animals, $p = 0.03$).

In the isotonic saline group, we treated 5 animals with Ringer's lactate and 3 with phosphate-buffered saline. We saw no differences between the treatments, and those animals were grouped for analysis. Overall, a nonsignificant trend toward more severe changes in ultrafiltration, in glucose transport, in submesothelial thickness, and in number of vessels was seen in animals treated with daily Dianeal 4.25% as compared with animals treated with isotonic saline (Table 1). Glucose-treated animals had a higher total TGFβ dialysate concentration as measured by ELISA (411 pg/mL vs 231 pg/mL, $p = 0.03$).

In animals treated with saline or glucose, the mass transport of glucose was inversely correlated with net ultrafiltration ($r = -0.712$, $p < 0.001$). The number of peritoneum-associated vessels per millimeter of peritoneum was also inversely correlated with net ultrafiltration ($r = -0.524$, $p = 0.03$). Finally, the total TGFβ1 concentration at week 3 was correlated with the thickness of the peritoneum as measured at week 5 ($r = 0.5$, $p = 0.03$).

We were able to identify uptake and expression of adenovirus by staining for β-galactosidase 4 days after transfection with AdLacZ (Figure 1).

DISCUSSION

We further explored the chronic inflammatory infusion model first described by Kim and colleagues (7). We noted impressive fibrosis and neovasculogenesis with associated ultrafiltration failure in animals treated with daily fluid infusion. The concentrations of profibrotic and vasculogenic cytokines in the dialysate were increased. The strong inverse correlation between glucose transport and net ultrafiltration, and between new peritoneal blood vessels and net ultrafiltration suggest that increased vascular surface area and the subsequently increased glucose transport leads to significantly decreased ultrafiltration. The dialysate concentration of TGFβ1 correlated with thickening of the peritoneum, but not with vascularity or transport properties, suggesting an important role for vasculogenesis, as compared with fibrogenesis, in the functional changes noted.

We saw fairly dramatic changes with infusion of isotonic saline. The reason for that finding is not clear, but infection is unlikely, given our extensive use of antibiotics. A foreign body reaction also does not ex-

TABLE 1
Functional and Histologic Changes in the Peritoneum—Daily Infusion Model

	No catheter (n=5)	Catheter only (n=3)	Isotonic saline (n=8)	4.25% Dianeal (n=11)
Net ultrafiltration (mL)	4.3±1.8	3.2±0.1	-5.9±2.4[a]	-8.0±3.9[a]
Mass transfer glucose (mmol/kg)	6.8±0.4	6.7±0.8	7.8±0.4[a]	8.0±0.9[a]
Vessels (/mm)	0.7±0.5	13±11	25±9[a]	30±33
Thickness (μm)	31±14	88±24[b]	118±50[a]	205±170
Hydroxyproline (μg/mg)	1.41±0.4	1.88±0.3	3.37±0.9[a]	2.83±1.2[a]
VEGF (pg/mL)				
Week 3	70±36	182±44	135±82	135±50[a]
Week 5	74±41	122±85	120±87	111±68
Total TGFβ (pg/mL)				
Week 3	247±44	221±150	231±91[c]	411±185[c]
Week 5	210±21	225±41	260±104	308±184

[a] $p < 0.05$ physiologic saline or Dianeal versus no catheter.
[b] $p < 0.05$ catheter only versus no catheter.
[c] $p < 0.05$ physiologic saline versus Dianeal.

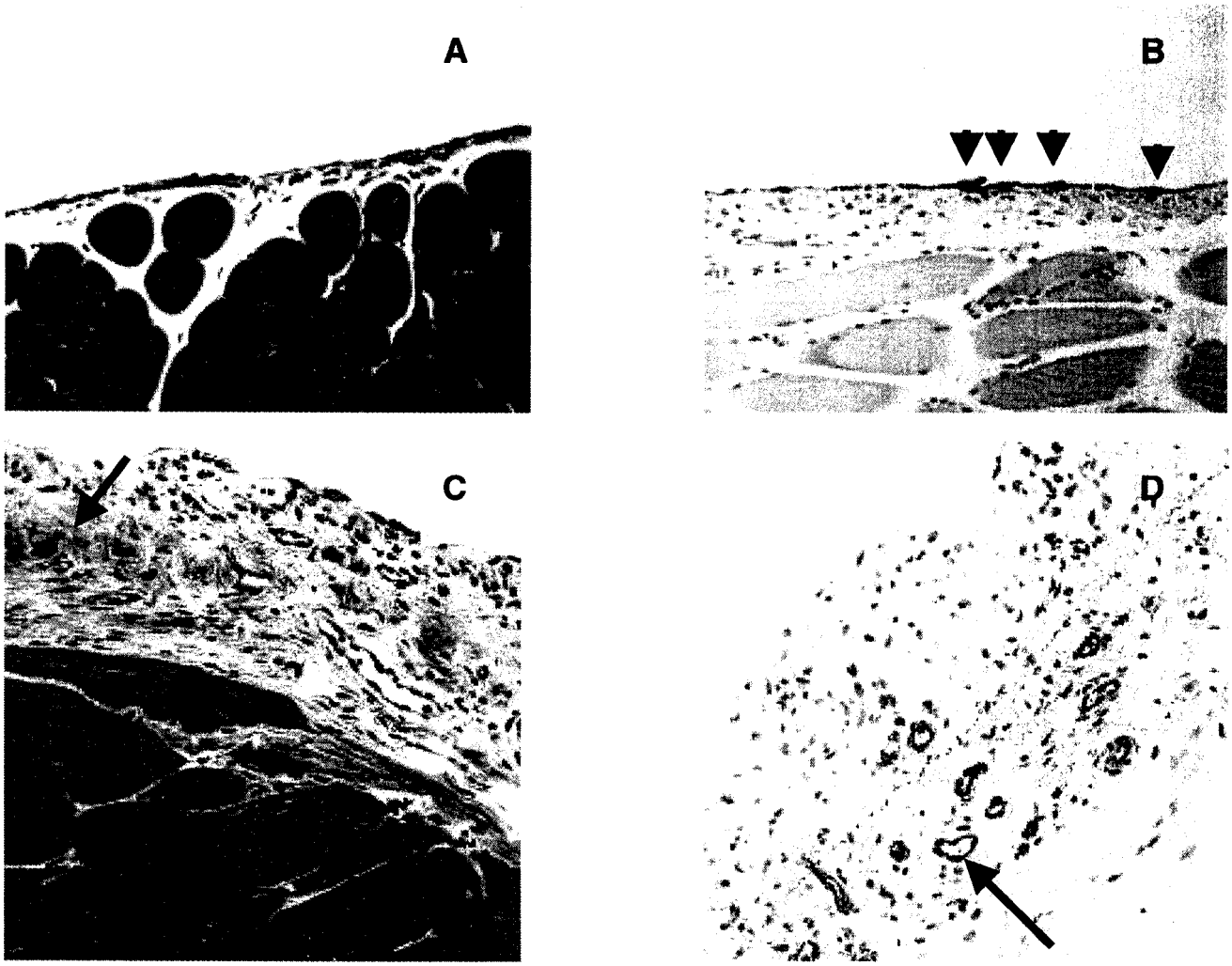

Figure 1 — (A) Normal anterior abdominal wall with peritoneal tissue from "no catheter" control animal. (B) Anterior abdominal wall from animal treated with adenovirus-mediated gene transfer of β-galactosidase and stained with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Arrowheads indicate mesothelial cells infected with adenovirus and expressing transgene product. (C) Peritoneum after 4 weeks of daily infusion with 4.25% Dianeal. Tissue is thickened with increased collagen deposition (arrow) and neovascularization. (D) Factor VIII–von Willebrand factor stain of same section as in (C) demonstrates neovascularization (arrow). All sections 125× magnification. (A) and (C) use Masson trichrome; (B) is counterstained with nuclear fast red.

plain the entire effect, because changes in the catheter-only animals were minimal.

We hypothesize that the trauma of catheter insertion initiates a fibroproliferative response that is rapidly contained in an otherwise untouched peritoneum. In the presence of daily fluid infusion, the ability of the peritoneum to respond to the insult is delayed, and the overall fibroproliferative effect is prolonged. A trend toward more pronounced effects was seen with Dianeal 4.25%. That finding suggests that glucose or other solution components may add to the already profibrotic state of the peritoneum after catheter insertion. However, the roles of LPS and glucose cannot be separated in the present experiments.

By 5 weeks, the VEGF and TGFβ1 dialysate concentrations decline (Table 1), which suggests that the acute fibroproliferative response would subside if the model were prolonged. Less dramatic, but clearly evident changes have been seen in 16-week to 20-week chronic infusion models (8), leading us to believe that an initial acute fibroproliferative phase occurs, subsides, and is then replaced by a more slowly developing chronic fibrosis and vascular injury.

Finally, we demonstrated that adenovirus-mediated gene transfer is possible in the present model. Adenovirus vectors have been engineered to transfer genes coding anti-inflammatory, anti-angiogenic (9), and anti-fibrotic (10) cytokines. The effect of those

agents can be studied in the present chronic inflammatory infusion model.

ACKNOWLEDGMENTS

The authors would like to thank Baxter Healthcare, the Kidney Foundation of Canada, and the Canadian Institutes of Health Research for their support. We also thank Mary Jo Smith for help with immunohistochemistry and histology.

REFERENCES

1. Davies SJ, Phillips L, Griffiths AM, Russell LH, Naish PF, Russell GI. What really happens to people on long-term peritoneal dialysis? *Kidney Int* 1998; 54:2207–17.
2. Honda K, Nitta K, Horita S, Yumura W, Nihei H, Nagai R, *et al.* Accumulation of advanced glycation end products in the peritoneal vasculature of continuous ambulatory peritoneal dialysis patients with low ultra-filtration. *Nephrol Dial Transplant* 1999; 14: 1541–9.
3. Plum J, Hermann S, Fussholler A, Schoenicke G, Donner A, Rohrborn A, *et al.* Peritoneal sclerosis in peritoneal dialysis patients related to dialysis settings and peritoneal transport properties. *Kidney Int* 2001; 59(Suppl 78):S42–7.
4. Lai KN, Lai KB, Lam CW, Chan TM, Li FK, Leung JC. Changes of cytokine profiles during peritonitis in patients on continuous ambulatory peritoneal dialysis. *Am J Kidney Dis* 2000; 35:644–52.
5. Zweers MM, de Waart DR, Smit W, Struijk DG, Krediet RT. Growth factors VEGF and TGF-β1 in peritoneal dialysis. *J Lab Clin Med* 1999; 134:124–32.
6. Margetts PJ, Galt T, Kolb M, Hoff CM, Shockley TR, Gauldie J. Adenovirus mediated gene transfer of TGF β1 to the rat peritoneum: effect on solute transport and VEGF expression (Abstract). *Perit Dial Int* 2000; 20:135.
7. Kim YL, Kim SH, Kim JH, Kim SJ, Kim CD, Cho DK, *et al.* Effects of peritoneal rest on peritoneal transport and peritoneal membrane thickening in continuous ambulatory peritoneal dialysis rats. *Perit Dial Int* 1999; 19(Suppl 2):S384–7.
8. Zweers M, Splint J, Krediet T, Struijk G. Ultrastructure of basement membranes of peritoneal capillaries in a chronic peritoneal infusion model in the rat. *Nephrol Dial Transplant* 2001; 16:651–4.
9. Gyorffy S, Palmer K, Podor TJ, Hitt M, Gauldie J. Combined treatment of a murine breast cancer model with type 5 adenovirus vectors expressing murine angiostatin and IL-12: a role for combined anti-angiogenesis and immunotherapy y. *J Immunol* 2001; 166:6212–17.
10. Kolb M, Margetts PJ, Galt T, Sime PJ, Xing Z, Schmidt M, *et al.* Transient transgene expression of decorin in the lung reduces the fibrotic response to bleomycin. *Am J Respir Crit Care Med* 2001; 163:770–7.

CHAPTER 5: ANTIANGIOGENIC AND ANTIFIBROTIC GENE THERAPY IN A CHRONIC INFUSION MODEL OF PERITONEAL DIALYSIS IN RATS

This final paper was published in the Journal of the American Society of Nephrology 2002; 13: 721-728. In this paper, we used our established daily dialysis model in rats and treated the rats with either antiangiogenic (AdAngiostatin) or antifibrotic (AdDecorin) gene therapy. This paper identified 2 main points. First, AdAngiostatin therapy significantly reduced the peritoneal vasculature. In places, we could see a dramatic apoptosis of endothelial cells. With this decrease in vasculature was an improvement (but not normalization) in UF function. Decorin, on the other hand, significantly reduced fibrosis measured by hydroxyproline concentration, but did not effect vascular density or UF function. As we had previously hypothesized that TGFβ1 is involved in angiogenesis in the peritoneum, this finding was somewhat contradictory. One possible explanation is that fibrogenic and angiogenic functions of TGFβ1 are separated by the type of activin-like kinase (ALK) receptor [172]. Decorin may block TGFβ1 signaling through ALK-5 and thus prevent fibrosis, but may allow signaling through ALK-1 and thus may not abrogate the angiogenic effect. This hypothesis requires further work to confirm.

The angiostatin adenovirus was created by Dr. Steve Gyorffy. The decorin adenovirus was created by Dr. Patricia Sime. I developed the concept and designed these experiments. The work was carried out by Lisa Yu with some assistance from Dr. Martin Kolb and myself. The paper was written by myself and was reviewed by Drs Gauldie, Hoff and Clifford Holmes.

Antiangiogenic and Antifibrotic Gene Therapy in a Chronic Infusion Model of Peritoneal Dialysis in Rats

PETER J. MARGETTS,*† STEVE GYORFFY,* MARTIN KOLB,* LISA YU,* CATHERINE M. HOFF,‡ CLIFFORD J. HOLMES,‡ and JACK GAULDIE*

**Centre for Gene Therapeutics, Department of Pathology and Molecular Medicine and †Division of Nephrology, McMaster University, Hamilton, Canada; and ‡Baxter Healthcare Renal Division Scientific Affairs, Baxter Healthcare Corporation, McGaw Park, Illinois*

Abstract. To identify the relative importance of peritoneal fibrosis and angiogenesis in peritoneal membrane dysfunction, adenoviral mediated gene transfer of angiostatin, a recognized angiogenesis inhibitor, and decorin, a transforming growth factor-β–inhibiting proteoglycan, were used in a daily infusion model of peritoneal dialysis. A peritoneal catheter and subcutaneous port were inserted in rats. Five and fourteen d after insertion, adenovirus-expressing angiostatin, decorin, or AdDL70, a null control virus, were administered. Daily infusion of 4.25% Baxter Dianeal was initiated 7 d after catheter insertion and continued until day 35. Three initial doses of lipopolysaccharide were administered on days 8, 10, and 12 to promote an inflammatory response. Net ultrafiltration was used as a measure of membrane function, and peritoneum-associated vasculature and mesenteric collagen content was quantified. Ultrafiltration dysfunction, angiogenesis, and fibrosis were observed in daily infusion control animals. Animals treated with AdAngiostatin demonstrated an improvement in net ultrafiltration (−3.1 *versus* −7.8 ml for control animals; $P = 0.0004$) with a significant reduction in vessel density. AdDecorin-treated animals showed a reduction in mesenteric collagen content (1.8 *versus* 2.9 μg/mg; $P = 0.04$); however, AdDecorin treatment had no effect on net ultrafiltration. In a rodent model of peritoneal membrane failure, net ultrafiltration was significantly improved and peritoneal-associated blood vessels were significantly reduced by using adenovirus-mediated gene transfer of angiostatin. Decorin, a transforming growth factor-β–inhibiting proteoglycan, reduced collagen content but did not affect net ultrafiltration. Improvement in the function of the peritoneum as a dialysis membrane after treatment with angiostatin has implications for treatment of peritoneal membrane dysfunction seen in patients on long-term dialysis.

