THE ROLE OF MESOTHELIAL CELL BIOLOGY

IN PERITONEAL FIBROSIS ON CAPD

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Abstract

THE ROLE OF MESOTHELIAL CELL BIOLOGY

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Worldwide 100,000 people are dependant on peritoneal dialysis (PD) for treatment of end-stage renal failure. Long term technique survival is limited by peritoneal membrane fibrosis and loss of membrane function. The human peritoneal mesothelial cell (HPMC) is the one of the most abundant cells in the peritoneal cavity, and is in direct contact with the peritoneal dialysate. The aim of these experiments was to investigate the role of the HPMC in the regulation of peritoneal fibrosis in the context of peritoneal dialysis.

Hyperosmolar glucose lactate-buffered dialysate is the most widely used dialysis solution. A culture system was developed to grow HPMC from uraemic patients undergoing PD catheter insertion. The effect exposure to a 50:50 mixture of dialysate and M199 for 12 hours was examined. Glucose was varied between 5-40mmol/L. Increases in glucose concentration caused a decrease in cell viability, a decrease in proliferation, and increase in fibronectin mRNA and protein amount.

The mechanism responsible for glucose induced increase in fibronectin was examined. Increasing glucose caused an increase in HPMC TGF-β protein amount. Exogenous TGF-β caused a dose dependant increase in HPMC fibronectin production, and increase mRNA for fibronectin and TGF-β itself. An anti TGF-β antibody prevented glucose induced HPMC fibronectin production.

Two alternative dialysis solutions were investigated; a different osmotic agent (amino acid dialysate), and a different dialysate buffer (bicarbonate). Amino acid dialysate showed less cytotoxicity, but inhibited proliferation, and caused TGF-β mediated fibronectin production. Although amino acid dialysate contains 3.3 mmol/L L-arginine, NO was not shown to mediate this response. Dialysate with bicarbonate:lactate buffer allowed greater HPMC proliferation, and no inhibition of proliferation with hyperosmolar glucose previously seen with lactate buffered dialysate.

These studies suggest the HPMC has a role in the production and regulation of ECM, and that TGF-β is an important intermediary.

Publications arising from this work

Manuscripts

Medcalf JF., Harris K., Walls J.
Preserving the peritoneum in CAPD. *Nephrol Dial Trans 1997;12:393-395*

Medcalf JF, Harris KPG, Walls J.
Long-term preservation of peritoneal membrane function in patients treated with CAPD.
Séminaires D'uro-néphrologie, Pitie-Salpetriere. Ed C.Chatelain & C. Jacobs. Paris (1998)

Medcalf JF, Pawluczyk IZA, Walls J, Harris KPG.
Effects of glucose dialysate on extra-cellular matrix production by human peritoneal mesothelial cells (HPMC) – the role of TGF-β.
Nephrology, Dialysis, Transplantation 2001 (In Press)

Oral presentations

Medcalf JF, Walls J, Harris KPG.
Effects of glucose, amino acid dialysate and icodextrin on fibronectin, TGF-β and cell viability of human peritoneal mesothelial cells.
Renal Association Autumn meeting, London, September 1997.

Medcalf JF, Walls J, Harris KPG.
The role of mesothelial cell extracellular matrix protein in peritoneal fibrosis on CAPD
Leicester Astra research prize, Leicester, March 1998 (2nd prize).

Medcalf JF, Walls J, Harris KPG.
Glucose dialysate effects on matrix production by human peritoneal mesothelial cells (HPMC) – the role of TGF-β.
Renal Association Spring Meeting, Bristol, March 1998.

Medcalf JF, Walls J, Harris KPG.
Increase in Fibronectin release by human peritoneal mesothelial cells (HPMC) is mediated through transforming growth-factor beta (TGF-β)
3rd European Peritoneal dialysis meeting, Edinburgh, April 1998.

Medcalf JF, Walls J, Harris KPG.
Glucose dialysate effects on matrix production by human peritoneal mesothelial cells (HPMC) – the role of TGF-β
8th Congress of the International Society of Peritoneal Dialysis, Seoul, Korea, 23-26th August 1998

Medcalf JF, Al-Jayyousi R, Walls J, Harris KPG
Effect of bicarbonate buffered peritoneal dialysate on human peritoneal mesothelial cell (HPMC) proliferation and extracellular matrix (ECM) production.
American Society of Nephrology, Toronto, Canada 13-16th October 2000

Poster presentations

Medcalf JF, Walls J, Harris KPG.
Effects of glucose, amino acid dialysate and icodextrin on fibronectin, TGF-β and cell viability of human peritoneal mesothelial cells.
American Society of Nephrology, San Antonio, November 1997.

Medcalf JF, Al-Jayyousi RH, Harris KPG.
Extra-cellular Matrix Production by Human Peritoneal Mesothelial Cell (HPMC) in Hyperosmolar Glucose Dialysate is Mediated via Aldose-reductase Pathway.
American Society of Nephrology, San Francisco, October 2001.

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Table of Contents

Index of figures and tables

Figures

Tables

Abbreviations

AGE	Advanced glycation end-products
ANOVA	Analysis of variance
APD	Automated peritoneal dialysis
BSA	Bovine serum albumin
CA125	Cancer antigen-125
CAPD	Continuous ambulatory peritoneal dialysis
CRF	Chronic renal failure
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
DPM	Disintegration per minute
ECM	Extra-cellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ESRF	End-stage renal failure
FBS	Foetal bovine serum
HBSS	Hanks balanced salt solution
HCl	Hydrochloric Acid
HD	Haemodialysis
HPMC	Human peritoneal mesothelial cell
HPTC	Human proximal tubular cells
HRP	Horse radish peroxidase
ICAM	Inter cellular adhesion molecule
IL	Interleukin
ITS	Insulin-transferrin-selanite
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
MOPS	3-(N-morpholino)pro-panesulfonic acid
MW	Molecular weight
NAD	Nicotinamide adenosine dinucleotide
NADH	Nicotinamide adenosine dinucleotide (reduced)
NaOH	Sodium Hydroxide
NIPD	Nocturnal intermittent peritoneal dialysis
NO	Nitric Oxide
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PD	Peritoneal dialysis
PDGF	Platelet derived growth factor
PMN	Polymorphonuclear cell
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SSC	Saline-sodium citrate
SSPE	Saline-sodium phosphate ethylenediaminetetra-acetic acid

TAE	Tris-acetic acid-EDTA
TBS	Tris buffered saline
TBST	Tris buffered saline - Tween
TGF-β	Transforming growth factor beta
TIMP	Tissue inhibitor of metalloproteinase
VCAM	Vascular cell adhesion molecule
WB	Wash buffer

1 Introduction

1.1 Description of CAPD

1.1.1 The development of CAPD

Peritoneal Dialysis (PD) is a technique by which patients with advanced chronic renal failure (CRF) use their peritoneal cavity to provide dialysis. Experiments to investigate the physiology of the lining of the peritoneal cavity, the peritoneum, began around the turn of the 20th Century when experiments in animals outlined basic physiological principles. It was in 1884 for example, that Starling and Tubby demonstrated that hypertonic intraperitoneal solutions increased, whilst hypotonic solutions decreased in volume with time in the peritoneal cavity [1]. It was not until the 1960s however, that doctors applied this knowledge to chronic peritoneal dialysis in patients with renal failure. Tenckhoff, one of the pioneers in early clinical trials described a year of successful peritoneal dialysis in one patient using a repeated abdominal puncture technique and an automated cycling machine to reduce the number of punctures [2]. However, it was only with the introduction of a safe, permanent indwelling catheter that the technique began to gain acceptance [3]. Unfortunately with intermittent daily peritoneal dialysis the technique was still limited by poor dialysis adequacy, and recurrent peritonitis.

The issue of inadequate dialysis dose was addressed in 1976, when continuous ambulatory peritoneal dialysis (CAPD) was first described by Polovitch and Moncrief in an abstract to the American Society of Artificial Organs meeting [4]. This was followed by the first clinical trial of its use in 1978 with nine patients using the technique for a period ranging between 5 to 26 weeks [5]. Since then the technique has expanded widely and currently over 100,000 patients world-wide use the technique of CAPD as a treatment for end-stage renal failure (ESRF). There is good reason for this popularity, and the technique offers

several advantages over longer established haemodialysis. Patients enjoy greater freedom from hospital and less strict dietary restrictions. In addition CAPD is thought to offer good steady state biochemical control, and potentially less cardiovascular stress than intermittent haemodialysis.

There have been considerable improvements in CAPD technique over the twenty years since 1978. Dialysis solutions were initially manufactured in glass bottles, and the technique was bedevilled by problems with peritonitis because of the difficulty keeping the solution sterile. The rate of peritonitis improved dramatically with the change to PVC bags for the dialysate, and with improvements in the connections between the bag and the patient.

Despite these advances in CAPD technique, the dialysate itself has changed very little. The standard dialysate in clinical practice remains a hypertonic glucose solution with a high lactate concentration. The solution is hypertonic in relation to uraemic plasma (344 vs 310 mOsmol/kg) to remove water from a potentially anuric dialysis patient by osmosis. The lactate is included in high concentration as a buffer. It is absorbed across the semi-permeable peritoneal membrane and is metabolised to bicarbonate in the liver of the CAPD patient. As will be discussed later this combination has theoretical and practical disadvantages, particularly to the cells lining the peritoneal cavity.

1.1.2 CAPD technique

Peritoneal dialysis (PD) has evolved from the original description of continuous ambulatory peritoneal dialysis to include a wide variety of different methods of draining dialysate in and out of the peritoneal cavity. Throughout this thesis the term CAPD will be considered synonymous with all other forms of PD. Although some authors suggest that automated PD (APD/NIPD) offer lower rates of peritonitis [6], no studies have

considered differences in the effect on the peritoneal membrane. CAPD involves a patient draining 1.5 to 3 litres of dialysis solution into their peritoneal cavity through a permanent indwelling silicone catheter. This is allowed to dwell in the peritoneal cavity for 4 to 6 hours, and then drained out and replaced with fresh dialysis solution. The majority of patients perform 4 exchanges per day, although variations in patient body size and degree of residual renal function may necessitate changes in fill volume or the number of dialysis exchanges per day. To produce dialysis the technique depends on the semi-permeable membrane of cells lining the peritoneal cavity. This facilitates diffusion, and also active transport of molecules between patient and dialysate. "Uraemic toxins" transfer from the patient to the dialysate by a combination of diffusion (predominantly low molecular weight molecules) and by convection with water (larger molecular weight molecules). Water shifts from the patient to the dialysate by osmosis from the low osmolality of the patient serum to the hyperosmolar dialysis solution.

1.1.3 Patient and technique survival

Patient survival on CAPD and haemodialysis (HD) is similar [7-11], although comparison is often complicated by different mix of patients on each type of dialysis. Considerable discrepancy exists however between the technique survival on the two methods of dialysis, with technique survival consistently worse on CAPD than HD [9-12]. Figure 1.1 shows the actuarial technique survival amongst a cohort of 60 patients who started on CAPD in Leicester during 1996. Loss of PD technique was defined as a necessary change away from PD, either to haemodialysis or transplantation. These results are similar to published data where technique survival is consistently estimated to be only 50-70% at five years [9,13]. The cause of this high technique loss is multifactorial, but loss of peritoneal membrane function is thought to be a key factor in 15-23.5% of patients who discontinue CAPD

[13,14]. In a recent study ultrafiltration failure was shown to be responsible for 51% of withdrawals from CAPD in patients who have been treated with the technique for more than six years [15]. Statistically peritonitis is the most common cause of technique failure accounting for 40-47% of patients who withdraw from CAPD [14]. Despite this, peritonitis does not appear to be a cause of progressive membrane failure, as rate of ultrafiltration failure (the most common failure of the semi-permeable membrane) was not influenced by the incidence of peritonitis in two large studies [16,17].

A significant limitation to long-term CAPD is therefore loss of peritoneal membrane function, most commonly manifest as ultrafiltration failure. Time on dialysis is not thought to cause a significant deterioration in osmotic clearance of small solutes (urea and creatinine) [18], but ultrafiltration failure affects 10% of patients in the first year, and 30% by the end of the second year of CAPD [16]. With time on CAPD ultrafiltration failure is therefore an increasing clinical problem, and the most likely aetiological factor for this is the constituents of the dialysis solutions themselves.

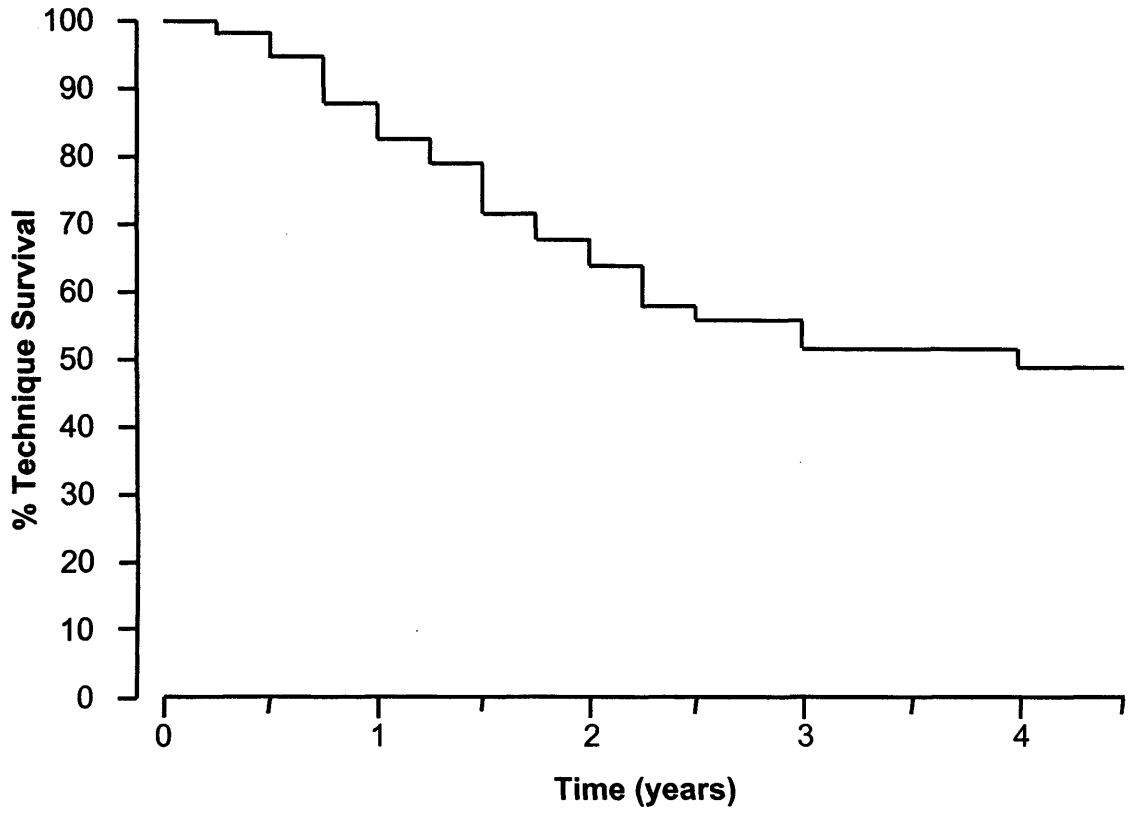


Figure 1.1 CAPD Technique survival

Proportion of patients remaining on CAPD from an initial cohort of 60 patients started on CAPD in Leicester in 1996. Loss of technique was defined as a necessary change to either haemodialysis or transplantation.

1.2 Peritoneal morphology

1.2.1 Normal

In health, the peritoneal cavity is lined by a even monolayer of mesothelial cells on a thin basement membrane (the peritoneum). No morphological differences appear to exist between the peritoneum from different areas of the peritoneal cavity (visceral or parietal), or between different species or from different body cavities [19]. The surface of the peritoneum is lined by mesothelial cells. Normal mesothelial cells are flat or discoid in cross-section and characteristically have a dense covering of microvilli on their apical surface. Adjoining cells are separated by overlapping cell junctions. These exhibit tight junctions superficially, and have desmosomes, and interdigitating processes towards their basal border [20]. The mesothelial cell cytoskeleton *in-vivo* consists of a combination of cytokeratin and vimentin. It has been suggested that the differential co-expression of these two intermediary filaments may reflect the degree of mesothelial cell maturation, with quickly dividing cells expressing greater vimentin, whilst non-dividing cells exhibit greater cytokeratin synthesis [21]. The underlying fine connective tissue stroma is approximately 1-2mm in thickness and consists of orientated bundles of collagen fibres in a matrix of ground substance. The peritoneum is relatively poorly vascularized, and the capillary bed in the normal peritoneum lies some distance from the surface lining [20].