During long-term peritoneal dialysis, the peritoneum undergoes histologic changes that include increased submesothelial collagen deposition, increased vascularization with vasculopathy, and loss of mesothelial cells (1,2). These changes suggest that both fibrogenic and angiogenic processes are active. Peritoneal concentration of vascular endothelial growth factor (VEGF) (3) and transforming growth factor–β (TGF-β) (4), key angiogenic and fibrogenic growth factors, have also been shown to correlate with peritoneal membrane function. These features strongly suggest that fibrogenic and angiogenic processes in the peritoneum are responsible for the alteration in peritoneal membrane function that is seen in patients on long-term peritoneal dialysis.

In these experiments, we used a short-term daily infusion model of peritoneal dialysis in rats. This model is associated with increased expression of VEGF and TGF-β, thickening of the submesothelial zone of the parietal peritoneum, increased angiogenesis, ultrafiltration failure, and increased transport of solutes (5). Similar models have previously been used to demonstrate the effect of peritoneal membrane rest (6) and systemic angiotensin-converting enzyme inhibition (7) on peritoneal membrane function.

Adenovirus-mediated gene transfer has been shown to be an effective tool for studying changes induced by transient overexpression of cytokines or growth factors on the peritoneal membrane. We have demonstrated that the adenovirus vector, when administered to the peritoneum, is highly infective for mesothelial cells. The transferred gene product is produced in high quantities and is expressed from approximately day 4 to day 14 after infection. We have previously demonstrated that adenovirus-mediated gene transfer of the active form of TGF-β_1 leads to fibrosis of the peritoneum with increased VEGF expression and vasculogenesis (8). This led to an associated change in function, including increased transport of glucose and decreased net ultrafiltration.

The antiangiogenic and antifibrotic approaches we used in the following experiments have been previously described. Angiostatin is a potent inhibitor of endothelial cell proliferation that was first described for its ability to inhibit cancer growth by inhibiting tumor neovascularization (9). The adenovirus carrying the gene product for angiostatin (AdAngiostatin) has been effective in several studies, including a direct tumor injection model (10).

Received August 30, 2001. Accepted November 5, 2001.
Correspondence to: Dr. Jack Gauldie, Chair, Department of Pathology and Molecular Medicine, McMaster University, 1200 Main St W, Room 2N16, Hamilton, Ontario, Canada L8N 3Z5. Phone: 905-521-2100 ext. 76332; Fax 905-577-0198; E-mail: gauldie@mcmaster.ca

1046-6673/1303-0721
Journal of the American Society of Nephrology

Decorin is a proteoglycan that binds and inactivates TGF-β. Decorin has been shown to be effective in direct application to prevent lung fibrosis (11) and as systemic gene therapy to modify renal fibrosis (12). We have shown adenovirus-mediated gene transfer of decorin (AdDecorin) to be an effective treatment in preventing pulmonary fibrosis in a murine bleomycin model (13).

In these experiments, we introduced AdAngiostatin, AdDecorin, or a control adenovirus (AdDL70) into a 4-wk, daily dialysate exposure model in rats. We demonstrate that animals treated with AdAngiostatin showed reduction in the parietal peritoneal vasculature, decreased glucose transport, and improved ultrafiltration. Animals treated with AdDecorin did not demonstrate alteration in peritoneal vasculature or ultrafiltration, but they did show reduced peritoneal fibrosis.

Materials and Methods

Animals

All animal studies were carried out according to the Canadian Council on Animal Care Guidelines. Sprague-Dawley rats, 200 to 250 mg (Harlan, Indianapolis, IN) were anesthetized with ketamine/xylazine. A 7 French silicone catheter (Access Systems, Newark, NJ) was inserted into the peritoneum through a small incision in the abdominal wall. The catheter was tunneled subcutaneously to an implanted port (Access Systems) on the back.

Animals were administered adenovirus (2×10^9 plaque-forming units [pfu] per animal) 5 d after catheter insertion with a repeat administration on day 14. Both administrations were given intraperitoneally via the dialysis catheter. Daily infusion of dialysate (4.25% Dianeal; Baxter Healthcare, McGaw Park, IL) was initiated 7 d after catheter insertion, beginning at 15 ml then quickly increasing to 25 ml daily. Animals also received intraperitoneal lipopolysaccharide (LPS–75 μg; Sigma, Oakville, ON) on days 8, 10, and 12. Animals were treated with rotating antibiotics consisting of intraperitoneal cefazolin, intraperitoneal ciprofloxacin, and oral sulfamethoxazole-trimethoprim.

Four groups of animals were studied, and all were killed after 5 wk. The first was a positive control group ($n = 11$) that received control virus (AdDL70). The second group ($n = 10$) received AdAngiostatin, and the third group ($n = 7$) received AdDecorin. For negative control, we used a group of animals ($n = 5$) with no catheter inserted and administered AdDL70 at initiation. At week 3 in all animals, a peritoneal fluid sample was taken 4 h after infusion of 20 ml of 2.5% Dianeal. At week 5, 20 ml of 2.5% Dianeal was infused, and 4 h later, the animal was killed, an accurate ultrafiltration volume was measured, and blood and tissue samples taken. In a subset of AdAngiostatin-treated animals, we took a peritoneal fluid sample 4 d after first administration of adenovirus to demonstrate effective gene transfer.

Whole blood was centrifuged at 5000 rpm for 10 min and the serum removed. Peritoneal fluid samples were centrifuged at 1500 rpm for 5 min. Samples were analyzed on a Hitachi 917 automated chemistry analyzer (Roche Diagnostics, Laval, Canada) for creatinine, glucose, and albumin. Mass transfer of glucose out of the peritoneum was calculated as (initial dialysate glucose $\times$ initial volume)$-$(final dialysate glucose $\times$ final volume). Creatinine and albumin clearances were calculated as mass transfer divided by the serum solute concentration. All values were corrected for animal weight at death.

To demonstrate the safety of adenovirus-mediated gene transfer of angiostatin, we studied 8 rats without peritoneal catheters. These animals were treated with 1 ($n = 4$) or 2 ($n = 4$) doses of AdAngiostatin (2×10^9 pfu intraperitoneally). Groups of 2 animals were sacrificed 4 and 7 d after treatment, and tissue samples were taken, fixed in neutral buffered formalin, embedded in paraffin, sectioned, and stained for hematoxylin/eosin and Masson trichrome. These sections were examined for pathologic changes in the peritoneum, liver, spleen, kidney, bowel, and ovaries.

Adenovirus

The cDNA for murine angiostatin was constructed from the four kringle regions of plasminogen ligated to the endogenous signal sequence as described previously (14). The construction of the adenovirus for human decorin (15) and control virus (AdDL70) (16) have been previously described. Adenovirus preparations were purified by CsCl gradient centrifugation and PD-10 Sephadex chromatography (Amersham Pharmacia, Baie d'Urfe, Quebec), and plaque was titered on 293 cells as described previously (17).

Histology

In daily dialysis-treated animals, tissue samples were taken at sacrifice from the lower anterior abdominal wall distant from the site of catheter placement. Sections from both sides of the midline were taken and fixed in a sufficient amount of 4% phosphate-buffered formaldehyde for 24 h. The tissue samples were then paraffin processed, embedded and 5 μm sections cut. Cut sections were then stained for Masson trichrome, and immunohistochemistry was carried out with antibodies to von Willebrand factor-factor VIII–related antigen (Dako Corporation, Carpenteria CA). Negative control sections were run in parallel. Sections were deparaffinized in xylene followed by 100% ethanol and then placed in a methanol H_2O_2 solution for 30 min to block endogenous peroxidase activity. After hydration to water with graded alcohols, the sections were placed in 0.05 M Tris-buffered saline (TBS), pH 7.6, digested with 0.05% Pronase (Sigma) in TBS with calcium chloride for 17 min at room temperature then blocked in 5% normal goat serum (NGS) followed by a 1 h incubation in the 1:500 rabbit anti-human factor VIII in 1% NGS. Sections were then incubated in a prediluted kit of a biotinylated goat anti-rabbit followed by a streptavidin/peroxidase conjugate (Zymed Labs, San Francisco, CA) as per manufacturer's instructions. Incubations were carried out at room temperature, and sections were washed in between incubations 3 $\times$ 5 min with 0.05 M TBS, pH 7.6, except before the addition of the primary antibody. All sections were rinsed in 0.05 M acetate buffer, pH 5.0, before development in an aminoethylcarbazole (AEC) chromogen substrate for 15 min. All sections were counterstained in Mayer's hematoxylin for 2 min before mounting with glycerin gelatin.

We stained for apoptosis with *in situ* labeling for free 3' hydroxy-DNA using terminal deoxynucletidyl transferase (Apotag, Intergen, Purchase NY) according to the manufacturer's direction. Briefly, sections were deparrafinized in xylene, treated with proteinase K (20 μg/ml) for 15 min, quenched with 3.0% hydrogen peroxide, and then exposed for 1 h to terminal deoxynucleotidyl transferase. Sections were further labeled with antidigoxigenin peroxidase conjugate, washed, and then developed with peroxidase substrate. Sections were counterstained with methyl green.

Blood Vessel Analysis

Sections of the anterior abdominal wall, immunostained for factor VIII, were studied in blinded fashion using two methods. First, we used a standardized microscope grid to count peritoneal-associated blood vessels and to measure the thickness of the submesothelial zone. Slides contained four separate transverse tissue sections taken from

the lower anterior abdominal wall, and each slide was examined at 12 random high-power views. All measurements were carried out at the same magnification. Second, we took the same sections and digitized 12 fields of view containing peritoneal tissues using a Leica DMR microscope (Leica Microsystems, Wetzlar, Germany). We then analyzed these images using Leica Qwin Image Processing Software (Leica Imaging Systems, Cambridge, England) with subroutines we created. In both methods, we limited the analysis to the submesothelial collagenous zone. Results for both analyses were reported as number of vessels/mm^2 of peritoneal tissue. We then multiplied these values by the thickness of the submesothelial zone to arrive at an estimate of the total vasculature measured as vessels/mm. Finally, our image-processing subroutine was able to estimate the total vessel cross-section area in each digitized image, and we could therefore calculate an average cross-sectional area per vessel for each slide analyzed.

We compared these two methods of evaluating blood vessels in histologic sections using regression analysis. This showed a very close correlation between the two methods ($r = 0.87$; $P < 0.0001$).

Hydroxyproline Assay

A portion of mesentery was taken and frozen for a hydroxyproline assay, modified from Woessner method (18). Tissues were weighed, homogenized in water, and centrifuged at 1000 rpm for 5 min, and the superficial fatty material was removed by vacuum suction. Solid material was precipitated with TCA with centrifugation at 1500 rpm for 15 min at 4°C. Samples were hydrolyzed overnight in 6 N HCl at 110°C. Hydroxyproline content is quantified by Erlich's reagent (Sigma) and assayed by measuring the optical density at 557 nm. A hydroxyproline standard sample (Sigma) was used to create a standard curve.

Western Blot Analysis

Peritoneal dialysis fluid taken at week 3 was analyzed for angiostatin and decorin. Equal volume of fluid was separated on a 15% sodium dodecyl sulfate–polyacrylamide gel under nonreducing conditions and transferred to Immobulon-P membranes (Millipore, Mississauga, ON). For angiostatin, the membrane was probed with a 1:500 dilution chicken anti-rabbit-plasminogen IgY antibody (14) (generous gift of Dr. Mark Hatton, McMaster University, Hamilton, ON) followed by a secondary rabbit anti-chicken alkaline phosphatase–conjugated antibody (Zymed). Decorin was detected by using a 1:2000 dilution of anti-decorin rabbit antibody (generous gift from Dr. Larry Fisher, National Institutes of Health, Bethesda, MD), followed by a secondary anti-rabbit alkaline phosphatase–conjugated antibody (Sigma). The probed membranes were developed by using NDT/BCIP (Promega, Madison, WI) according to the manufacturer's instructions. Standard molecular weight markers (Life Technologies, Burlington, ON) were used.

Statistical Analyses

Data is presented ± SD unless otherwise noted. Comparison between groups was made by *t* test. Regression analysis was used to look for correlation between blood vessel number/mm, net ultrafiltration, and solute transport.

Results

Adenoviral Expression and Safety of Repeated Dose of AdAngiostatin

We have previously demonstrated that adenovirus is efficiently taken up and expressed by mesothelial cells in the peritoneum after intraperitoneal delivery (8). We have also previously shown that the control virus, AdDL70, had little effect on the peritoneum after a mild initial inflammation lasting approximately 48 h (19). In the experiments reported here, we investigated the adenoviral gene product expression by Western blot analysis of peritoneal dialysis fluid. We showed expression of angiostatin after both the first and second administration of adenovirus (Figure 1A). Likewise, AdDecorin-treated animals showed increased presence of decorin in the peritoneal dialysate (Figure 1B), suggesting effective infection and adenoviral transgene expression in this model. We have previously shown the *in vitro* efficacy of both AdAngiostatin (14) and AdDecorin (13).