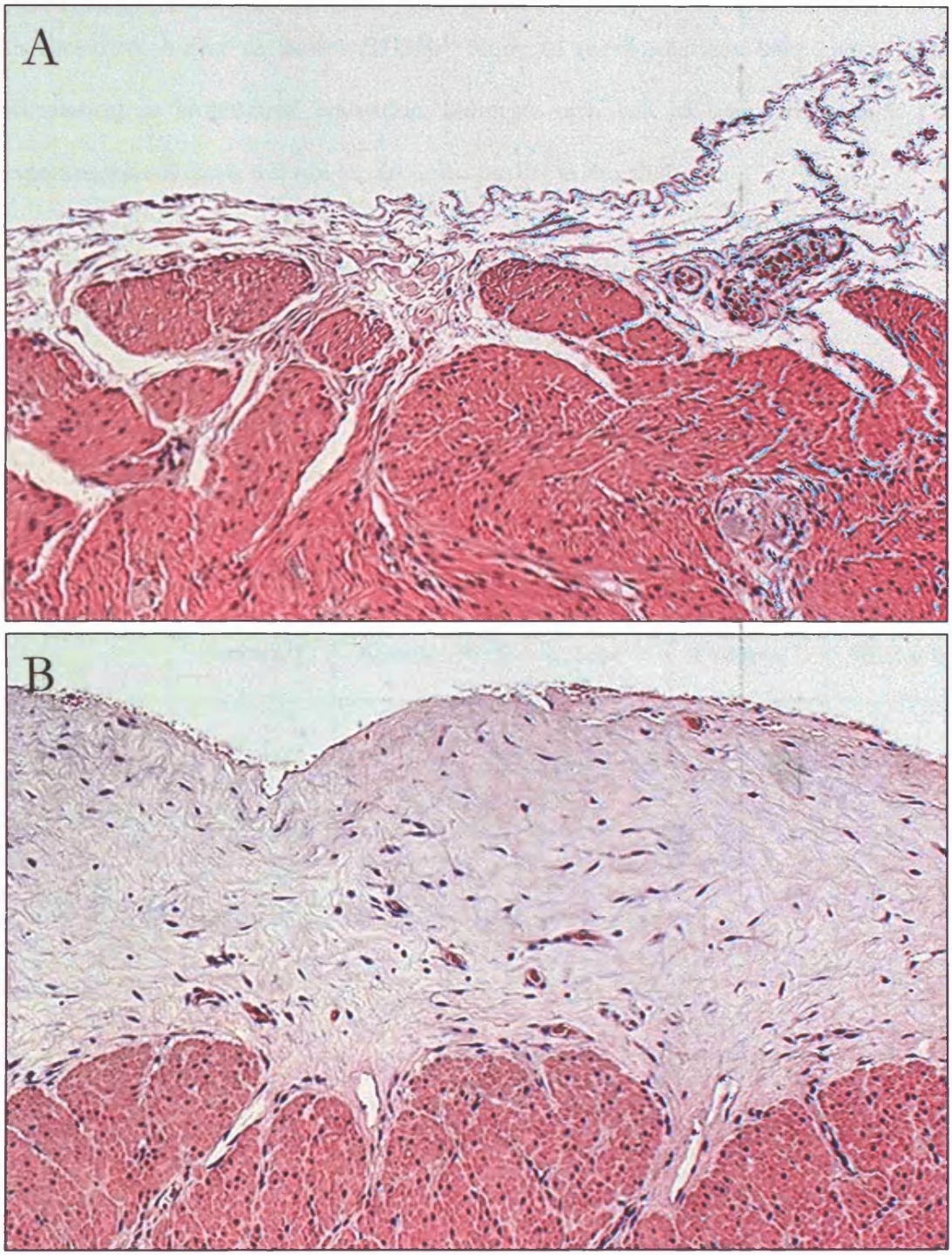
1.2.2 Effect of CAPD

The technique of CAPD results in many morphological changes to the peritoneum, which have been characterised in both animals [22] and uraemic humans [23-26]. Over the first months on CAPD the mesothelial cells progressively loose their apical microvilli,

cytoplasmic vacuoles appear, and the inter-cellular junctions widen. Advanced glycation endproducts (AGE), formed by non-enzymatic glycation of proteins are deposited in the loose connective tissue and peritoneal blood vessel walls [27,28]. Small foreign bodies can also be seen in all layers of the peritoneum, and are presumed to be plastic residues from the dialysate bags. Mesothelial cells lining the peritoneum are gradually denuded from the surface of the peritoneum with time on dialysis. After one year of CAPD the majority of patients have partial or total disappearance of mesothelium, and the submesothelial tissue becomes fibrous and thickened [29]. An example of this process is shown in Figure 1.2. Repeated peritonitis is an established cause of this progressive fibrosis, but fibrosis has also been shown to occur in the absence of infective episodes [29]. In these patients the most likely cause of the fibrosis is repeated insults from the unphysiological dialysis solutions.

Figure 1.2 Morphology of the peritoneum in-vivo

A – A – Normal peritoneal morphology showing a fine mesothelial layer on a thin layer of loose connective tissue and muscle. B – Effect of 5 years CAPD. The loose connective tissue is replaced by dense, avascular fibrous tissue. Both shown courtesy of Dr. P. Furness, Department of Histopathology, LGH at 10X magnification.



1.3 Composition of dialysis solutions

The compositions of the current commercial peritoneal dialysis solutions available in the U.K. are listed in Table 1-1. There are also several experimental dialysis solutions. Examples include using glycerol as osmotic agent [30], or dialysate containing pyruvate as an alternative buffer to lactate [31-33]. None of these solutions have gained general acceptance, or commercial realisation, although each has its own proponents. These experimental solutions will not be discussed further in this thesis.

	1.36%PD4	**3.86% PD4**	**Physioneal ®**	**Nutrineal ®**	**Icodextrin ®**
Osmotic agent	Glucose 76mmol/L	Glucose 210mmol/L	Glucose 76mmol/L	1.1% amino acid solution	Glucose polymer
Buffer	Lactate 40mmol/L	Lactate 40mmol/L	Bicarbonate 25mmol/L Lactate 15mmol/L	Lactate 40mmol/L	Lactate 40mmol/L
pH	5.2	5.2	7.0-7.4	6.5	5.2
Osmolality	344	478	344	365	284

Table 1-1 Composition of different commercial dialysate

1.3.1 Lactate based hyperosmolar glucose

As previously discussed the most widely used dialysis solution for CAPD is hyperosmolar glucose, lactate buffered dialysate. This contains 40mmol/L L-Lactate and is manufactured at three glucose concentrations (1.36, 2.27 and 3.86% glucose). The vast majority of longitudinal studies of technique survival and peritoneal morphology have been conducted on patients using this solution.

1.3.2 Bicarbonate-lactate based hyperosmolar glucose

Similar to the standard lactate dialysate this solution uses a combination of lactate and bicarbonate as the buffering agent. Because peritoneal dialysis solutions contain calcium (in the form of the soluble chloride salt) this dialysate is supplied as two separate solutions which are combined before use. The buffer is kept separate from the calcium salt in order to prevent precipitation of poorly soluble calcium bicarbonate. Potential advantages of this solution are an initial pH of 7.4, and a lower (although still supra-physiological) lactate concentration. In one of the few studies considering the effect of dialysate in-vivo it has been suggested that this dialysate induces the less production of ECM. Patients who had all received at least 3 months CAPD were randomised to either receive bicarbonate/lactate dialysate or continue PD4 for 20 weeks. Patients on bicarbonate/lactate dialysate had lower concentration of both pro-collagen I and TGF-β in the PD effluent expressed for CA125 amount [34,35].

1.3.3 Amino-acid dialysate (Nutrineal®)

Amino acid containing dialysate has been shown to have potential beneficial effects on patient nutrition [36]. The solution uses a solution of 20 amino acids, of which 8 are essential amino acids, and 3 other considered essential in patients on dialysis (Table 5-1). A

theoretical concern over worsening metabolic acidosis from this dialysate formulation appears unfounded in clinical trials, although serum urea does rise [36]. There is no data published that considers the effect of this solution on peritoneal function either *in-vivo* or *in-vitro.*

1.3.4 Glucose polymer solution icodextrin (Extraneal®)

The glucose polymer peritoneal dialysis solution, icodextrin has been shown to minimise water absorption compared with standard hyperosmolar glucose dialysate over a long (usually overnight) PD dwell. Glucose has a low molecular size (180Da) and is therefore absorbed by the patient over the time of a CAPD dwell. Consequently, it ceases to form an osmotic gradient after approximately 6-8 hours in most patients. The glucose polymer icodextrin has a molecular weight of 12-20 kDa, and is therefore absorbed at a much slower rate than glucose across the peritoneal membrane. In addition it achieves ultrafiltration by a process of "colloid osmosis", similar to albumin in plasma and therefore produces effective ultrafiltration when manufactured at a osmolality comparable to plasma [37]. Clinical studies have shown the solution to be as effective at ultrafiltrating water as a 3.86% glucose dialysis solution over a long overnight dwell [38]. In addition, it does not use small water channels to ultrafiltrate water, and it has been shown to improve technique survival of individual patients with ultrafiltration failure [39]. As with amino acid dialysate, there is no data published that considers the effect of this solution on peritoneal function either *in-vivo* or *in-vitro.*

1.4 Functional characteristics of the peritoneum *in-vivo*

It is unfortunate that there is little published information on the physiological role of the peritoneal cells *in-vivo.* Morphological similarities have been drawn between peritoneal

mesothelial cells and other cell types [19]. Assumptions have then been made about their potential biological role. No work exists considering the *in-vivo* role of peritoneal fibroblasts, endothelial cells or inflammatory cells, and it is presumed that they are all relatively quiescent in health.

The peritoneal mesothelial cell has been studied the most *in-vivo*. Studies of mesothelial morphology have shown that the cells contain prominent rough endoplasmic reticulum, well-developed Golgi regions and an abundance of mitochondria [19]. In this regard they have been likened to type II pneumocytes, and are presumably responsible for lubricating the peritoneum to allow the abdominal viscera to move free over each other [40]. Mesothelial cells also express ICAM and VCAM on their apical surface [26], which is likely to enable them to provide an attachment for inflammatory cells during peritonitis.

It is probable that mesothelial cells synthesise small amounts of many other products, but their measurement is hampered by technical constraints. Fibronectin [41] and transforming growth factor beta (TGF-β) [42] can be detected in peritoneal dialysis effluents from patients on CAPD, and their concentration appears to increase during episodes of peritonitis. This had led to the conclusion that there was significant intraperitoneal synthesis of these factors during times of peritoneal inflammation. However, these proteins readily cross into peritoneal dialysate from plasma, and dialysate levels are further confounded by the already higher serum fibronectin levels in patients with chronic renal failure [43]. Many proteins (albumin included) appear in higher concentration in the dialysate during peritonitis because of damage to the mesothelium and endothelial leakage. Although detection of these substances in the dialysate does not exclude local production, it is likely that this is masked by a much larger amount transported across the peritoneum from plasma [44].

In an attempt to investigate the effect of CAPD on the mesothelium *in-vivo* an animal model of CAPD has been developed in the mouse [45]. Peritoneal imprints were taken from animals that had received either one IP injection of glucose dialysis solution per day for 30 days, or a sham injection. Exposure to glucose dialysate caused a decrease in cell density, and decrease in mesothelial cell viability assessed by trypan blue exclusion in a concentration dependant manner [22]. In separate experiments increase in both cell membrane and cytoplasmic enzymes was observed, including a moderate increase in glucose-6-phosphatase assessed by histochemical staining [46]. These are the only experiments that have been able to correlate exposure to dialysis solutions and the biological effect on the mesothelium *in-vivo.*

1.5 Functional characteristics of the peritoneum *in-vitro*

A far greater number of studies have been conducted on the cells of the peritoneum *in-vitro.* Studies have concentrated on the cellular response to infection (CAPD peritonitis), expression and synthesis of cytokines by peritoneal cells, and the inhibitory effects of dialysis solutions on these responses.

1.5.1 CAPD peritonitis

Peritonitis is a significant contributory factor to CAPD technique failure, and also patient morbidity and mortality on CAPD. This is commonly, but not exclusively caused by contamination of the peritoneum with gram-positive bacteria of the patients' normal skin flora. Streptococci and Staphylococci are the most common organisms, but peritonitis can also be caused by gram-negative organisms (either from the skin or by migration across the intestinal wall), fungi and yeast

1.5.1.1 Peritoneal response to infection

In anatomical terms, the peritoneal mesothelial cell mono-layer forms the first line of defence in microbial contamination of the peritoneum, and the interaction between the mesothelial cell and peritoneal macrophage is though to be pivotal in the response to infection. Peritoneal macrophages can be cultured from spent dialysate from an overnight CAPD dwell. They are known to synthesise interleukin 1β (IL-1β), and TNF-α [47]. *In-vitro* the production of these cytokines can be increased by stimulation with Staphylococcus *Epidermidis* [48]. Usually present in only small numbers in health, the macrophage would therefore appear to initiate the peritoneal inflammatory response, either directly, or by stimulating the close-by mesothelial cell population.

Human peritoneal mesothelial cells are known to produce interleukin 1α and 1β (IL-1α and 1β) [49], interleukin 6 (IL-6) [50,51], interleukin-8 (IL-8) [50,52,53], and the prostaglandin 6-keto-$PGF_{1\alpha}$ [54-56]. *In-vitro* IL-1ß and TNF-α have been shown to increase synthesis of most of these cytokines [52]. These cytokines and chemokines enable the human peritoneal mesothelial cell to recruit other inflammatory cells to the peritoneal cavity. Because the mesothelial cell is the most abundant cell type in the peritoneal cavity, the production of mesothelial cytokines is likely to be as significant as those produced by the smaller population of peritoneal macrophages.

An important prerequisite to successful cell mediated defence against infection is the attachment of leukocytes to a fixed surface to co-ordinate cell recruitment and activation. The mechanism by which this take place has been characterised in the endothelial cell and occurs via the inter-cellular adhesion molecules (ICAM-1, 2 and 3) and the vascular cell adhesion molecule (VCAM-1). The mesothelial cell expresses both ICAM-1 and VCAM-1 [57], and in the peritoneal cavity therefore appears to perform a role similar to the

endothelial cell in leukocyte attachment. *In-vitro* expression of both ICAM-1 and VCAM-1 by human peritoneal mesothelial cells was increased by exposure to both TNF-α or interferon gamma, and less consistently by IL-1 [57]. As described above, all of these cytokines are produced *in-vitro* by either peritoneal macrophages or mesothelial cells in response to an infective stimulus.

1.5.1.2 The effect of peritoneal dialysate on host defence

Unlike surgical causes of peritonitis, the response to infection in the peritoneum of patients on CAPD is modulated by the presence of dialysate within the peritoneal cavity. It has consistently been shown that the peritoneal dialysate is inhibitory to an effective cellular response to infection. Glucose dialysate is inhibitory to PBMC IL-6 and TNF-α release, and this increases rather than decreases with duration of intra-peritoneal dwell [58]. The same dialysate is inhibitory to polymorphonuclear cell respiratory burst, essential for effective killing of bacteria [59]. In addition lactate-based glucose dialysate has also been shown to impair IL-1ß induced IL-6 release by human peritoneal mesothelial cell [60]. Finally, it could be speculated that the removal and replacement of dialysis solution (as occurs in CAPD) would remove inflammatory cells and cytokines necessary for effective defence against infection. Peritonitis is common on CAPD (currently occurring approximately once per 18 patient months), and it is clear that peritoneal dialysate inhibits an effective host response through a variety of different mechanisms.

1.5.2 The non-infected peritoneum

The morphological similarity between mesothelial cells and pneumocytes has been discussed. *In-vitro* mesothelial cells have been shown to produce phosphatidylcholine [61], consistent with the role of producing lubrication for the opposing surfaces of the

peritoneum. As will be discussed, they are also capable of synthesising and secreting components of the extra-cellular matrix. Just as the peritoneal dialysate modulates the cellular responses to infection, so it is also capable of affecting these other mesothelial cell functions.