We used 2×10^9 pfu of intraperitoneally delivered adenovirus, as we have shown high levels of transgene product at this dose with little persisting adenovirus effect (8). In this model, we used two doses of adenovirus vector delivered 9 d apart. There is an immunologic response to the adenovirus that makes second administration less effective but still demonstrable (20). Others have demonstrated effective repeated intraperitoneal administration of adenovirus in a tumor model (21). In our model, we studied animals administered one or two doses of

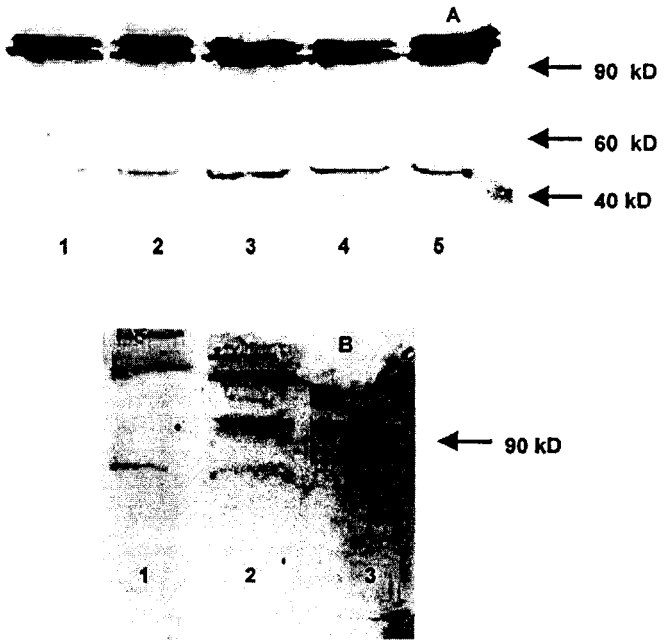

Figure 1. Western blot analysis of peritoneal dialysis fluid. (A) Angiostatin: band at 95 kD is plasminogen. Lighter band at 50 kD is angiostatin. Angiostatin is detectable in AdDL70-treated animals 7 d after 2nd virus administration (lane 1) but is greatly enhanced 4 d after 1st administration of AdAngiostatin (lanes 2 and 3) and still present 7 d after 2nd administration (lanes 4 and 5). (B) Decorin: decorin (approximately 102 kD band) was not detectable in control animals (lane 1), detectable in AdDL70, daily dialysate animals (lane 2), and enhanced after administration of AdDecorin (lane 3). Samples were separated on a 15% polyacrylamide gel, transferred to a nylon membrane, and then probed with antiplasminogen antibody (A) or antidecorin antibody (B). Molecular markers are shown to the right of the gels.

AdAngiostatin with no daily dialysis. Histologically, we could not detect any effects of angiostatin on kidneys, spleen, liver, or ovaries. After the second administration of angiostatin, there was a mild peritoneal response with occasional eosinophils and neutrophils present in the submesothelial tissue. The mesothelial cells appeared rounded up and activated (data not shown). The effect of this peritoneal inflammatory response to adenovirus in the setting of our chronic, inflammatory model is uncertain.

Changes in Structure of the Peritoneum

We compared the histology of control animals (AdDL70, no catheter) with animals treated with 4 wk of daily dialysis, initial LPS, and either AdDL70, AdAngiostatin, or AdDecorin. Daily dialysate infusion and LPS exposure significantly altered the structure of the parietal peritoneum. As we have previously demonstrated (5), daily dialysate infusion leads to thickening of the submesothelial collagenous zone with increased total number and density of blood vessels (Figure 2; Table 1). Hydroxyproline content of mesenteric tissue is also significantly increased in animals treated with daily dialysate.

Administration of AdDecorin had significant impact on fibrogenesis in this model. There was a significant decrease in the mesenteric hydroxyproline concentration (1.8 *versus* 2.8 μg/mg; $P = 0.04$) (Figure 2; Table 1) compared with AdDL70, daily infusion animals. AdAngiostatin had an impact on the vascularity of the peritoneum with a significant decrease in the number ($P = 0.03$) and density ($P = 0.02$) of peritoneal vessels (Table 1). We also saw a significant decrease ($P = 0.03$) in the average cross-sectional area of blood vessels after AdAngiostatin treatment. The submesothelial thickness was not significantly reduced, but the hydroxyproline concentration of the mesentery was unchanged after AdAngiostatin treatment (Table 1).

Histologic sections of AdAngiostatin-treated animals showed areas in which blood vessels appeared to have been lost from the parietal peritoneum, and residual cells demonstrated small, condensed nuclei, suggesting apoptosis or necrosis (Figure 3). We further carried out staining for fragmented DNA using terminal deoxynucleotidyl transferase labeling and could identify an number of cells undergoing apoptosis, including endothelial cells, in sections from animals treated with AdAngiostatin. Sections from animals treated with AdDL70 did not show significant apoptosis in the submesothelial tissue (Figure 3).

Changes in Peritoneal Function

Daily dialysate also had a significant effect on the function of the peritoneum as a dialysis membrane (Table 2). Specifically, we saw a significant impairment in ultrafiltration with associated increased mass transport of glucose out of the peritoneum. There was an increase in albumin clearance along with a decrease in clearance of creatinine. When we grouped all animals studied, we identified a significant inverse correlation between the number of vessels seen in the submesothelial tissue and net ultrafiltration ($r = -0.61$; $P = 0.0002$; Figure 4). We also saw a positive correlation between the number of

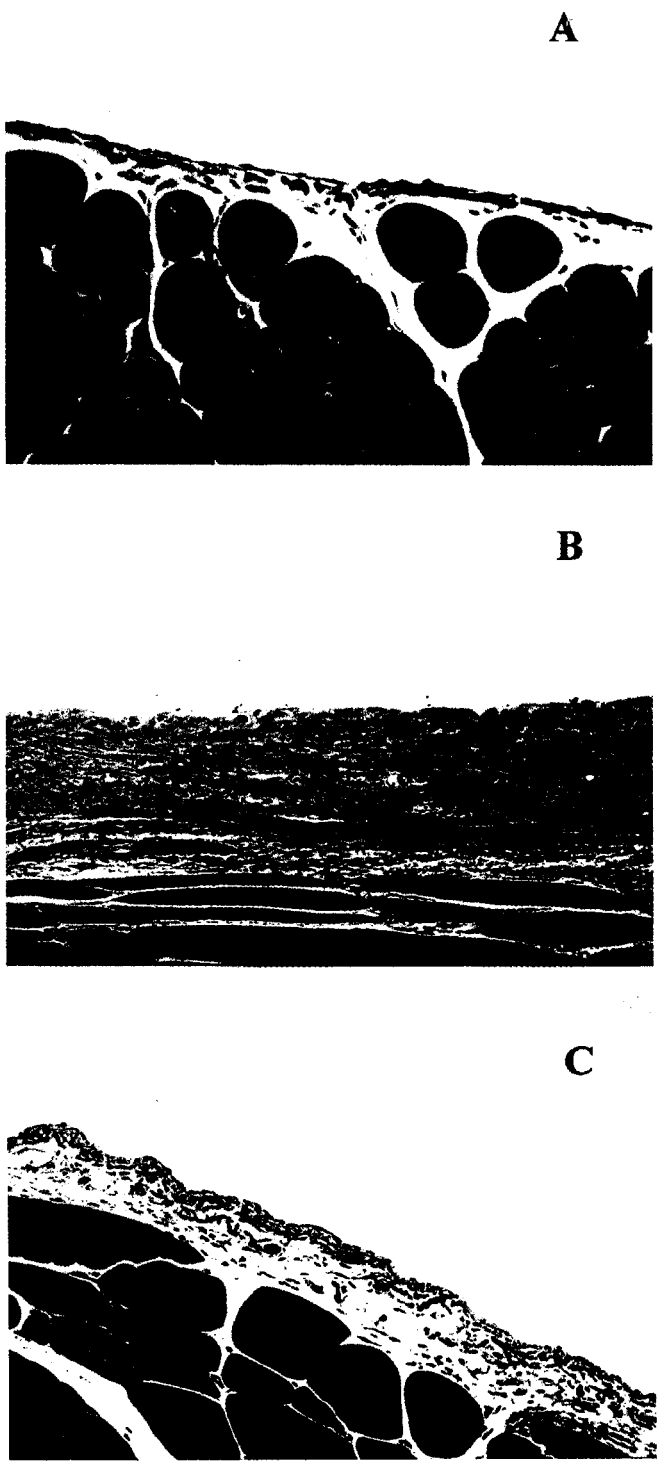

Figure 2. Histology of the anterior abdominal wall. (A) Control animal treated with AdDL70, no catheter, and no daily dialysate. Section shows normal histology with thin submesothelial zone above the abdominal wall muscles stained in red. (B) Animal treated with AdDL70, catheter, and daily dialysate. Histology is remarkably altered with thick, dense submesothelial zone and increased vascularization. (C) Animal treated with AdDecorin and daily dialysate. Section shows increased submesothelial thickening but somewhat reduced collagen deposition but similar vascularization to daily dialysate control animals. Masson trichrome stain. Magnification, ×50.

Table 1. Structural changes in the peritoneum after treatment with daily dialysate and null, angiostatin, or decorin adenovirus-mediated gene transfer compared with control (no catheter) animals[a]

Parameter	AdDL70, No Catheter (n = 5)	AdDL70 + Catheter (n = 11)	AdAngiostatin (n = 10)	AdDecorin (n = 7)
Thickness (μm)	30.8 ± 13.5	193 ± 134[b]	122 ± 38[b]	200 ± 88[b]
Vessels/mm	0.4 ± 0.3	12 ± 9[b]	5 ± 4[b,c]	11 ± 7[b]
Vessels/mm^2	12 ± 10	58 ± 21[b]	36 ± 19[c]	43 ± 16
Area/vessel (μm^2)	ND	277 ± 96	180 ± 86[c]	285 ± 128
Hydroxyproline (μg/mg)	1.4 ± 0.3	2.8 ± 1.0[b]	3.2 ± 1.9	1.8 ± 0.9[c]

[a] ND, no data.
[b] $P < 0.05$ compared with AdDL70, no catheter control.
[c] $P < 0.05$ compared with AdDL70 + catheter control.

submesothelial vessels and the glucose transport (r = 0.55; P = 0.0023) but no association with creatinine clearance (P = 0.3) or albumin clearance (P = 0.7).

AdAngiostatin treatment appeared to have a significant positive impact on peritoneal function. There was an improvement in the ultrafiltration dysfunction (P = 0.0004) with a decrease in glucose transport (P = 0.003). The creatinine and albumin clearances were unchanged compared with the daily infusion control animals (Table 2). AdAngiostatin treatment did not completely reverse the ultrafiltration dysfunction, and the ultrafiltration in these animals was still significantly reduced compared with no catheter control animals. AdDecorin treatment had little impact on peritoneal function (Table 2). Ultrafiltration dysfunction and glucose transport were unchanged. Albumin clearance was slightly increased, but this change was not statistically significant.

Discussion

These experiments have allowed us to investigate antifibrotic and antiangiogenic intervention in an acute fibroproliferative model of peritoneal dialysis. This model employed a daily infusion of high-concentration glucose dialysate over a 4-wk period with an initial exposure to LPS and resulted in changes in the peritoneum similar to those seen in patients on long-term peritoneal dialysis. The submesothelium becomes thickened, and there is increased collagen deposition with angiogenesis. We observed associated changes in peritoneal function, including ultrafiltration dysfunction and increased glucose transport. The processes involved in peritoneal changes in patients on chronic dialysis are obviously slower and more progressive. Certain features, such as loss of mesothelium and vasculopathy seen in patients on long-term dialysis, are not seen in our model. The extent to which this daily infusion model mimics what may happen in chronic peritoneal dialysis patients is yet to be determined.

Angiogenesis is an important element of fibrosing diseases (22). We have previously shown AdAngiostatin to effectively reduce angiogenesis in a matrigel model (14) and in a direct tumor injection model (10) and to reduce lung metastases in a murine breast cancer model (14). In this work, we demonstrated a reduction in peritoneal-associated vessels with an improvement in ultrafiltration dysfunction and decreased glucose absorption after administration of AdAngiostatin. Interestingly, we observed a reduction in submesothelial thickening that was not statistically significant, but no change in hydroxyproline concentration of the mesenteric tissue. These experiments were not designed to measure the effect of antiangiogenic therapy on fibrosis. Previous work has identified an inhibition of lung fibrosis by blocking proangiogenic CXC chemokines (23) or peritoneal adhesions by directly blocking angiogenesis (24). In both these experiments, the total amount of fibrotic tissue was assessed, whereas we measured the concentration of tissue collagen, which was not effected by angiostatin. The nonsignificant reduction of submesothelial thickness may be an indication of reduced total fibrotic tissue and therefore in agreement with previous work noted above. A larger sample size would be necessary to prove this hypothesis in our model.

The importance of angiogenesis in the alteration in peritoneal membrane function in patients on long-term dialysis has become increasingly clear (25). The interstitium of the peritoneum may have a role as a barrier to water reabsorption and larger molecule transport (26), but the capillary wall and the total vascular surface area likely have the greatest impact on transport of small solutes and ultrafiltration. In agreement with this hypothesis, our experiments demonstrated that inhibition of angiogenesis improved ultrafiltration and reduced small solute transport, but inhibition of fibrosis, through treatment with AdDecorin, did not alter peritoneal function. Also, in all animals treated with daily dialysis, we saw a strong inverse correlation between number of blood vessels and net ultrafiltration.

Decorin is a member of the small leucine-rich proteoglycan family (27). Previous work has demonstrated that decorin can alleviate organ fibrosis in several animal models (12,13). This is in agreement with the present experiments, where we show that treatment with AdDecorin in our model significantly reduced hydroxyproline concentration of mesenteric tissue, but did not significantly impact membrane-associated vessels or peritoneal function. We have previously demonstrated that the intraperitoneal delivery of AdTGF-β_1 to the peritoneum increased VEGF expression and induced peritoneal angiogenesis