1.5.2.1 Direct cytotoxicity of glucose dialysis solutions

It is well recognised that neat, unmodified glucose-based dialysate is toxic to cultured human peritoneal mesothelial cells [62,63], fibroblasts [64] and peripheral blood mononuclear cells (PBMC) [65]. Studies using lactate dehydrogenase (LDH) release as a marker of cell viability have shown that it is the combination of low pH and high lactate concentration which appears to be toxic to the cultured mesothelial cell [66]. With cultured PBMC, cell viability can be improved by adjusting the pH of the solution from 5.2 to 7.3 [65]. Others have shown that a short (fifteen minutes) intra-peritoneal dwell is also sufficient to neutralise the toxicity of the neat glucose dialysate to mesothelial cells [67]. Although the pH of dialysis is known to rapidly rise to a physiological pH once instilled into the peritoneal cavity, the toxicity of neat dialysis solution is apparent after similarly short exposure times. Finally in addition to frank cytotoxicity, glucose dialysate has also been shown to accelerate programmed cell death. Apoptosis increased in human peritoneal mesothelial cells incubated in hyperosmolar glucose dialysate for 48hours on well inserts [68].

1.5.2.2 Cytotoxicity of other dialysis solutions

Reference to the composition of the different dialysis solutions (Table 1-1) reveals that they share many similarities. Functionally it appears that icodextrin is less inhibitory to granulocyte and monocyte function *in-vitro*, but as inhibitory as hyperosmolar glucose to

mesothelial cell viability [69]. As with the glucose-based dialysate, mesothelial cell viability can be improved in icodextrin by a short intra-peritoneal dwell [67], and PBMC function can be restored by correcting the pH [65].

By adding different amino acids singly as supplements to culture medium it appears that some amino-acids are capable of inhibiting proliferation, and also increasing LDH release [70]. It appeared that when used alone L-typtophan was particularly toxic to human peritoneal mesothelial cells. The commercial amino-acid dialysate (Nutrineal®) is a 1.1% solution of mixed amino-acids. *In-vitro* studies are conflicting whether this has a less suppressive effect on mesothelial cell proliferation [71] or is no different to glucose based dialysate [72]. Amino acid dialysate may induce less mesothelial cell collagen synthesis than glucose based dialysate [73], but render them more susceptible to damage by free radicals [71].

1.5.2.3 Advanced glycation end-products (AGE)

Advanced glycation end-products (AGE) are derived from the non-enzymatic glycation of long-lived proteins. They are produced when proteins are exposed to high glucose concentrations for prolonged periods of time. It has been known for some time that AGE are partly responsible for the endothelial dysfunction in patients with diabetes mellitus [74]. In CAPD, the cells of the peritoneal cavity as similarly exposed to the high glucose concentrations of peritoneal dialysate for long periods of time. It is perhaps not unexpected therefore, that AGE can be detected in spent dialysate [75]. AGE have also been shown histologically to be deposited within the peritoneum, predominantly in the vascular walls [27]. Predictably, the degree of AGE deposition has been shown to increase with time spent on peritoneal dialysis [27].

In-vitro a 1.36% glucose solution is capable of glycating human serum albumin, and this is accelerated by heat sterilisation of the solution [76]. AGE have been shown to be inhibitory to rat peritoneal mesothelial cell proliferation and protein synthesis [77]. In a comparative study icodextrin produced slower *in-vitro* glycation of human serum albumin compared with 1.36% glucose [76], although like glucose the process of heat sterilisation promotes significantly more rapid production of AGE [78]

1.6 Production and regulation of extra-cellular matrix

All multi-cellular organisms are constructed from an intricate combination of cells with a scaffold of extra-cellular matrix (ECM) proteins to hold the specialist cells in correct alignment. The major component of this scaffold is collagen, named from the Greek words meaning "to produce glue". Present to some extent in nearly all organs its basic structure is modified to meet the specialised needs of each tissue. Collagen is accompanied by other ECM proteins such as elastin and fibronectin. In combination with the proteoglycans of the ground substance they form the extra-cellular matrix.

Fibronectin is believed to have a special role in cell interactions, particularly cell migration, cell adhesion and wound healing. Fibronectin consists of two 250 kDa polypeptide chains linked by a disulphide bond near their carboxyl termini. It is both secreted and also expressed on the surface of cells. Fibronectin specifically binds to fibrin in clot to allow remodelling by fibroblasts, and via a heparin-binding domain to other many cells.

Several of the resident cells of the peritoneum are involved in production and regulation of ECM. In health the HPMC forms the major cell component of the peritoneum and *in-vitro* have been shown to produce the extracellular matrix proteins fibronectin, laminin and collagen types I and III [79]. Fibroblasts are present in the loose connective tissue stroma

supporting the mesothelium. Peritoneal fibroblasts have not been widely studied, but there is no reason to suppose that they differ from other fibroblasts in their ability to synthesis ECM. Peritoneal macrophages also secrete fibronectin to opsonize bacteria for phagocytosis [80]. However, they form are a relatively small population in comparison to the much larger population of mesothelial cells. By secreting pro-fibrotic growth factors such as TGF-β and PDGF-like peptides [81] macrophages are likely to regulate peritoneal ECM production, especially during peritonitis.

In-vivo the amount of extra-cellular matrix present is the product of the balance between production and degradation. Production of extra-cellular matrix is known to be regulated by many different cytokines and hormones including platelet derived growth factor (PDGF), transforming growth factor beta (TGF-β), interleukin-1 (IL-1), and tumour necrosis factor alpha (TNF-α). In addition, exogenous factors such as the bacterial cell wall protein lipopolysaccharide (LPS) also modulate ECM production. These factors are known to affect ECM production at many different levels, with effects on gene expression, mRNA stabilisation, and gene translation.

The balance of ECM amount is also tightly regulated by a set of specific enzymes capable of ECM degradation, the metalloproteinases, and their specific inhibitors the tissue inhibitors of metalloproteinase (TIMPs). There are at least nine metalloproteinases, each with a different range of matrix substrates, but are broadly divided into collagenases (MMP3) and gelatinases (MMP2 and 9) to aid categorisation. TIMPs are highly specific inhibitors of metalloproteinase, but less specific regarding the different sub-types. They form 1:1 non-covalent complexes with MMPs, blocking access of substrates to the catalytic site. TIMP-1 is a non-inducible protein, and TIMP2 a constitutive protein. Both are

widely distributed in all body fluids. TIMP-3 is confined to the extra-cellular matrix itself, and TIMP-4 largely to cardiac tissue.

The role of the mesothelial cell in ECM production in CAPD is poorly characterised. Glucose, present in high concentration in dialysis solution, has been shown in other cell systems to influence ECM amounts, particularly in the context of diabetes mellitus. The role of glucose, and the pro-fibrotic growth factor TGF-β will be discussed more fully in each relevant chapter.

1.7 Hypothesis and Experimental aims

The aim of these experiments was to investigate the role of the human peritoneal mesothelial cell in the production and regulation of extra-cellular matrix with particular reference to the effect and mechanism of action of different dialysis solution, and the role of TGF-β.

2 Methods

2.1 Cell Culture

Cell culture work was carried out in a dedicated cell culture laboratory. All work was performed aseptically in a type II laminar flow hood. Cells were cultured in a humidified 5% CO_2 atmosphere at 37°C.

2.1.1 Source of mesothelial cells

Human omentum was obtained under general anaesthetic at the time of new CAPD catheter insertion according to the terms of the Leicestershire Ethics Committee approval and following informed consent (see Appendix, Chapter 8.3). Complete culture medium was medium 199 (M199) (Life Technologies 31150-022) with 10% vol:vol FBS (Life Technologies), 100IU/ml penicillin, 100μg/ml streptomycin (supplied combined Life Technologies 15140-114), 5μg/ml insulin, 5μg/ml transferrin, 5ng/ml selanite (supplied combined Sigma I1884), 2mM L-glutamine (Life Technologies 25030-024) and 0.4μg/ml hydrocortisone (Sigma H0396).

2.1.2 Coating of cell culture plates

All culture plates and flasks were pre-treated with collagen in-order to mimic cell basement membrane conditions and improve cell attachment and differentiation. Bovine calf skin collagen type I (Sigma C8919) was diluted to 50μg/ml in 0.1M acetic acid (BDH AnalaR). 2.5ml of this solution was added to each 25cm^2 flask, or 380μl to each well of a 12 well culture plate, and left overnight in the laminar flow hood. The following morning any remaining collagen solution was discarded and the plates rinsed three times with HBSS at slightly greater volume than the original collagen volume. Plates were stored for upto 1 week re-sealed in their original wrap at 4°C.