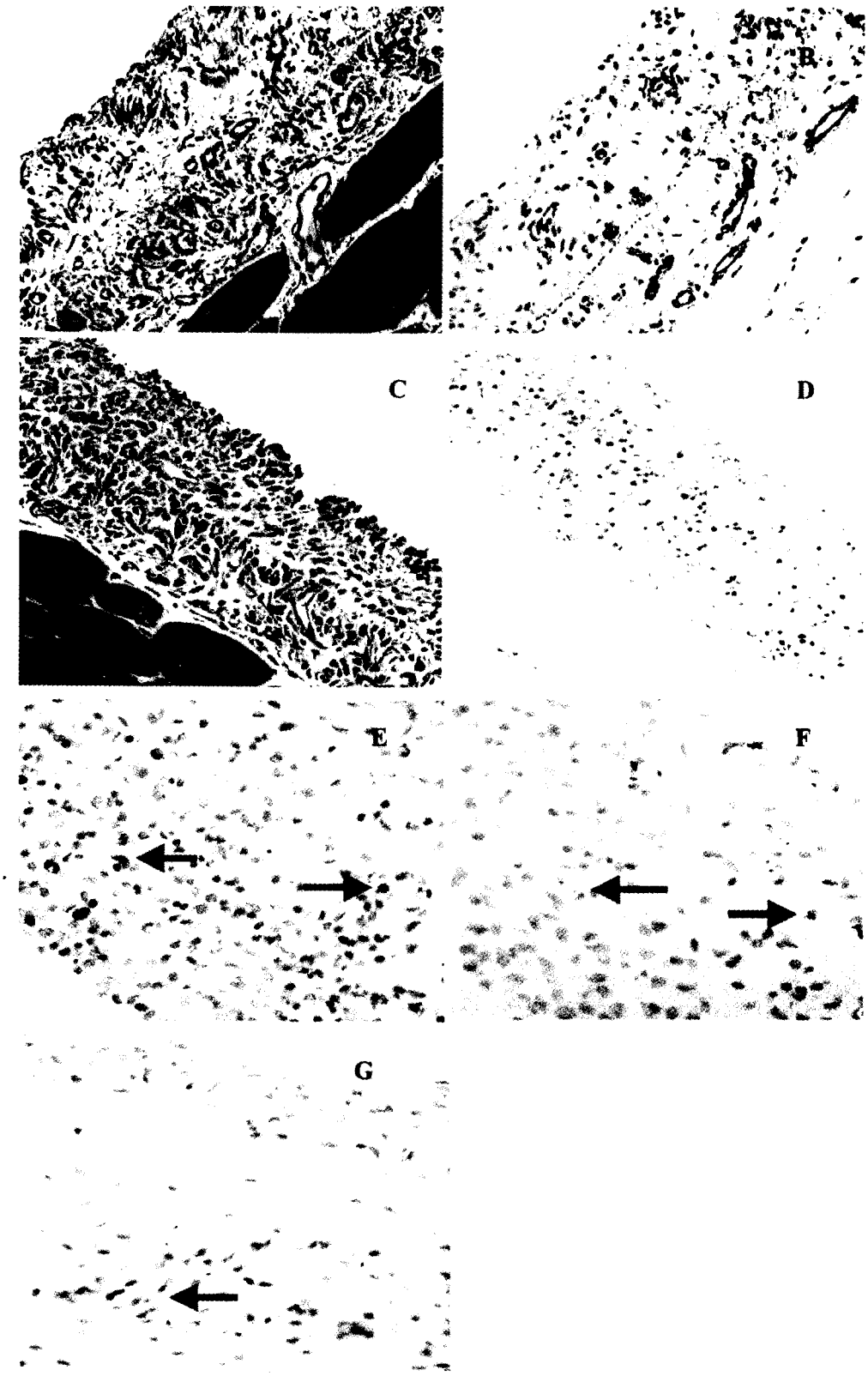

Figure 3. Histology of the anterior abdominal wall. (A) Section from animal treated with AdDL70 and daily dialysate shows submesothelial thickening and collagen deposition with increased vascularization shown by factor VIII-stained section in panel B. (C) Section from animal treated with AdAngiostatin and daily dialysate shows increased thickness and hypercellularity but a significant loss of vascularity, especially noticeable in factor VIII-stained section in panel D. (E) Staining for DNA fragmentation in animals treated with AdAngiostatin shows apoptotic cells, including endothelial cells (arrows) with corresponding factor VIII-stained section shown in panel F. (G) DNA fragmentation in animals treated with AdDL70 and daily dialysis shows virtually no apoptotic cells. Panels A and C are Masson trichrome; panels B, D, F are factor VIII; panels E and G are labeled with terminal deoxynucleotidyl transferase. Magnifications: ×100 in A through D; ×200 in E through G.

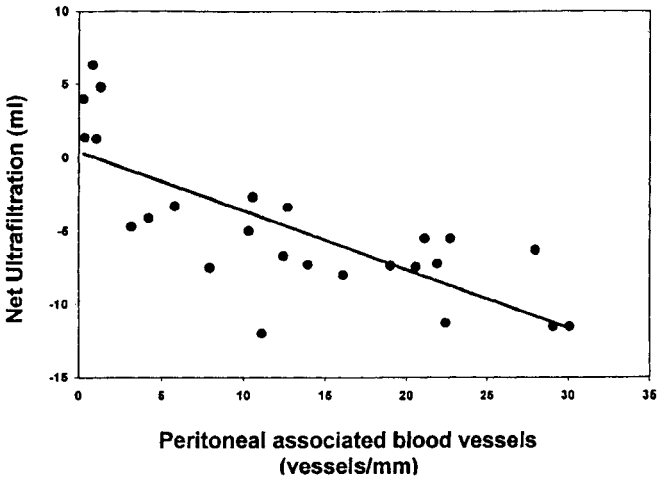

Figure 4. Inverse correlation between peritoneal-associated blood vessels counted on factor VIII-stained sections and net ultrafiltration measured after 4 wk of daily dialysate infusion. $r = -.61$; $P < 0.001$.

(8). There are likely other mechanisms in our model that lead to VEGF expression and angiogenesis, such as indirect induction through inflammatory cytokines, or direct induction through exposure to components of dialysate. TGF-β_1 may have a relatively minor role to play in the induction of angiogenesis in our model. This suggests that fibrosis does not have a significant impact on peritoneal dysfunction and again indicates the importance of the vasculature as a cause of dysfunction in peritoneal dialysis. We did see a nonsignificant increase in albumin transport in animals treated with AdDecorin. It is possible that, through alteration in the composition of the interstitium of the peritoneum that large molecule transport was affected. Further work is required to explore this possibility.

We were unable to completely reverse the effects of peritoneal dysfunction with AdAngiostatin in this model. Clearly, even high and repeated doses of angiostatin were not sufficient to block a substantial component of angiogenesis. This suggests either that the angiogenic stimuli are very strong or that other factors are present which inhibit the ability of angiostatin to induce widespread endothelial apoptosis

We believe that the results from these studies have direct therapeutic implications for patients on peritoneal dialysis. Our findings support the hypothesis that new blood vessel formation is a key component of peritoneal membrane dysfunction. Therapeutic strategies that reduce the peritoneal vasculature should be developed. We noted that creatinine clearance, although decreased in our model compared with untreated animals, was not changed after treatment with AdAngiostatin compared with AdDL70-positive control animals. This suggests that antiangiogenic therapy may not decrease the amount of dialysis provided by the peritoneum. The decreased vascular surface area and small solute transport were balanced by increased dialysate volume; therefore, creatinine clearance was preserved.

Previous work has demonstrated that anti-VEGF antibodies can prevent angiogenesis and peritoneal dysfunction in a hyperglycemic animal model (28). Other strategies targeting vascular growth factors and angiogenesis have been developed for the treatment of cancer and may be applicable to the problem of increased solute transport and ultrafiltration failure in the peritoneum (29). The most effective treatment will likely be prevention. Angiogenesis is likely driven by uremia, inflammation, and nonphysiologic dialysate. Optimizing dialysis treatment to avoid these elements may prevent peritoneal angiogenesis and best protect the peritoneum as a long-term dialysis membrane.

Acknowledgments

This paper was presented in abstract form at the World Congress of Nephrology, San Francisco, CA, October 2001. The research was funded by Baxter Healthcare, McGaw Park, Illinois, Canadian Institutes of Health Research, and the Kidney Foundation of Canada. We would like to acknowledge the technical support of Duncan Chong and Xueya Feng. We also thank Mary Jo Smith for her histology and immunohistochemistry expertise.

References

1. Mateijsen MA, van der Wal AC, Hendriks PM, Zweers MM, Mulder J, Struijk DG, Krediet RT: Vascular and interstitial changes in the peritoneum of CAPD patients with peritoneal sclerosis. *Perit Dial Int* 19: 517–525, 1999
2. Plum J, Hermann S, Fussholler A, Schoenicke G, Donner A, Rohrborn A, Grabensee B: Peritoneal sclerosis in peritoneal

Table 2. Functional properties of the peritoneal membrane in control animals (no catheter) and animals treated with daily dialysate and adenovirus

Parameter	AdDL70, No Catheter ($n = 5$)	AdDL70 + Catheter ($n = 11$)	AdAngiostatin ($n = 10$)	AdDecorin ($n = 7$)
Net ultrafiltration (ml)	4.3 ± 1.8	−8.9 ± 3.3[a]	−3.2 ± 2.7[a,b]	−9.0 ± 3.5[a]
Glucose transport (mmol/kg)	6.8 ± 0.4	8.0 ± 0.6[a]	7.1 ± 0.4[c]	7.7 ± 0.2[a]
Creatinine clearance (L/g)	49.3 ± 6.8	22 ± 10[a]	24 ± 8[a]	22 ± 8[a]
Albumin clearance (L/g)	1 ± 1.3	2.9 ± 2.0[a]	4.3 ± 2[a]	4.9 ± 2.3[a]

[a] $P < 0.01$ compared with AdDL70, no catheter control.
[b] $P = 0.0004$ compared with AdDL70 + catheter control.
[c] $P = 0.0025$ compared with AdDL70 + catheter control.

dialysis patients related to dialysis settings and peritoneal transport properties. *Kidney Int* 59(suppl 78): S42–S47, 2001
3. Zweers MM, de Waart DR, Smit W, Struijk DG, Krediet RT: Growth factors VEGF and TGF-beta1 in peritoneal dialysis. *J Lab Clin Med* 134: 124–132, 1999
4. Lai KN, Lai KB, Lam CW, Chan TM, Li FK, Leung JC: Changes of cytokine profiles during peritonitis in patients on continuous ambulatory peritoneal dialysis. *Am J Kidney Dis* 35: 644–652, 2000
5. Margetts PJ, Kolb M, Yu L, Hoff CM, Gauldie, J: A chronic inflammatory infusion model of peritoneal dialysis in rats. *Perit Dial Int* 2002: in press
6. Kim YL, Kim SH, Kim JH, Kim SJ, Kim CD, Cho DK, Kim YJ, Moberly JB: Effects of peritoneal rest on peritoneal transport and peritoneal membrane thickening in continuous ambulatory peritoneal dialysis rats. *Perit Dial Int* 19(suppl 2): S384–S387, 1999
7. Duman S, Gunal AI, Sen S, Asci G, Ozkahya M, Terzioglu E, Akcicek F, Atabay G: Does enalapril prevent peritoneal fibrosis induced by hypertonic (3.86%) peritoneal dialysis solution? *Perit Dial Int* 21: 219–224, 2001
8. Margetts PJ, Kolb M, Hoff CM, Shockley TR, Gauldie J: Gene transfer of transforming growth factor beta 1 to the rat peritoneum: Effects on membrane function. *J Am Soc Nephrol* 12: 2029–2039, 2001
9. O'Reilly MS, Holmgren L, Shing Y, Chen C, Rosenthal RA, Moses M, Lane WS, Cao Y, Sage EH, Folkman J: Angiostatin: A novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. *Cell* 79: 315–328, 1994
10. Gyorffy S, Palmer K, Podor TJ, Hitt M, Gauldie J: Combined treatment of a murine breast cancer model with type 5 adenovirus vectors expressing murine angiostatin and il-12: A role for combined anti-angiogenesis and immunotherapy. *J Immunol* 166: 6212–6217, 2001
11. Giri SN, Hyde DM, Braun RK, Gaarde W, Harper JR, Pierschbacher MD: Antifibrotic effect of decorin in a bleomycin hamster model of lung fibrosis. *Biochem Pharmacol* 54: 1205–1216, 1997
12. Isaka Y, Brees DK, Ikegaya K, Kaneda Y, Imai E, Noble NA, Border WA: Gene therapy by skeletal muscle expression of decorin prevents fibrotic disease in rat kidney. *Nat Med* 2: 418–423, 1996
13. Kolb M, Margetts PJ, Galt T, Sime PJ, Xing Z, Schmidt M, Gauldie J: Transient transgene expression of decorin in the lung reduces the fibrotic response to bleomycin. *Am J Respir Crit Care Med* 163: 770–777, 2001
14. Gyorffy S, Palmer K, Gauldie J: Adenoviral vector expressing murine angiostatin inhibits a model of breast cancer metastatic growth in the lungs of mice. *Am J Pathol* 159: 1137–1147, 2001
15. Zhao J, Sime PJ, Bringas P Jr, Gauldie J, Warburton D: Adenovirus-mediated decorin gene transfer prevents TGF-beta-induced inhibition of lung morphogenesis. *Am J Physiol* 277: L412–L422, 1999
16. Bett AJ, Haddara W, Prevec L, Graham FL: An efficient and flexible system for construction of adenovirus vectors with insertions or deletions in early regions 1 and 3. *Proc Natl Acad Sci USA* 91: 8802–8806, 1994
17. Xing Z, Ohkawara Y, Jordana M, Graham F, Gauldie J: Transfer of granulocyte-macrophage colony-stimulating factor gene to rat lung induces eosinophilia, monocytosis, and fibrotic reactions. *J Clin Invest* 97: 1102–1110, 1996
18. Woessner JF Jr, Bashey RI, Boucek RJ: Collagen development in heart and skin of the chick embryo. *Biochim Biophys Acta* 140: 329–338, 1967
19. Hoff CM: Ex vivo and in vivo gene transfer to the peritoneal membrane in a rat model. *Nephrol Dial Transplant* 16: 666–668, 2001
20. Kolb M, Inman M, Margetts PJ, Galt T, Gauldie J: Budesonide enhances repeated gene transfer and expression in the lung with adenoviral vectors. *Am J Respir Crit Care Med* 164: 866–872, 2001
21. Lambright ES, Force SD, Lanuti ME, Wasfi DS, Amin KM, Albelda SM, Kaiser LR: Efficacy of repeated adenoviral suicide gene therapy in a localized murine tumor model. *Ann Thorac Surg* 70: 1865–1870, 2000
22. Kalluri R, Sukhatme VP: Fibrosis and angiogenesis. *Curr Opin Nephrol Hypertens* 9: 413–418, 2000
23. Belperio JA, Keane MP, Arenberg DA, Addison CL, Ehlert JE, Burdick MD, Strieter RM: CXC chemokines in angiogenesis. *J Leukoc Biol* 68: 1–8, 2000
24. Chiang SC, Cheng CH, Moulton KS, Kasznica JM, Moulton SL: TNP-470 inhibits intraabdominal adhesion formation. *J Pediatr Surg* 35:189–196, 2000
25. Krediet RT, Zweers MM, van der Wal AC, Struijk DG: Neoangiogenesis in the peritoneal membrane. *Perit Dial Int* 20(suppl 2):S19–S25, 2000
26. Flessner MF: Changes in the peritoneal interstitium and their effect on peritoneal transport. *Perit Dial Int* 19(suppl 2):S77–S82, 1999
27. Iozzo RV: The family of the small leucine-rich proteoglycans: key regulators of matrix assembly and cellular growth. *Crit Rev Biochem Mol Biol* 32: 141–174, 1997
28. Vriese AS, Tilton RG, Stephan CC, Lameire NH: Vascular endothelial growth factor is essential for hyperglycemia-induced structural and functional alterations of the peritoneal membrane. *J Am Soc Nephrol* 12: 1734–1741, 2001
29. Kuo CJ, Farnebo F, Yu EY, Christofferson R, Swearingen RA, Carter R, von Recum HA, Yuan J, Kamihara J, Flynn E, D'Amato R, Folkman J, Mulligan RC: Comparative evaluation of the antitumor activity of antiangiogenic proteins delivered by gene transfer. *Proc Natl Acad Sci USA* 98: 4605–4610, 2001

DISCUSSION

Peritoneal dialysis (PD) is a widely used, effective therapy for patients with end stage renal disease. Ultrafiltration (UF) dysfunction is a common complication of this therapy caused by systemic factors including uremia and inflammation, and local factors including non-physiological dialysis solutions and recurrent peritonitis. The morphological changes correlating with UF dysfunction include fibrosis and angiogenesis in the peritoneal tissues.