2.1.3 Primary culture

Human peritoneal mesothelial cells were cultured using a modification of the original techniques previously described [79]. Subsequent studies have shown have shown that HPMC are more reliably cultured using medium 199 (M199) in place of Hams-F12 medium [32,49,56,60,62,82-84]. M199 was therefore was used in place of Hams-F12 for all stages of the cell culture process. A $6cm^2$ piece of omentum was washed three times in sterile PBS, and then incubated with 15ml of a solution containing 0.125% trypsin; 0.05% EDTA (1:4 dilution of 10X trypsin-EDTA solution (Sigma T4174) with PBS) for 20 minutes at 37°C. Continuous agitation was achieved using an autoclaved stir-bar and magnetic stirrer. After incubation the suspension was centrifuged at 1000rpm for 5 minutes at 4°C. The supernatant and digested omentum was discarded and the cell pellet re-suspended in culture medium with all additives. Cells were seeded in 5ml of medium in $25cm^2$ canted neck, vented cap tissue culture flasks (Corning 25103). Medium was changed after 24 hours to remove non-adherent cells (predominantly red blood cells), and thereafter every 3 days till confluent (approximately 7 to 10 days).

2.1.4 Passaging cells

Confluent cells were washed once with HBSS. 2.5ml 0.05% wt:vol trypsin, 0.02% wt:vol EDTA in Pucks' modified saline (Life Technologies 45300-019) was added to each $25cm^2$ flask and incubated for 10-15 minutes at 37°C until all the cells had detached as assessed by microscopy. The trypsin was inactivated by adding 10ml of standard medium containing 10% vol:vol FBS, and centrifuged at 1000 rpm for 5 minutes at 4°C. The supernatant was discarded and the cell pellet re-suspended in complete medium. Cell concentration was measured using a modified Neubauer chamber and the cells seeded at 1-2 x 10^{-4} cells / cm^2 in $25cm^2$ flasks or 12 well culture plates (Costar 3512).

2.2 Cell characterisation and immunocytochemistry

Confluent HPMC have a polygonal "cobblestone" like in appearance in culture. In this regard they can clearly be differentiated from cultured fibroblast cells (Figure 2.1). HPMC are known to co-express the intermediate filaments cytokeratin and vimentin, characteristic of epithelial and mesodermal cells [79]. HPMC do not express either von Willebrand factor (factor VIIIc) surface antigen or the common leukocyte antigen CD45 enabling them to be differentiated from endothelial cells, or monocyte/macrophages respectively.

Mesothelial cells were grown in an eight well culture plate consisting of a removable plastic surround on a glass microscope slide (Labtech chamber slide). Culture medium was removed and the cells fixed using a 50:50 mixture of acetone:methanol for 90 seconds. The cells were washed 3 times with tris-buffered saline (TBS), and then incubated with the correct dilution of the antibody of interest at room temperature for 2 hours. Antibodies used were mouse anti-human vimentin (Dako Ltd. M725, 1:10 dilution), anti-cytokeratin (M821, 1:40), anti-CD45 (Dako Ltd. 1:25) and anti-von Willebrand factor (Dako Ltd. M616, 1:25). Human umbilical vein endothelial cells were used as a positive control for anti-von Willebrand factor antibody, and a whole blood film used as a positive control for the anti-CD45 antibody. The cells were washed again three times with TBS before addition of a rabbit anti-mouse alkaline phosphate conjugated antibody (Dako Ltd. D314) diluted 1:20. Slides were flooded with AP solution (Appendix, chapter 8.1) and incubated at room temperature for 20 minutes. Naturally occurring alkaline phosphatase was inhibited by the levamasole contained in this solution. Slides were washed three times in TBS and then counter-stained by incubating with haematoxalin before mounting with aquafix™. Results of the immunocytochemistry are shown in Figure 2.2. This confirms that the cultured cells are of mesothelial cell type.

Figure 2.1 Morphology of cultured HPMC

A – Confluent human peritoneal mesothelial cells (primary culture). B – Confluent human peritoneal fibroblasts (primary culture)

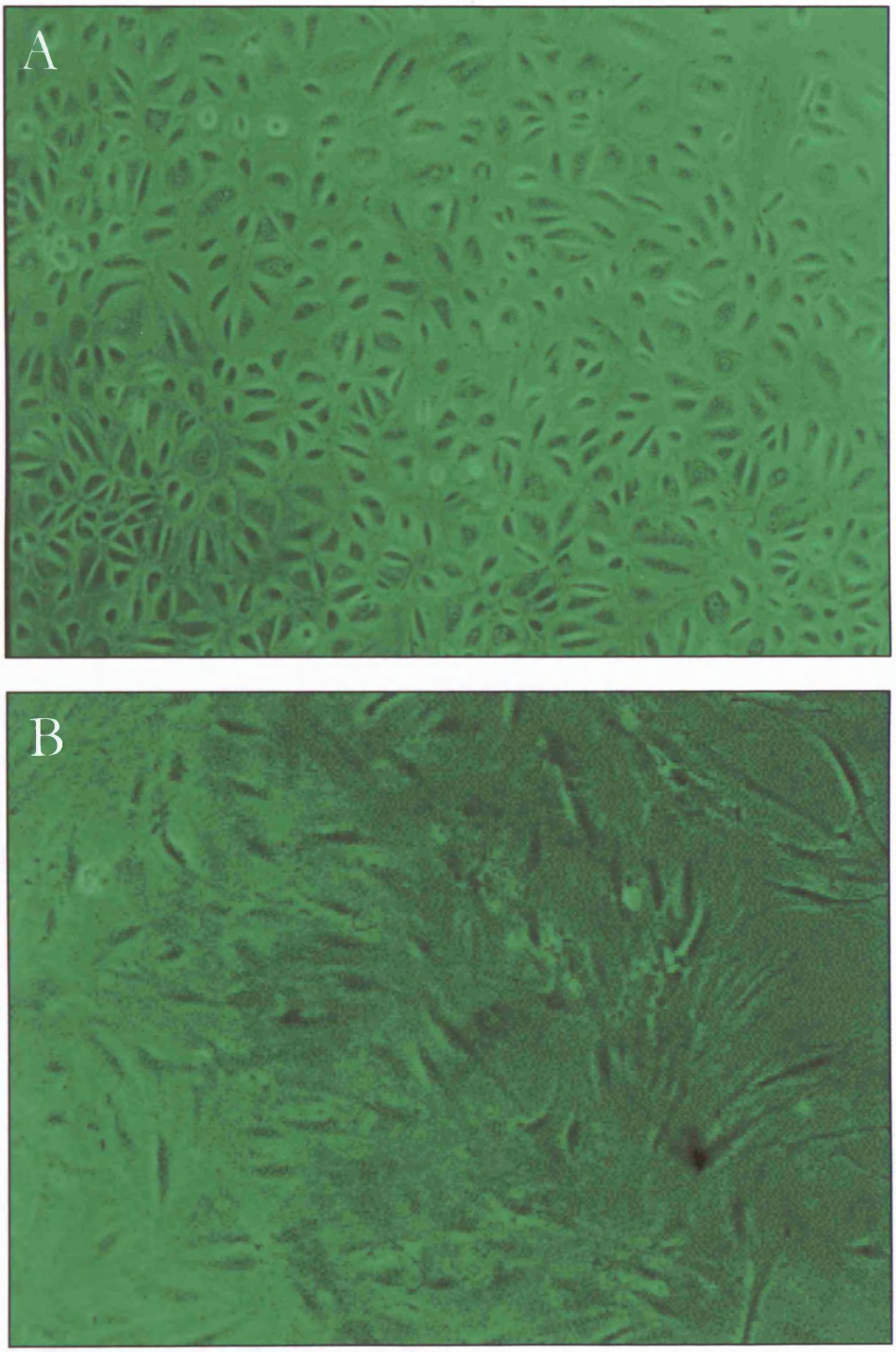
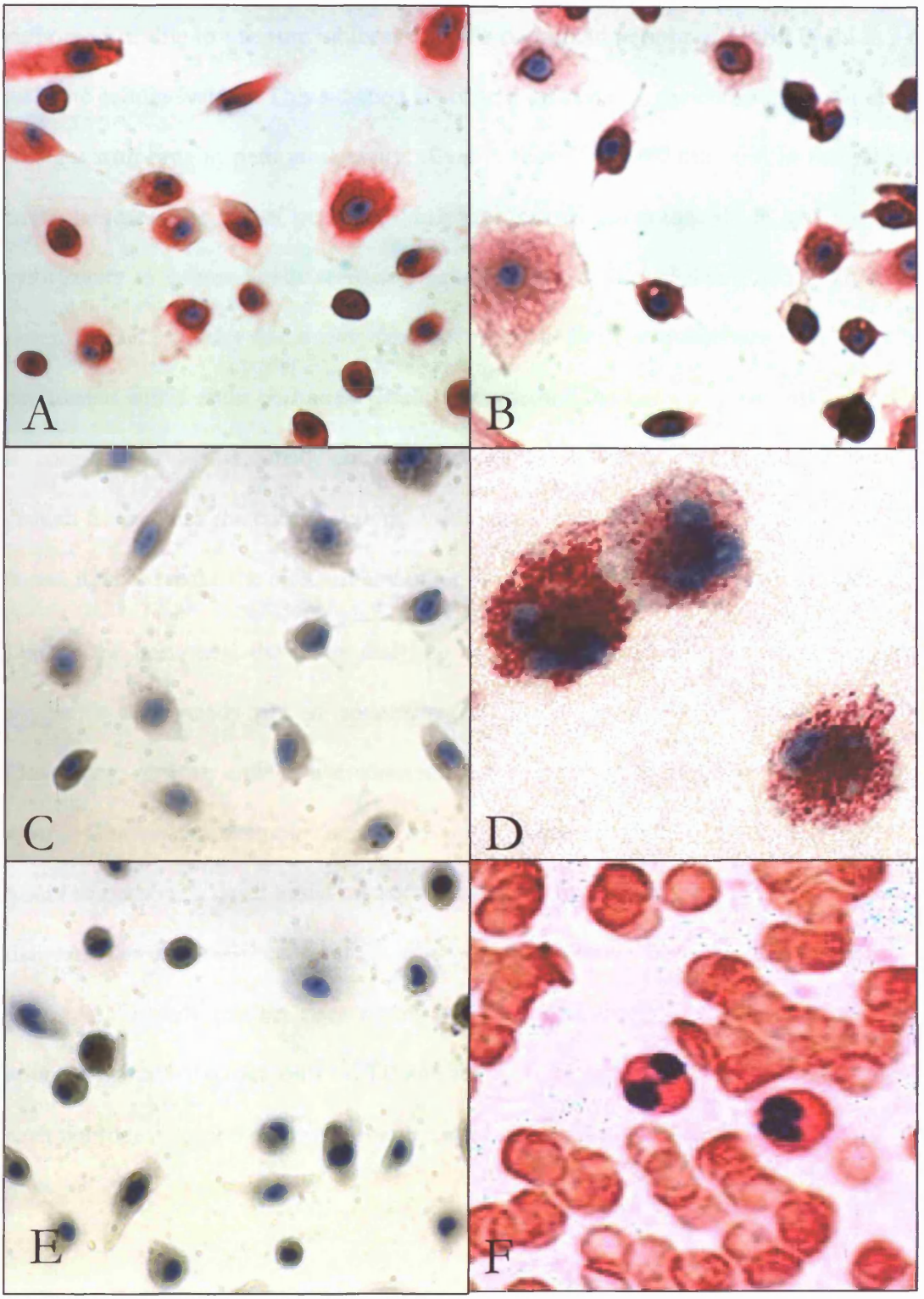


Figure 2.2 Immunocytochemisty staining of HPMC

A - HPMC positive staining for cytokeratin, B - HPMC positive staining for vimentin, C- HPMC negative staining for vWF, D - Human umbilical vein endothelial cell positive control for vWF, E - HPMC negative staining for CD45, F - Peripheral blood film; Monocyte stained with CD45



2.3 Development of the experimental design

Several attempts have been made to develop a suitable culture system to mimic the equilibration that occurs in the peritoneum during a CAPD dwell. Initial work suggested that neat glucose dialysate is cytotoxic to cultured HPMC [59], although despite this the authors were able to measure inhibitory effects on human peripheral blood PMN in a neat dialysate culture system. This situation is artificial however, as the composition of dialysate changes with time in peritoneal cavity. Even a short dwell (30 minutes) in the peritoneal cavity increases the pH of peritoneal dialysate towards physiological pH and reduces the cytotoxicity to cultured cells seen with cells exposed to neat dialysate [85]. Therefore a potential methodology for *in-vitro* experiment would be to use dialysate which had been pre-treated with a short peritoneal dwell. This method has been used successfully [56] but is complicated by potentially introducing biologically active molecules, cytokines and growth factors into the culture system. Although this mimics the equilibration that occurs *in-vivo,* it would make the effect of any other test conditions very difficult to interpret.

During the peritoneal dwell the dialysate pH rises over the first 60 minutes, and then plateaux's at a steady pH of approximately 7.1 for the remainder of the dwell [86]. Osmolality, glucose and lactate concentrations decline over this time in the peritoneal cavity. Glucose, for example declines to approximately 50% after 2 hours, and 38% after 4 hours of peritoneal dwell based on an "average" peritoneal transport [87]. In addition, the dialysate also changes because of the transport of molecules from the patients' serum. In particular dialysate protein rises during the peritoneal dwell. Peritoneal protein loss is approximately 5-15g/day on CAPD, and the final protein concentration at the end of a each peritoneal dwell therefore ranges between approximately 1.9 and 5.6 g/L.

A cell culture method for investigating the effects on HPMC during CAPD therefore needs to be consistent with the composition present in the peritoneum during the majority of the peritoneal dwell. Some authors argue that a dynamic system, which starts with cells exposed to neat dialysate, and then supplementing this with culture medium and BSA, might be the most physiological system [86]. However this method requires the culture vessel to be continually removed from the steady state atmosphere of the tissue culture incubator, and it is likely that temperature and CO_2 equilibrium is never attained. In addition, there has been no work published successfully using this technique.

An alternative system uses a culture well inserts. Cells are grown on a semi-permeable support with their baso-lateral aspect in culture medium, and their apical surface bathed in dialysate. This has been used successfully to demonstrate an increase in apoptosis with glucose dialysate [68], and allows the dialysate to be changed several times in each 24 hours to mimic the *in-vivo* situation on CAPD. However, for this method to work successfully a perfect cell monolayer is required to prevent passive movement between the two chambers which is difficult or impossible to achieve consistently (personal observations). In addition, the buffering capacity of the culture medium is quickly lost after 2-3 changes of dialysate, resulting in a system which progressively decreases in pH.

For these reasons two alternative static cell culture systems were considered.

2.3.1 Effect of neat dialysis solutions on HPMC

The aim of this experiment therefore was to investigate a potential culture model using neat glucose dialysate. In order to allow for the rapid initial correction of the dialysate pH exposure to dialysate was kept short (30 minutes). In this way it was intended to mimic the immediate effects of exposure to dialysate *in-vivo* before the rapid correction of pH that is

seen early in clinical practice. The effect of correcting dialysate pH was examined by adding NaOH to the neat dialysate.

2.3.1.1 Methods

HPMC were cultured as previously described. Cells from passages 2-4 were grown to confluence on a 12 well tissue culture plate that had previously been coated with type I collagen. After a period of 48hrs in medium containing 0.1% FBS to render the cells quiescent the cells were exposed to the following test conditions

A 30minute exposure to 1.36% glucose PD4

B 30minute exposure to 1.36% glucose PD4 with pH corrected to 7.4 with 1M NaOH

C 30minute exposure to culture medium M199 (control)

In all cases this was followed by 11½ hours in M199. The supernatant and cell digests were then assayed for LDH activity as described in section 2.9.

2.3.1.2 Results

The results of experiment 1 are shown in Table 2-1, and illustrated in Figure 2.3. Even a short (30minute) expose to neat dialysis solution results in a substantial decrease in cell viability. A percentage LDH release of 100% would represent total cell death (all the LDH in the culture medium). Exposure to neat 1.36% glucose dialysate without pH correction resulted in 80.5% LDH release and therefore is overtly toxic to HPMC. Correcting the pH of the 1.36% glucose dialysate to the physiological range resulted in similar LDH release to

cells exposure to culture medium alone. This suggests that the cytotoxicity is a direct result of the low pH of the dialysate.