About 14% [7] of PD patients will have to stop using PD therapy and switch to an alternate renal replacement therapy because of UF failure. More importantly, there is increasing evidence that subtle UF dysfunction goes unrecognized clinically and patients become volume expanded [24;182] and this leads to hypertension [25], hypoalbuminemia [27], and poor cardiovascular outcomes. Therefore, fibrosis and angiogenesis in the peritoneal tissues have important functional outcomes in PD patients that may lead to technique failure and increased morbidity and mortality. Solute removal was previously emphasized as a goal for PD therapy [183]. However recent evidence suggests total solute removal is less important in PD outcomes [184;185], and the importance of maintaining the peritoneal membrane and maintaining UF function has been recently highlighted [186].

Our work has demonstrated the importance of angiogenesis in UF failure with the observation of an inverse correlation between vascularization of the peritoneal tissues and net UF [40]. More importantly, we demonstrated that the use of adenovirus mediated angiostatin therapy in the chronic daily infusion model of PD led to a reduction in the number of blood vessels, and an improvement in net UF [40].

This observation not only firmly established the role of angiogenesis in the etiology of UF dysfunction, but it also points towards potential therapies for established peritoneal membrane failure. Adenovirus mediated gene therapy leads to transient gene expression, and so may not be suited for a chronic process such as peritoneal membrane alteration in PD therapy. Safety issues are also a concern. There may, however, still be other forms of gene therapy suitable to the peritoneum to modify both local processes and to deliver longer term systemic therapies [116].

Our demonstration of the efficacy of anti-angiogenic therapy on improving membrane function opens the door for other potential non-gene therapy approaches. Other anti-angiogenic therapies are available and some are being used clinically for applications as novel cancer therapies. There is an interest in using thalidomide in PD patients, both for its anti-inflammatory and anti-angiogenic properties. Other agents such as angiostatin [187], endostatin [188], TNP-470 [118], and novel vascular endothelial growth factor (VEGF) receptor tyrosine kinase inhibitors [119] are available and could be tested in an animal model for the impact on peritoneal membrane function.

Previously, the transient effect of peritonitis on peritoneal membrane function was felt to be due to vasogenic factors such as nitric oxide (NO) [45] or prostaglandins [189]. Using adenovirus mediated gene transfer of the inflammatory cytokines interleukin (IL)-1β and tumor necrosis factor (TNF)-α, we have demonstrated a direct angiogenic response in the peritoneum to these agents [81]. A single episode of peritonitis has little [72] or no [50] effect on long term peritoneal transport whereas severe or recurring peritonitis does [2]. Our study identified a differential fibrotic, angiogenic, and functional outcome with transient overexpression of TNFα or IL-1β. IL-1β exposure led to a prolonged

fibrogenic and angiogenic response with prolonged membrane dysfunction. TNFα exposure led to a very transient response [81]. It has yet to be shown whether severe peritonitis, or peritonitis caused by gram negative organisms, elicits a differential inflammatory response with increased expression of IL-1 and whether this explains the different functional changes observed after an episode of peritonitis. If IL-1 is found to be associated with persistent peritoneal membrane dysfunction in patients as it was in our animal study, then this suggests that combining prompt antibiotic therapy with IL-1 inhibition, perhaps through IL-1 receptor antagonist [89], may preserve the peritoneal membrane function better than antibiotics alone.

Several previous studies have demonstrated that the profibrotic cytokine transforming growth factor (TGF)-β is likely involved in angiogenesis. TGFβ appears to have direct effects through regulation of VEGF, platelet derived growth factor, and fibroblast growth factors, along with secondary vascular modification through angiopoietin (ANG) -1[160;170]. We have clearly demonstrated the role of TGFβ1 in the angiogenic response. Transient overexpression of TGFβ1 led to significant peritoneal fibrosis associated with increased vascularization [113]. This increased angiogenesis was associated with persistent UF dysfunction. TGFβ1 appeared to upregulate VEGF in vivo and in vitro in mesothelial cell culture. These results have recently been replicated in other experimental systems [169;190].

Studies of the peritoneum have allowed us to gain some insight into the fibrogenic process. It is clear that TGFβ1 is a key profibrotic cytokine and alone can initiate and maintain peritoneal fibrosis [113]. What was also of interest was our observation that, 7 days after peritoneal infection with adenovirus, TNFα caused a similar upregulation of TGFβ1

and deposition of collagen in peritoneal tissues as IL-1β [81]. However, at later time points, the fibrotic process was maintained in animals exposed to IL-1β. We found that tissue inhibitor of metalloproteinase (TIMP)-1 was upregulated by IL-1β but not by TNFα. TIMP-1 inhibits the metalloproteinases (MMP) which in turn are responsible for collagenolysis. These results suggest that initiation of fibrosis is due to inflammation and TGFβ1, whereas persisting fibrosis is due to an imbalance between collagen deposition and degradation. We have similar findings in an analysis of fibrosis-resistant and fibrosis-prone mice strains [191]. Fibrosis-resistant mice demonstrate a similar expression of collagen after exposure to TGFβ as fibrosis-prone mice, but the expression of TIMP-1 is much lower.

These observations have therapeutic implications. Anti-fibrotic therapy in general has been targeted toward inhibiting the initial inflammatory response to injury or activity of TGFβ [192]. Often, this initial phase of the fibrogenic process is not recognized and patients present with established fibrosis. Our findings suggest that, in this setting, altering the collagen metabolic balance towards net collagenolysis may have therapeutic benefits [81;191].

In the peritoneum, the formation and modification of collagen in response to cytokine stimulation or injury is not well studied. It is known that the composition of the interstitium has an effect on the hydrodynamic properties of the peritoneum and will affect fluid absorption and macromolecular transport by tissues and lymphatics [48]. Albumin transport appears to correlate inversely with hydroxyproline concentration in the peritoneal tissues. This suggests that fibrosis may impede macromolecular transport. Our model of adenovirus mediated TGFβ1 induced peritoneal fibrosis will allow the further

study of alteration in macromolecular transport, fluid absorption, tissue collagens and hyaluronan concentrations. These studies are important as pharmacologic intervention, such as hyaluronan [193], may have an effect on the peritoneal interstitium.

The different angiogenic responses we have observed after transient adenovirus mediated cytokine overexpression are intriguing and are a focus of ongoing and future research. While, angiogenesis has been extensively studied in the setting of malignancy, not a great deal is known about angiogenesis in the setting of fibrosis [143]. Various cytokines such as TNFα induce a transient angiogenesis. The blood vessels induced after over expression of TNFα demonstrate increased permeability to macromolecules such as albumin. TGFβ1 and IL-1β both induce a persisting peritoneal vasculature with comparatively less albumin transport. This suggests that the fibrogenic environment is associated with a more mature vascular phenotype. I therefore suggest that the fibrogenic response is associated with upregulation of ANG-1 which interacts with endothelial cells to reduce susceptibility to apoptosis [194] and decrease macromolecular transport [145]. Preliminary data suggests that in vivo, TGFβ1 is associated with increased ANG-1 expression compared with VEGF, which appears to upregulate ANG-2 [159]. TGFβ may also act through the receptor activin receptor like kinase (ALK) -1 which has been shown in knock out mice to be important in blood vessel maturation [172].

This finding has implications for PD research and therapy. We were unable to fully reverse angiogenesis and functional changes in our chronic model even with high doses of adenovirus mediated gene transfer of angiostatin [40]. This may partially be a result of the fibrogenic response generated in this model and the possibility that this could upregulate ANG-1 and protect the vasculature. Decorin, a proteoglycan that binds to and

inhibits TGFβ1, did not have an impact alone on the vasculature but did reduce the collagen content of the peritoneal tissue [40]. It is possible that the combination of therapy, anti-angiogenic and anti-fibrotic, is necessary to further impact on peritoneal angiogenesis. Also, altering the angiopoietins balance in favor of ANG-2 may make the endothelium more sensitive to the inhibiting effect of angiostatin.

TGFβ is associated with poor prognosis in malignancy and this has been thought to be due to its immunomodulating effects or an effect on cell cycle regulation [134]. More recently, it has been observed that TGFβ may have a role in the progression of malignancy through its synergistic effect with hypoxia on VEGF expression and angiogenesis [131]. TGFβ has been associated with worse prognosis and increased vascular density in non-small cell lung cancer [195]. Angiopoietins also are involved in the angiogenesis related to malignancy [196]. The effect of TGFβ on angiopoietins expression in malignancy has not yet been fully explored.

Adenovirus mediated gene transfer of VEGF has been used to treat ischemic heart disease in humans in several clinical trials [197]. One of the potential side effects has been a vascular leakage phenomenon and the transient nature of the angiogenic response. Future improvements may include combined angiogenic therapy using VEGF and ANG-1 to mature and prolong the vascularization.

Our work has introduced a new animal model for research in PD. Adenovirus mediated gene transfer into the peritoneum is very effective with a transient, high level expression of adenovirus [113]. This technique has been used in combination with a chronic infusion model to demonstrate potential therapeutic options [40]. Our results have led to insights into the structure and function of the peritoneum as a dialysis membrane and may

eventually lead to improvements in PD therapy that can benefit patients with prolongation of therapy and reduced morbidity and mortality. The importance of the angiogenic response in alteration of the function of the peritoneal membrane has provided a unique opportunity to develop techniques to study blood vessel growth in the setting of fibrogenesis. We have provided some early evidence of the role of TGFβ1 and other inflammatory cytokines in the induction and modification of angiogenesis.

Future work will include further evaluation of the role of TGFβ1 in angiogenesis through its regulation of ANG-1. The peritoneum also provides a simple environment to study the relative contribution of TIMPs and MMPs to the maintenance of fibrosis and the interaction between TIMPs, MMPs and angiogenesis. This model, combined with more detailed functional studies of the peritoneum, will allow the assessment of the relative contribution of vascularization, aquaporin dysfunction, and interstitial / lymphatic reabsorption to UF dysfunction after a fibrogenic stimulus. These results will continue to provide insights and new possible therapies for patients on PD and may provide further insight into angiogenesis in other disease processes.

Reference List

1. Gokal R: Taking peritoneal dialysis beyond the year 2000. Peritoneal Dialysis International 19 Suppl 3:S35-S42, 1999
2. Davies SJ, Bryan J, Phillips L, Russell GI: Longitudinal changes in peritoneal kinetics: the effects of peritoneal dialysis and peritonitis. Nephrol.Dial.Transplant. 11:498-506, 1996
3. Krediet RT, Lindholm B, Rippe B: Pathophysiology of peritoneal membrane failure. [Review] [153 refs]. Peritoneal Dialysis International 20 Suppl 4:S22-S42, 2000
4. Mactier RA, Khanna R, Twardowski ZJ, Nolph KD: Ultrafiltration failure in continuous ambulatory peritoneal dialysis due to excessive peritoneal cavity lymphatic absorption. American Journal of Kidney Diseases 10:461-466, 1987
5. Devuyst O, Nielsen S, Cosyns JP, Smith BL, Agre P, Squifflet JP, Pouthier D, Goffin E: Aquaporin-1 and endothelial nitric oxide synthase expression in capillary endothelia of human peritoneum. Am.J.Physiol 275:H234-H242, 1998
6. Maiorca R, Cancarini GC, Zubani R, Camerini C, Manili L, Brunori G, Movilli E: CAPD viability: a long-term comparison with hemodialysis. Perit.Dial.Int. 16:276-287, 1996
7. Kawaguchi Y: National comparisons: optimal peritoneal dialysis outcomes among Japanese patients. Peritoneal Dialysis International 19 Suppl 3:S9-16, 1999
8. Slingeneyer A, Canaud B, Mion C: Permanent loss of ultrafiltration capacity of the peritoneum in long-term peritoneal dialysis: an epidemiological study. Nephron 33:133-138, 1983
9. Heimburger O, Waniewski J, Werynski A, Tranaeus A, Lindholm B: Peritoneal transport in CAPD patients with permanent loss of ultrafiltration capacity. Kidney Int. 38:495-506, 1990
10. Ho-dac-Pannekeet MM, Atasever B, Struijk DG, Krediet RT: Analysis of ultrafiltration failure in peritoneal dialysis patients by means of standard peritoneal permeability analysis. Peritoneal Dialysis International 17:144-150, 1997
11. De Vriese AS, Mortier S, Lameire NH: What happens to the peritoneal membrane in long-term peritoneal dialysis? Perit.Dial.Int. 21 Suppl 3:S9-18, 2001
12. Heimburger O, Wang T, Lindholm B: Alterations in water and solute transport with time on peritoneal dialysis. Perit.Dial.Int. 19 Suppl 2:S83-90.:S83-S90, 1999
13. Struijk DG, Krediet RT, Koomen GC, Boeschoten EW, Hoek FJ, Arisz L: A prospective study of peritoneal transport in CAPD patients. Kidney Int. 45:1739-1744, 1994
14. Lo WK, Brendolan A, Prowant BF, Moore HL, Khanna R, Twardowski ZJ, Nolph KD: Changes in the peritoneal equilibration test in selected chronic peritoneal dialysis patients. J.Am.Soc.Nephrol. 4:1466-1474, 1994
15. Churchill DN, Thorpe KE, Nolph KD, Keshaviah PR, Oreopoulos DG, Page D: Increased peritoneal membrane transport is associated with decreased patient and technique survival for continuous peritoneal dialysis patients. The Canada-USA (CANUSA) Peritoneal Dialysis Study Group. J.Am.Soc.Nephrol. 9:1285-1292, 1998

16. Davies SJ, Phillips L, Russell GI: Peritoneal solute transport predicts survival on CAPD independently of residual renal function. Nephrology Dialysis Transplantation 13:962-968, 1998
17. Wang T, Heimburger O, Waniewski J, Bergstrom J, Lindholm B: Increased peritoneal permeability is associated with decreased fluid and small-solute removal and higher mortality in CAPD patients. Nephrology Dialysis Transplantation 13:1242-1249, 1998
18. Fried L: Higher membrane permeability predicts poorer patient survival. [see comments.]. Peritoneal Dialysis International 17:387-389, 1997
19. Davies SJ, Phillips L, Griffiths AM, Russell LH, Naish PF, Russell GI: What really happens to people on long-term peritoneal dialysis? Kidney Int. 54:2207-2217, 1998
20. Jager KJ, Merkus MP, Dekker FW, Boeschoten EW, Tijssen JG, Stevens P, Bos WJ, Krediet RT: Mortality and technique failure in patients starting chronic peritoneal dialysis: results of The Netherlands Cooperative Study on the Adequacy of Dialysis. NECOSAD Study Group. Kidney International 55:1476-1485, 1999
21. Chung SH, Chu WS, Lee HA, Kim YH, Lee IS, Lindholm B, Lee HB: Peritoneal transport characteristics, comorbid diseases and survival in CAPD patients. Peritoneal Dialysis International 20:541-547, 2000
22. Park HC, Kang SW, Choi KH, Ha SK, Han DS, Lee HY: Clinical outcome in continuous ambulatory peritoneal dialysis patients is not influenced by high peritoneal transport status. Perit.Dial.Int. 21 Suppl 3:S80-S85, 2001
23. Ates K, Nergizoglu G, Keven K, Sen A, Kutlay S, Erturk S, Duman N, Karatan O, Ertug AE: Effect of fluid and sodium removal on mortality in peritoneal dialysis patients. Kidney Int. 60:767-776, 2001
24. Tzamaloukas AH, Saddler MC, Murata GH, Malhotra D, Sena P, Simon D, Hawkins KL, Morgan K, Nevarez M, Wood B, .: Symptomatic fluid retention in patients on continuous peritoneal dialysis. J.Am.Soc.Nephrol. 6:198-206, 1995
25. Bos WJ, Struijk DG, van Olden RW, Arisz L, Krediet RT: Elevated 24-hour blood pressure in peritoneal dialysis patients with ultrafiltration failure. Advances in Peritoneal Dialysis 14:108-110, 1998
26. Jones CH, Smye SW, Newstead CG, Will EJ, Davison AM: Extracellular fluid volume determined by bioelectric impedance and serum albumin in CAPD patients. Nephrol.Dial.Transplant 13:393-397, 1998
27. Jones CH, Wells L, Stoves J, Farquhar F, Woodrow G: Can a reduction in extracellular fluid volume result in increased serum albumin in peritoneal dialysis patients? Am.J.Kidney Dis. 39:872-875, 2002
28. Garosi G, Di Paolo N: Peritoneal sclerosis: one or two nosological entities? Semin.Dial. 13:297-308, 2000
29. Pollock CA: Diagnosis and management of encapsulating peritoneal sclerosis. Perit.Dial.Int. 21 Suppl 3:S61-S66, 2001
30. Kawaguchi Y, Kawanishi H, Mujais S, Topley N, Oreopoulos DG: Encapsulating peritoneal sclerosis: definition, etiology, diagnosis, and treatment. International Society for Peritoneal Dialysis Ad Hoc Committee on Ultrafiltration Management

in Peritoneal Dialysis. [Review] [202 refs]. Peritoneal Dialysis International 20 Suppl 4:S43-S55, 2000

31. Garosi G, Di Paolo N: Inflammation and gross vascular alterations are characteristic histological features of sclerosing peritonitis. Perit.Dial.Int. 21:417-418, 2001
32. Krediet RT: Peritoneal physiology--impact on solute and fluid clearance. [Review] [76 refs]. Advances in Renal Replacement Therapy 7:271-279, 2000
33. Rippe B, Stelin G, Haraldsson B: Computer simulations of peritoneal fluid transport in CAPD. Kidney Int. 40:315-325, 1991
34. Rippe B, de Arteaga J, Venturoli D: Aquaporins are unlikely to be affected in marked ultrafiltration failure: results from a computer simulation. Perit.Dial.Int. 21 Suppl 3:S30-S34, 2001
35. Williams JD, Craig KJ, Topley N, Von Ruhland C, Fallon M, Newman GR, Mackenzie RK, Williams GT, Peritoneal Biopsy Study Group.: Morphologic changes in the peritoneal membrane of patients with renal disease. Journal of the American Society of Nephrology 13:470-479, 2002
36. Honda K, Nitta K, Horita S, Yumura W, Nihei H: Morphological changes in the peritoneal vasculature of patients on CAPD with ultrafiltration failure. Nephron 72:171-176, 1996
37. Mateijsen MA, van der Wal AC, Hendriks PM, Zweers MM, Mulder J, Struijk DG, Krediet RT: Vascular and interstitial changes in the peritoneum of CAPD patients with peritoneal sclerosis. Peritoneal Dialysis International 19:517-525, 1999
38. Plum J, Hermann S, Fussholler A, Schoenicke G, Donner A, Rohrborn A, Grabensee B: Peritoneal sclerosis in peritoneal dialysis patients related to dialysis settings and peritoneal transport properties. Kidney Int. 59 Suppl 78:S42-7.:S42-S47, 2001
39. Combet S, Miyata T, Moulin P, Pouthier D, Goffin E, Devuyst O: Vascular proliferation and enhanced expression of endothelial nitric oxide synthase in human peritoneum exposed to long-term peritoneal dialysis. J.Am.Soc.Nephrol. 11:717-728, 2000
40. Margetts PJ, Gyorffy S, Kolb M, Yu L, Hoff CM, Holmes CJ, Gauldie J: Antiangiogenic and antifibrotic gene therapy in a chronic infusion model of peritoneal dialysis in rats. J.Am.Soc.Nephrol. 13:721-728, 2002
41. Zweers MM, de Waart DR, Smit W, Struijk DG, Krediet RT: Growth factors VEGF and TGF-beta1 in peritoneal dialysis. [see comments.]. Journal of Laboratory & Clinical Medicine 134:124-132, 1999
42. Monquil MC, Imholz AL, Struijk DG, Krediet RT: Does impaired transcellular water transport contribute to net ultrafiltration failure during CAPD? Peritoneal Dialysis International 15:42-48, 1995
43. Yang B, Folkesson HG, Yang J, Matthay MA, Ma T, Verkman AS: Reduced osmotic water permeability of the peritoneal barrier in aquaporin-1 knockout mice. Am.J.Physiol 276:C76-C81, 1999
44. Zweers MM, Imholz AL, Struijk DG, Krediet RT: Correction of sodium sieving for diffusion from the circulation. Advances in Peritoneal Dialysis 15:65-72, 1999

45. Combet S, Van Landschoot M, Moulin P, Piech A, Verbavatz JM, Goffin E, Balligand JL, Lameire N, Devuyst O: Regulation of aquaporin-1 and nitric oxide synthase isoforms in a rat model of acute peritonitis. J.Am.Soc.Nephrol. 10:2185-2196, 1999
46. Goffin E, Combet S, Jamar F, Cosyns JP, Devuyst O: Expression of aquaporin-1 in a long-term peritoneal dialysis patient with impaired transcellular water transport. American Journal of Kidney Diseases [computer file] 33:383-388, 1999
47. Mactier RA, Khanna R, Twardowski Z, Moore H, Nolph KD: Contribution of lymphatic absorption to loss of ultrafiltration and solute clearances in continuous ambulatory peritoneal dialysis. Journal of Clinical Investigation 80:1311-1316, 1987
48. Flessner MF: Changes in the peritoneal interstitium and their effect on peritoneal transport. Perit.Dial.Int. 19 Suppl 2:S77-82.:S77-S82, 1999
49. Rosengren BI, Carlsson O, Rippe B: Hyaluronan and peritoneal ultrafiltration: a test of the "filter-cake" hypothesis. Am.J.Kidney Dis. 37:1277-1285, 2001
50. Fussholler A, zur NS, Grabensee B, Plum J: Peritoneal fluid and solute transport: influence of treatment time, peritoneal dialysis modality, and peritonitis incidence. J.Am.Soc.Nephrol. 13:1055-1060, 2002
51. Ota K, Mineshima M, Watanabe N, Naganuma S: Functional deterioration of the peritoneum: does it occur in the absence of peritonitis? Nephrol.Dial.Transplant 2:30-33, 1987
52. Margetts PJ, Kolb M, Yu L, Hoff CM, Gauldie J: A chronic inflammatory infusion model of peritoneal dialysis in rats. Perit.Dial.Int. 21 Suppl 3:S368-72.:S368-S372, 2001
53. Zweers MM, Splint LJ, Krediet RT, Struijk DG: Ultrastructure of basement membranes of peritoneal capillaries in a chronic peritoneal infusion model in the rat. Nephrol.Dial.Transplant 16:651-654, 2001
54. Davies SJ, Phillips L, Naish PF, Russell GI: Peritoneal glucose exposure and changes in membrane solute transport with time on peritoneal dialysis. Journal of the American Society of Nephrology 12:1046-1051, 2001
55. Mortier S, De Vriese AS, Van d, V, Schaub TP, Passlick-Deetjen J, Lameire NH: Hemodynamic effects of peritoneal dialysis solutions on the rat peritoneal membrane: role of acidity, buffer choice, glucose concentration, and glucose degradation products. J.Am.Soc.Nephrol. 13:480-489, 2002
56. Selgas R, Bajo MA, Castro MJ, del Peso G, Aguilera A, Fernandez-Perpen A, Cirugeda A, Sanchez-Tomero JA: Risk factors responsible for ultrafiltration failure in early stages of peritoneal dialysis. Peritoneal Dialysis International 20:631-636, 2000
57. Zweers MM, Struijk DG, Smit W, Krediet RT: Vascular endothelial growth factor in peritoneal dialysis: a longitudinal follow-up. J.Lab Clin.Med. 137:125-132, 2001
58. Devuyst O, Topley N, Williams JD: Morphological and functional changes in the dialysed peritoneal cavity: impact of more biocompatible solutions. Nephrol.Dial.Transplant. 17 Suppl 3:12-5.:12-15, 2002
59. Tauer A, Knerr T, Niwa T, Schaub TP, Lage C, Passlick-Deetjen J, Pischetsrieder M: In vitro formation of N(epsilon)-(carboxymethyl)lysine and imidazolones

under conditions similar to continuous ambulatory peritoneal dialysis. Biochem.Biophys.Res.Commun. 280:1408-1414, 2001

60. Witowski J, Wisniewska J, Korybalska K, Bender TO, Breborowicz A, Gahl GM, Frei U, Passlick-Deetjen J, Jorres A: Prolonged exposure to glucose degradation products impairs viability and function of human peritoneal mesothelial cells. J.Am.Soc.Nephrol. 12:2434-2441, 2001
61. Inagi R, Miyata T, Yamamoto T, Suzuki D, Urakami K, Saito A, van Ypersele dS, Kurokawa K: Glucose degradation product methylglyoxal enhances the production of vascular endothelial growth factor in peritoneal cells: role in the functional and morphological alterations of peritoneal membranes in peritoneal dialysis. FEBS Lett. 463:260-264, 1999
62. Musi B, Carlsson O, Rippe A, Wieslander A, Rippe B: Effects of acidity, glucose degradation products, and dialysis fluid buffer choice on peritoneal solute and fluid transport in rats. Perit.Dial.Int. 18:303-310, 1998
63. Devuyst O: New insights in the molecular mechanisms regulating peritoneal permeability. Nephrol.Dial.Transplant 17:548-551, 2002
64. Boulanger E, Wautier MP, Wautier JL, Boval B, Panis Y, Wernert N, Danze PM, Dequiedt P: AGEs bind to mesothelial cells via RAGE and stimulate VCAM-1 expression. Kidney Int. 61:148-156, 2002
65. Lamb EJ, Cattell WR, Dawnay AB: In vitro formation of advanced glycation end products in peritoneal dialysis fluid. Kidney International 47:1768-1774, 1995
66. Friedlander MA, Wu YC, Elgawish A, Monnier VM: Early and advanced glycosylation end products. Kinetics of formation and clearance in peritoneal dialysis. J.Clin.Invest 97:728-735, 1996
67. Ho-dac-Pannekeet MM, Weiss MF, de Waart DR, Erhard P, Hiralall JK, Krediet RT: Analysis of non enzymatic glycosylation in vivo: impact of different dialysis solutions. Peritoneal Dialysis International 19 Suppl ?:S68-S74, 1999
68. Nakamura S, Miyazaki S, Sakai S, Morita T, Hirasawa Y, Niwa T: Localization of imidazolone in the peritoneum of capd patients: a factor for a loss of ultrafiltration. Am.J.Kidney Dis. 38:S107-S110, 2001
69. Honda K, Nitta K, Horita S, Yumura W, Nihei H, Nagai R, Ikeda K, Horiuchi S: Accumulation of advanced glycation end products in the peritoneal vasculature of continuous ambulatory peritoneal dialysis patients with low ultra-filtration. Nephrol.Dial.Transplant. 14:1541-1549, 1999
70. Park MS, Lee HA, Chu WS, Yang DH, Hwang SD: Peritoneal accumulation of AGE and peritoneal membrane permeability. Peritoneal Dialysis International 20:452-460, 2000
71. Faller B, Lameire N: Evolution of clinical parameters and peritoneal function in a cohort of CAPD patients followed over 7 years. Nephrology Dialysis Transplantation 9:280-286, 1994
72. Ates K, Koc R, Nergizoglu G, Erturk S, Keven K, Sen A, Karatan O: The longitudinal effect of a single peritonitis episode on peritoneal membrane transport in CAPD patients. Perit.Dial.Int. 20:220-226, 2000
73. Wong TY, Szeto CC, Lai KB, Lam CW, Lai KN, Li PK: Longitudinal study of peritoneal membrane function in continuous ambulatory peritoneal dialysis: relationship with peritonitis and fibrosing factors. Perit.Dial.Int. 20:679-685, 2000

74. Selgas R, Munoz J, Cigarran S, Ramos P, Revuelta K, Escuin F, Miguel JL: Peritoneal functional parameters after five years on continuous ambulatory peritoneal dialysis (CAPD): the effect of late peritonitis. Peritoneal Dialysis International 9:329-332, 1989
75. Selgas R, Fernandez-Reyes MJ, Bosque E, Bajo MA, Borrego F, Jimenez C, del Peso G, de Alvaro F: Functional longevity of the human peritoneum: how long is continuous peritoneal dialysis possible? Results of a prospective medium long-term study. Am.J.Kidney Dis. 23:64-73, 1994
76. Andreoli SP, Leiser J, Warady BA, Schlichting L, Brewer ED, Watkins SL: Adverse effect of peritonitis on peritoneal membrane function in children on dialysis. Pediatric Nephrology 13:1-6, 1999
77. Topley N, Liberek T, Davenport A, Li FK, Fear H, Williams JD: Activation of inflammation and leukocyte recruitment into the peritoneal cavity. Kidney Int.Suppl 56:S17-21.:S17-S21, 1996
78. Topley N, Jorres A, Luttmann W, Petersen MM, Lang MJ, Thierauch KH, Muller C, Coles GA, Davies M, Williams JD: Human peritoneal mesothelial cells synthesize interleukin-6: induction by IL-1 beta and TNF alpha. Kidney Int. 43:226-233, 1993
79. Lai KN, Lai KB, Lam CW, Chan TM, Li FK, Leung JC: Changes of cytokine profiles during peritonitis in patients on continuous ambulatory peritoneal dialysis. Am.J.Kidney Dis. 35:644-652, 2000
80. Mlambo NC, Hylander B, Brauner A: Increased levels of transforming growth factor beta 1 and basic fibroblast growth factor in patients on CAPD: a study during non-infected steady state and peritonitis. Inflammation 23:131-139, 1999
81. Margetts PJ, Kolb M, Yu L, Hoff CM, Holmes CJ, Anthony DC, Gauldie J: Inflammatory cytokines, angiogenesis, and fibrosis in the rat peritoneum. Am.J.Pathol. 160:2285-2294, 2002
82. Yang WS, Kim BS, Lee SK, Park JS, Kim SB: Interleukin-1beta stimulates the production of extracellular matrix in cultured human peritoneal mesothelial cells. Perit.Dial.Int. 19:211-220, 1999
83. Mandl-Weber S, Haslinger B, Lederer SR, Sitter T: Heat-killed microorganisms induce PAI-1 expression in human peritoneal mesothelial cells: role of interleukin-1alpha. Am.J.Kidney Dis. 37:815-819, 2001
84. Yung S, Coles GA, Davies M: IL-1 beta, a major stimulator of hyaluronan synthesis in vitro of human peritoneal mesothelial cells: relevance to peritonitis in CAPD. Kidney Int. 50:1337-1343, 1996
85. Visser CE, Brouwer-Steenbergen JJ, Struijk G, Krediet RT, Beelen RH: Production of IL-1 beta and TNF-alpha by peritoneal macrophages depends on the bacterial species and the inoculum. Adv.Perit.Dial. 13:201-4.:201-204, 1997
86. Ben Av P, Crofford LJ, Wilder RL, Hla T: Induction of vascular endothelial growth factor expression in synovial fibroblasts by prostaglandin E and interleukin-1: a potential mechanism for inflammatory angiogenesis. FEBS Lett. 372:83-87, 1995
87. Li J, Perrella MA, Tsai JC, Yet SF, Hsieh CM, Yoshizumi M, Patterson C, Endege WO, Zhou F, Lee ME: Induction of vascular endothelial growth factor

gene expression by interleukin-1 beta in rat aortic smooth muscle cells. J.Biol.Chem. 270:308-312, 1995
88. Norrby K: Interleukin-1-alpha and de novo mammalian angiogenesis. Microvasc.Res. 54:58-64, 1997
89. Abramson SB, Amin A: Blocking the effects of IL-1 in rheumatoid arthritis protects bone and cartilage. Rheumatology.(Oxford) 41:972-980, 2002
90. Miyata T, van Ypersele dS, Kurokawa K, Baynes JW: Alterations in nonenzymatic biochemistry in uremia: origin and significance of "carbonyl stress" in long-term uremic complications. Kidney Int. 55:389-399, 1999
91. Combet S, Ferrier ML, Van Landschoot M, Stoenoiu M, Moulin P, Miyata T, Lameire N, Devuyst O: Chronic uremia induces permeability changes, increased nitric oxide synthase expression, and structural modifications in the peritoneum. J.Am.Soc.Nephrol. 12:2146-2157, 2001
92. Stenvinkel P, Heimburger O, Paultre F, Diczfalusy U, Wang T, Berglund L, Jogestrand T: Strong association between malnutrition, inflammation, and atherosclerosis in chronic renal failure. Kidney Int. 55:1899-1911, 1999
93. Wang T, Heimburger O, Cheng HH, Bergstrom J, Lindholm B: Does a high peritoneal transport rate reflect a state of chronic inflammation? Perit.Dial.Int. 19:17-22, 1999
94. Stenvinkel P, Chung SH, Heimburger O, Lindholm B: Malnutrition, inflammation, and atherosclerosis in peritoneal dialysis patients. Perit.Dial.Int. 21 Suppl 3:S157-S162, 2001
95. Chung SH, Heimburger O, Stenvinkel P, Bergstrom J, Lindholm B: Association between inflammation and changes in residual renal function and peritoneal transport rate during the first year of dialysis. Nephrol.Dial.Transplant 16:2240-2245, 2001
96. Margetts PJ, McMullin JP, Rabbat CG, Churchill DN: Peritoneal membrane transport and hypoalbuminemia: cause or effect? Perit.Dial.Int. 20:14-18, 2000
97. Ronco C, Feriani M, Chiaramonte S, Brendolan A, Bragantini L, Conz P, Dell'Aquila R, Milan M, La Greca G: Pathophysiology of ultrafiltration in peritoneal dialysis. [Review] [43 refs]. Peritoneal Dialysis International 10:119-126, 1990
98. Bazzato G, Coli U, Landini S, Lucatello S, Fracasso A, Righetto F, Scanferla F, Morachiello P: Restoration of ultrafiltration capacity of peritoneal membrane in patients on CAPD. International Journal of Artificial Organs 7:93-96, 1984
99. Mujais S, Nolph K, Gokal R, Blake P, Burkart J, Coles G, Kawaguchi Y, Kawanishi H, Korbet S, Krediet R, Lindholm B, Oreopoulos D, Rippe B, Selgas R: Evaluation and management of ultrafiltration problems in peritoneal dialysis. International Society for Peritoneal Dialysis Ad Hoc Committee on Ultrafiltration Management in Peritoneal Dialysis. Perit.Dial.Int. 20 Suppl 4:S5-21, 2000
100. Gokal R, Mistry CD, Peers EM: Peritonitis occurrence in a multicenter study of icodextrin and glucose in CAPD. MIDAS Study Group. Multicenter Investigation of Icodextrin in Ambulatory Dialysis. Perit.Dial.Int. 15:226-230, 1995
101. Mistry CD, Gokal R, Peers E: A randomized multicenter clinical trial comparing isosmolar icodextrin with hyperosmolar glucose solutions in CAPD. MIDAS

Study Group. Multicenter Investigation of Icodextrin in Ambulatory Peritoneal Dialysis. Kidney Int. 46:496-503, 1994

102. Posthuma N, ter Wee PM, Verbrugh HA, Oe PL, Peers E, Sayers J, Donker AJ: Icodextrin instead of glucose during the daytime dwell in CCPD increases ultrafiltration and 24-h dialysate creatinine clearance. Nephrology Dialysis Transplantation 12:550-553, 1997
103. Johnson DW, Arndt M, O'Shea A, Watt R, Hamilton J, Vincent K: Icodextrin as salvage therapy in peritoneal dialysis patients with refractory fluid overload. BMC.Nephrol. 2:2, 2001
104. Jones S, Holmes CJ, Mackenzie RK, Stead R, Coles GA, Williams JD, Faict D, Topley N: Continuous dialysis with bicarbonate/lactate-buffered peritoneal dialysis fluids results in a long-term improvement in ex vivo peritoneal macrophage function. Journal of the American Society of Nephrology 13 Suppl 1:S97-103, 2002
105. Park MS, Kim JK, Holmes C, Weiss MF: Effects of bicarbonate/lactate solution on peritoneal advanced glycosylation end-product accumulation. Perit.Dial.Int. 20 Suppl 5:S33-S38, 2000
106. Tranaeus A: A long-term study of a bicarbonate/lactate-based peritoneal dialysis solution--clinical benefits. The Bicarbonate/Lactate Study Group. Peritoneal Dialysis International 20:516-523, 2000
107. Rippe B, Simonsen O, Heimburger O, Christensson A, Haraldsson B, Stelin G, Weiss L, Nielsen FD, Bro S, Friedberg M, Wieslander A: Long-term clinical effects of a peritoneal dialysis fluid with less glucose degradation products. Kidney International 59:348-357, 2001
108. Jones S, Holmes CJ, Krediet RT, Mackenzie R, Faict D, Tranaeus A, Williams JD, Coles GA, Topley N, Bicarbonate/Lactate Study Group.: Bicarbonate/lactate-based peritoneal dialysis solution increases cancer antigen 125 and decreases hyaluronic acid levels. Kidney International 59:1529-1538, 2001
109. Breborowicz A, Polubinska A, Moberly J, Ogle K, Martis L, Oreopoulos D: Hyaluronan modifies inflammatory response and peritoneal permeability during peritonitis in rats. American Journal of Kidney Diseases [computer file] 37:594-600, 2001
110. Guo QY, Peng WX, Cheng HH, Ye RG, Lindholm B, Wang T: Hyaluronan preserves peritoneal membrane transport properties. Perit.Dial.Int. 21:136-142, 2001
111. Chan H, Abraham G, Oreopoulos DG: Oral lecithin improves ultrafiltration in patients on peritoneal dialysis. [see comments.]. Peritoneal Dialysis International 9:203-205, 1989
112. Krack G, Viglino G, Cavalli PL, Gandolfo C, Magliano G, Cantaluppi A, Peluso F: Intraperitoneal administration of phosphatidylcholine improves ultrafiltration in continuous ambulatory peritoneal dialysis patients. [see comments.]. Peritoneal Dialysis International 12:359-364, 1992
113. Margetts PJ, Kolb M, Galt T, Hoff CM, Shockley TR, Gauldie J: Gene transfer of transforming growth factor-beta1 to the rat peritoneum: effects on membrane function. J.Am.Soc.Nephrol. 12:2029-2039, 2001

114. Bett AJ, Haddara W, Prevec L, Graham FL: An efficient and flexible system for construction of adenovirus vectors with insertions or deletions in early regions 1 and 3. Proc.Natl.Acad.Sci.U.S.A 91:8802-8806, 1994
115. Kolb M, Inman M, Margetts PJ, Galt T, Gauldie J: Budesonide enhances repeated gene transfer and expression in the lung with adenoviral vectors. Am.J.Respir.Crit Care Med. 164:866-872, 2001
116. Hoff CM: Ex vivo and in vivo gene transfer to the peritoneal membrane in a rat model. Nephrol.Dial.Transplant. 16:666-668, 2001
117. Belo AV, Ferreira MA, Bosco AA, Machado RD, Andrade SP: Differential effects of thalidomide on angiogenesis and tumor growth in mice. Inflammation 25:91-96, 2001
118. Chiang SC, Cheng CH, Moulton KS, Kasznica JM, Moulton SL: TNP-470 inhibits intraabdominal adhesion formation. J.Pediatr.Surg. 35:189-196, 2000
119. Lin B, Podar K, Gupta D, Tai YT, Li S, Weller E, Hideshima T, Lentzsch S, Davies F, Li C, Weisberg E, Schlossman RL, Richardson PG, Griffin JD, Wood J, Munshi NC, Anderson KC: The vascular endothelial growth factor receptor tyrosine kinase inhibitor PTK787/ZK222584 inhibits growth and migration of multiple myeloma cells in the bone marrow microenvironment. Cancer Res. 62:5019-5026, 2002
120. Mutsaers SE, Bishop JE, McGrouther G, Laurent GJ: Mechanisms of tissue repair: from wound healing to fibrosis. Int.J.Biochem.Cell Biol. 29:5-17, 1997
121. el Nahas AM, Muchaneta-Kubara EC, Essawy M, Soylemezoglu O: Renal fibrosis: insights into pathogenesis and treatment. Int.J.Biochem.Cell Biol. 29:55-62, 1997
122. Sime PJ, O'Reilly KM: Fibrosis of the lung and other tissues: new concepts in pathogenesis and treatment. Clin.Immunol. 99:308-319, 2001
123. Border WA, Noble NA: Transforming growth factor beta in tissue fibrosis. N.Engl.J.Med. 331:1286-1292, 1994
124. Ha H, Yu MR, Lee HB: High glucose-induced PKC activation mediates TGF-beta 1 and fibronectin synthesis by peritoneal mesothelial cells. Kidney Int. 59:463-470, 2001
125. Wang T, Ghen YG, Ye RG, Mai WY, Zhen ZH, Li HQ: Enhanced expression of TGF-beta 1 by peritoneal macrophages in CAPD patients. Advances in Peritoneal Dialysis 11:11-14, 1995
126. Kang DH, Hong YS, Lim HJ, Choi JH, Han DS, Yoon KI: High glucose solution and spent dialysate stimulate the synthesis of transforming growth factor-beta1 of human peritoneal mesothelial cells: effect of cytokine costimulation. Perit.Dial.Int. 19:221-230, 1999
127. Moussad EE, Brigstock DR: Connective tissue growth factor: what's in a name? Mol.Genet.Metab 71:276-292, 2000
128. Rougier JP, Guia S, Hagege J, Nguyen G, Ronco PM: PAI-1 secretion and matrix deposition in human peritoneal mesothelial cell cultures: transcriptional regulation by TGF-beta 1. Kidney Int. 54:87-98, 1998
129. Tomasek JJ, Gabbiani G, Hinz B, Chaponnier C, Brown RA: Myofibroblasts and mechano-regulation of connective tissue remodelling. Nat.Rev.Mol.Cell Biol. 3:349-363, 2002

130. Iglesias-De La Cruz MC, Ziyadeh FN, Isono M, Kouahou M, Han DC, Kalluri R, Mundel P, Chen S: Effects of high glucose and TGF-beta1 on the expression of collagen IV and vascular endothelial growth factor in mouse podocytes. Kidney Int. 62:901-913, 2002
131. Breier G, Blum S, Peli J, Groot M, Wild C, Risau W, Reichmann E: Transforming growth factor-beta and Ras regulate the VEGF/VEGF-receptor system during tumor angiogenesis. Int.J.Cancer 97:142-148, 2002
132. Gajdusek CM, Luo Z, Mayberg MR: Basic fibroblast growth factor and transforming growth factor beta-1: synergistic mediators of angiogenesis in vitro. J.Cell Physiol 157:133-144, 1993
133. Pepper MS: Transforming growth factor-beta: vasculogenesis, angiogenesis, and vessel wall integrity. Cytokine Growth Factor Rev. 8:21-43, 1997
134. Dennler S, Goumans MJ, ten Dijke P: Transforming growth factor beta signal transduction. J.Leukoc.Biol. 71:731-740, 2002
135. Moustakas A, Pardali K, Gaal A, Heldin CH: Mechanisms of TGF-beta signaling in regulation of cell growth and differentiation. Immunol.Lett. 82:85-91, 2002
136. Gorelik L, Flavell RA: Transforming growth factor-beta in T-cell biology. Nat.Rev.Immunol. 2:46-53, 2002
137. Li DY, Sorensen LK, Brooke BS, Urness LD, Davis EC, Taylor DG, Boak BB, Wendel DP: Defective angiogenesis in mice lacking endoglin. Science 284:1534-1537, 1999
138. Attisano L, Wrana JL: Signal transduction by the TGF-beta superfamily. Science 296:1646-1647, 2002
139. Kolb M, Margetts PJ, Sime PJ, Gauldie J: Proteoglycans decorin and biglycan differentially modulate TGF-beta-mediated fibrotic responses in the lung. Am.J.Physiol Lung Cell Mol.Physiol 280:L1327-L1334, 2001
140. Abreu JG, Ketpura NI, Reversade B, De Robertis EM: Connective-tissue growth factor (CTGF) modulates cell signalling by BMP and TGF-beta. Nat.Cell Biol. 4:599-604, 2002
141. Mateijsen MA, van der Wal AC, Hendriks PM, Zweers MM, Mulder J, Struijk DG, Krediet RT: Vascular and interstitial changes in the peritoneum of CAPD patients with peritoneal sclerosis. Perit.Dial.Int. 19:517-525, 1999
142. Papetti M, Herman IM: Mechanisms of normal and tumor-derived angiogenesis. Am.J.Physiol Cell Physiol 282:C947-C970, 2002
143. Kalluri R, Sukhatme VP: Fibrosis and angiogenesis. Curr.Opin.Nephrol.Hypertens. 9:413-418, 2000
144. Carmeliet P: Mechanisms of angiogenesis and arteriogenesis. Nat.Med. 6:389-395, 2000
145. Thurston G, Rudge JS, Ioffe E, Zhou H, Ross L, Croll SD, Glazer N, Holash J, McDonald DM, Yancopoulos GD: Angiopoietin-1 protects the adult vasculature against plasma leakage. Nat.Med. 6:460-463, 2000
146. Carmeliet P, Moons L, Luttun A, Vincenti V, Compernolle V, De Mol M, Wu Y, Bono F, Devy L, Beck H, Scholz D, Acker T, DiPalma T, Dewerchin M, Noel A, Stalmans I, Barra A, Blacher S, Vandendriessche T, Ponten A, Eriksson U, Plate KH, Foidart JM, Schaper W, Charnock-Jones DS, Hicklin DJ, Herbert JM, Collen D, Persico MG: Synergism between vascular endothelial growth factor and

placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. Nat.Med. 7:575-583, 2001
147. Ferrier ML, Combet S, Van Landschoot M, Stoenoiu MS, Cnops Y, Lameire N, Devuyst O: Inhibition of nitric oxide synthase reverses changes in peritoneal permeability in a rat model of acute peritonitis. Kidney Int. 60:2343-2350, 2001
148. Peng H, Cheung AK, Reimer LG, Kamerath CD, Leypoldt JK: Effect of indomethacin on peritoneal protein loss in a rabbit model of peritonitis. Kidney Int. 59:44-51, 2001
149. Liu W, Ahmad SA, Reinmuth N, Shaheen RM, Jung YD, Fan F, Ellis LM: Endothelial cell survival and apoptosis in the tumor vasculature. Apoptosis. 5:323-328, 2000
150. Lindahl P, Johansson BR, Leveen P, Betsholtz C: Pericyte loss and microaneurysm formation in PDGF-B-deficient mice. Science 277:242-245, 1997
151. Papapetropoulos A, Garcia-Cardena G, Dengler TJ, Maisonpierre PC, Yancopoulos GD, Sessa WC: Direct actions of angiopoietin-1 on human endothelium: evidence for network stabilization, cell survival, and interaction with other angiogenic growth factors. Lab Invest 79:213-223, 1999
152. Ferrara N: Role of vascular endothelial growth factor in the regulation of angiogenesis. Kidney Int. 56:794-814, 1999
153. Neufeld G, Cohen T, Gengrinovitch S, Poltorak Z: Vascular endothelial growth factor (VEGF) and its receptors. FASEB J. 13:9-22, 1999
154. Inoki I, Shiomi T, Hashimoto G, Enomoto H, Nakamura H, Makino K, Ikeda E, Takata S, Kobayashi K, Okada Y: Connective tissue growth factor binds vascular endothelial growth factor (VEGF) and inhibits VEGF-induced angiogenesis. FASEB J. 16:219-221, 2002
155. Pepper MS, Ferrara N, Orci L, Montesano R: Potent synergism between vascular endothelial growth factor and basic fibroblast growth factor in the induction of angiogenesis in vitro. Biochem.Biophys.Res.Commun. 189:824-831, 1992
156. Holash J, Wiegand SJ, Yancopoulos GD: New model of tumor angiogenesis: dynamic balance between vessel regression and growth mediated by angiopoietins and VEGF. Oncogene %20;18:5356-5362, 1999
157. Yancopoulos GD, Davis S, Gale NW, Rudge JS, Wiegand SJ, Holash J: Vascular-specific growth factors and blood vessel formation. Nature 407:242-248, 2000
158. Sundberg C, Kowanetz M, Brown LF, Detmar M, Dvorak HF: Stable expression of angiopoietin-1 and other markers by cultured pericytes: phenotypic similarities to a subpopulation of cells in maturing vessels during later stages of angiogenesis in vivo. Lab Invest 82:387-401, 2002
159. Margetts PJ, Kolb M, Hoff CM, Gauldie J: The role of angiopoietins in resolution of angiogenesis resulting from adenovirual mediated gene transfer of TGFβ1 or VEGF to the rat peritoneum [Abstract]. J.Am.Soc.Nephrol. 12:433A, 2001
160. Scott BB, Zaratin PF, Colombo A, Hansbury MJ, Winkler JD, Jackson JR: Constitutive expression of angiopoietin-1 and -2 and modulation of their expression by inflammatory cytokines in rheumatoid arthritis synovial fibroblasts. J.Rheumatol. 29:230-239, 2002

161. Mandriota SJ, Pepper MS: Regulation of angiopoietin-2 mRNA levels in bovine microvascular endothelial cells by cytokines and hypoxia. Circ.Res. 83:852-859, 1998
162. Fujikawa K, de AS, I, Jain SK, Presman E, Christensen RA, Varticovski L: Role of PI 3-kinase in angiopoietin-1-mediated migration and attachment-dependent survival of endothelial cells. Exp.Cell Res. 253:663-672, 1999
163. Firestein GS: Starving the synovium: angiogenesis and inflammation in rheumatoid arthritis. J.Clin.Invest 103:3-4, 1999
164. Keane MP, Belperio JA, Arenberg DA, Burdick MD, Xu ZJ, Xue YY, Strieter RM: IFN-gamma-inducible protein-10 attenuates bleomycin-induced pulmonary fibrosis via inhibition of angiogenesis. J.Immunol. 163:5686-5692, 1999
165. Fukumura D, Gohongi T, Kadambi A, Izumi Y, Ang J, Yun CO, Buerk DG, Huang PL, Jain RK: Predominant role of endothelial nitric oxide synthase in vascular endothelial growth factor-induced angiogenesis and vascular permeability. Proc.Natl.Acad.Sci.U.S.A 98:2604-2609, 2001
166. Kolb M, Margetts PJ, Anthony DC, Pitossi F, Gauldie J: Transient expression of IL-1beta induces acute lung injury and chronic repair leading to pulmonary fibrosis. J.Clin.Invest 107:1529-1536, 2001
167. Krane SM, Goldring MB: Potential role for interleukin-1 in fibrosis associated with chronic ambulatory peritoneal dialysis. [Review] [25 refs]. Blood Purification 6:173-177, 1988
168. Sanchez-Elsner T, Botella LM, Velasco B, Corbi A, Attisano L, Bernabeu C: Synergistic cooperation between hypoxia and transforming growth factor-beta pathways on human vascular endothelial growth factor gene expression. J.Biol.Chem. 276:38527-38535, 2001
169. Pertovaara L, Kaipainen A, Mustonen T, Orpana A, Ferrara N, Saksela O, Alitalo K: Vascular endothelial growth factor is induced in response to transforming growth factor-beta in fibroblastic and epithelial cells. J.Biol.Chem. 269:6271-6274, 1994
170. Margetts PJ, Kolb M, Hoff CM, Gauldie J: Different fibrogenic and angiogenic responses to transient overexpression of TNFα or IL-1β by adenoviral mediated gene transfer to the rat peritoneum [Abstract]. J.Am.Soc.Nephrol. 12:433A, 2001
171. Daniel TO, Abrahamson D: Endothelial signal integration in vascular assembly. Annu.Rev.Physiol 62:649-671, 2000
172. Oh SP, Seki T, Goss KA, Imamura T, Yi Y, Donahoe PK, Li L, Miyazono K, ten Dijke P, Kim S, Li E: Activin receptor-like kinase 1 modulates transforming growth factor-beta 1 signaling in the regulation of angiogenesis. Proc.Natl.Acad.Sci.U.S.A 97:2626-2631, 2000
173. Folkman J, D'Amore PA: Blood vessel formation: what is its molecular basis? Cell 87:1153-1155, 1996
174. Dickson MC, Martin JS, Cousins FM, Kulkarni AB, Karlsson S, Akhurst RJ: Defective haematopoiesis and vasculogenesis in transforming growth factor-beta 1 knock out mice. Development 121:1845-1854, 1995
175. Corpechot C, Barbu V, Wendum D, Kinnman N, Rey C, Poupon R, Housset C, Rosmorduc O: Hypoxia-induced VEGF and collagen I expressions are associated

with angiogenesis and fibrogenesis in experimental cirrhosis. Hepatology 35:1010-1021, 2002
176. Visconti RP, Richardson CD, Sato TN: Orchestration of angiogenesis and arteriovenous contribution by angiopoietins and vascular endothelial growth factor (VEGF). Proc.Natl.Acad.Sci.U.S.A 99:8219-8224, 2002
177. Lameire N, Van Biesen W, Van Landschoot M, Wang T, Heimburger O, Bergstrom J, Lindholm B, Hekking LP, Havenith CE, Beelen RH: Experimental models in peritoneal dialysis: a European experience. Kidney Int. 54:2194-2206, 1998
178. Pawlaczyk K, Kuzlan-Pawlaczyk M, Anderstam B, Heimburger O, Bergstrom J, Waniewski J, Breborowicz A, Lindholm B: Effects of intraperitoneal heparin on peritoneal transport in a chronic animal model of peritoneal dialysis. Nephrology Dialysis Transplantation 16:669-671, 2001
179. Breborowicz A, Wieczorowska-Tobis K, Korybalska K, Polubinska A, Radkowski M, Oreopoulos DG: The effect of a nitric oxide inhibitor (L-NAME) on peritoneal transport during dialysis in rats. Peritoneal Dialysis International 18:188-192, 1998
180. Zweers M, Splint J, Krediet T, Struijk G: Ultrastructure of basement membranes of peritoneal capillaries in a chronic peritoneal infusion model in the rat. Nephrol.Dial.Transplant. 16:651-654, 2001
181. Wieczorowska-Tobis K, Polubinska A, Schaub TP, Schilling H, Wisniewska J, Witowski J, Passlick-Deetjen J, Breborowicz A: Influence of neutral-pH dialysis solutions on the peritoneal membrane: a long-term investigation in rats. Perit.Dial.Int. 21 Suppl 3:S108-13.:S108-S113, 2001
182. Plum J, Schoenicke G, Kleophas W, Kulas W, Steffens F, Azem A, Grabensee B: Comparison of body fluid distribution between chronic haemodialysis and peritoneal dialysis patients as assessed by biophysical and biochemical methods. Nephrol.Dial.Transplant 16:2378-2385, 2001
183. Churchill DN, Thorpe KE, Keshaviah PR: Adequacy of dialysis and nutrition in continuous peritoneal dialysis: association with clinical outcomes. Canada-USA (CANUSA) Peritoneal Dialysis Study Group. J.Am.Soc.Nephrol. 7:198-207, 1996
184. Paniagua R, Amato D, Vonesh E, Correa-Rotter R, Ramos A, Moran J, Mujais S: Effects of Increased Peritoneal Clearances on Mortality Rates in Peritoneal Dialysis: ADEMEX, a Prospective, Randomized, Controlled Trial. J.Am.Soc.Nephrol. 13:1307-1320, 2002
185. Churchill DN: The ADEMEX Study: Make Haste Slowly. J.Am.Soc.Nephrol. 13:1415-1418, 2002
186. Coles GA: Have we underestimated the importance of fluid balance for the survival of PD patients? Perit.Dial.Int. 17:321-326, 1997
187. O'Reilly MS, Holmgren L, Shing Y, Chen C, Rosenthal RA, Moses M, Lane WS, Cao Y, Sage EH, Folkman J: Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. Cell 79:315-328, 1994

188. Bloch W, Huggel K, Sasaki T, Grose R, Bugnon P, Addicks K, Timpl R, Werner S: The angiogenesis inhibitor endostatin impairs blood vessel maturation during wound healing. FASEB J. 14:2373-2376, 2000
189. Steinhauer HB, Schollmeyer P: Prostaglandin-mediated loss of proteins during peritonitis in continuous ambulatory peritoneal dialysis. Kidney Int. 29:584-590, 1986
190. Gary Lee YC, Melkerneker D, Thompson PJ, Light RW, Lane KB: Transforming growth factor beta induces vascular endothelial growth factor elaboration from pleural mesothelial cells in vivo and in vitro. Am.J.Respir.Crit Care Med. 165:88-94, 2002
191. Kolb M, Bonniaud P, Galt T, Sime PJ, Kelly M.K, Margetts PJ, Gauldie J: Differences in the fibrogenic response after transfer of active TGFbeta1 gene to lungs of a 'fibrosis-prone' and 'resistant' mouse strain. Am.J.Respir.Cell Mol.Physiol 27:141-150, 2002
192. Gambaro G, Weigert C, Ceol M, Schleicher ED: Inhibition of transforming growth factor-beta 1 gene overexpression as a strategy to prevent fibrosis. Contrib.Nephrol.107-113, 2001
193. Breborowicz A, Polubinska A, Pawlaczyk K, Kuzlan-Pawlaczyk M, Moberly J, Martis L, Oreopoulos D: Intraperitoneal hyaluronan administration in conscious rats: absorption, metabolism, and effects on peritoneal fluid dynamics. Perit.Dial.Int. 21:130-135, 2001
194. Kim I, Kim HG, So JN, Kim JH, Kwak HJ, Koh GY: Angiopoietin-1 regulates endothelial cell survival through the phosphatidylinositol 3'-Kinase/Akt signal transduction pathway. Circ.Res. 86:24-29, 2000
195. Hasegawa Y, Takanashi S, Kanehira Y, Tsushima T, Imai T, Okumura K: Transforming growth factor-beta1 level correlates with angiogenesis, tumor progression, and prognosis in patients with nonsmall cell lung carcinoma. Cancer 91:964-971, 2001
196. Hawighorst T, Skobe M, Streit M, Hong YK, Velasco P, Brown LF, Riccardi L, Lange-Asschenfeldt B, Detmar M: Activation of the tie2 receptor by angiopoietin-1 enhances tumor vessel maturation and impairs squamous cell carcinoma growth. Am.J.Pathol. 160:1381-1392, 2002
197. Rosengart TK, Lee LY, Patel SR, Sanborn TA, Parikh M, Bergman GW, Hachamovitch R, Szulc M, Kligfield PD, Okin PM, Hahn RT, Devereux RB, Post MR, Hackett NR, Foster T, Grasso TM, Lesser ML, Isom OW, Crystal RG: Angiogenesis gene therapy: phase I assessment of direct intramyocardial administration of an adenovirus vector expressing VEGF121 cDNA to individuals with clinically significant severe coronary artery disease. Circulation 100:468-474, 1999