30 minute test medium	% LDH release
1.36% PD4 pH 5.2	80.8 ± 7.20
1.36% PD4 pH 7.4	5.10 ± 1.65
Culture medium	8.70 ± 2.14

Table 2-1 Effects of neat dialysate on LDH release by HPMC

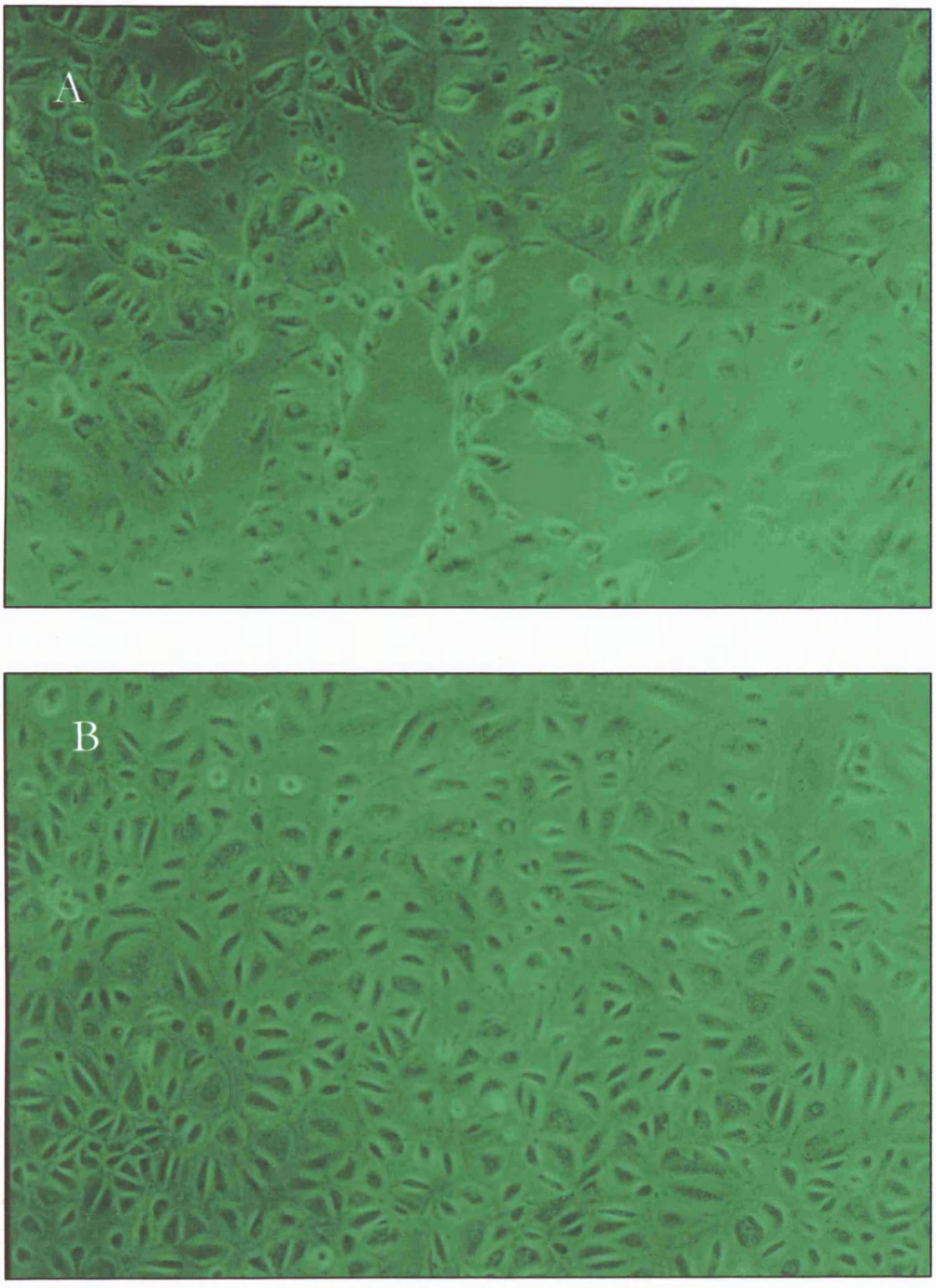
Results are percentage LDH activity in culture supernatant / total LDH activity expressed as means ± SEM of three experiments in duplicate. Neat 1.36% glucose dialysate is overtly cytotoxic to HPMC after only short exposure. The effect disappears with correction of the pH to 7.4

2.3.1.3 Conclusion

This experiment demonstrated that even a short exposure to neat dialysate resulted in an unacceptable degree of cytotoxicity for a useful cell culture system. These observations may however in part explains the progressive loss of mesothelial cells from the peritoneal cavity with time on dialysis *in-vivo*. As a culture system however it would not allow further assessment of mesothelial cell function beyond cytotoxicity, and hence was not developed further.

Figure 2.3 Exposure of HPMC to neat dialysate

Quiescent HPMC in culture were exposed to test medium for 30 minutes before this was removed and replaced with culture medium for 11½ hours. Test medium was A – neat 1.36% glucose PD4 dialysate (pH 5.2), B – 1.36% glucose PD4 with pH corrected to 7.4 by addition of NaOH.



2.3.2 Cell viability with 50:50 dialysate and M199

A potential culture system using a 50:50 mixture of dialysate with culture medium (M199) was considered. In addition the effect of differing concentrations of FBS were examined. *In-vitro* FBS is known to modulate the function and characteristics of cultured cells, with high concentrations stimulating cell proliferation, and small concentrations usually necessary to preserve cell viability. A range of different concentrations of FBS was therefore tested in order to determine a system which would optimise HPMC viability.

2.3.2.1 Methods

Confluent HPMC in 12 well culture plates from passages 2-4 were rendered quiescent by incubation in M199 with 0.1% FBS for 48 hours. The medium was then changed to a 50:50 mixture of dialysate with M199. The dialysate used was a laboratory manufactured dialysate of identical composition to PD4, but with no D-glucose. The chemical constituents of PD4 are documented on the commercial product and the composition is summarised in chapter 8.1. A non-sterile 10 times solution was manufactured in nanopure water. This solution was then diluted 10 fold and filter-sterilised before cell culture use. The final D-glucose concentration in the test medium was adjusted to either 5mmol/L or 20mmol/L by adding additional filter sterilised D-glucose. The two test medium were then supplemented with either none, 0.1 or 0.5% FBS. After 12 hours the pH of the cell culture supernatant was measured as described in section 2.7. In a separate experiment cell viability was assessed after 24 hours by measuring LDH release in the cell supernatant and cell lysate as described in section 2.9.

2.3.2.2 Results

The pH of the 50:50 mixture of dialysate and M199 5mmol/L glucose after 12 hours at 5% CO_2 = 7.07 ± 0.04. The pH was unaffected by increasing concentrations of D-glucose. In the absence of FBS an increase in glucose concentration resulted in a doubling of LDH release and hence significant decrease in HPMC viability. The presence of even small (0.1%) concentrations of FBS prevented this, with no increase in LDH release seen in the high glucose test condition compared with control in the presence of either 0.1% or 0.5% FBS.

FBS	5mmol/L D-glucose	20mmol/L D-glucose
None	10.0 ± 0.86	21.1 ± 4.3
0.1%	11.1 ± 2.2	9.6 ± 2.4
0.5%	8.0 ± 0.88	6.0 ± 1.2

Table 2-2 Effect of different concentrations of FBS on HPMC viability

Results are percentage LDH activity in culture supernatant / total LDH activity expressed as means ± SEM of three experiments in duplicate. Cell viability is reduced in the presence of hyper-osmolar glucose, but can be prevented by the addition of a low concentration of FBS.

2.3.2.3 Conclusion

These results suggest that the test medium of 50:50 dialysate and M199 equilibrates to a pH comparable to the pH *in-vivo* during the peritoneal dwell [86]. In addition, it would

appear that a low concentration of FBS is necessary in-order to preserve cell viability particularly at higher D-glucose concentration. The culture system adopted for all subsequent experiments was therefore a 50:50 mixture of dialysate with M199 with 0.1% FBS.

2.4 ELISA

2.4.1 Fibronectin ELISA

An enzyme-linked immunosorbent assay (ELISA) was used which had previously been developed and validated for the measurement of human fibronectin in tissue culture supernatants [88]. Immunoplates (Nunc) were coated using a polyclonal rabbit anti-human fibronectin antibody (Sigma F3648) diluted 1:1000 in carbonate/bicarbonate coating buffer (appendix1). 100µl of antibody solution was added to each well and the plate incubated overnight at +4°C. The plates were washed four times in wash buffer (WB) (Appendix, chapter 8.1), and non-specific protein binding sites blocked by incubating the plate for 1 hour at room temperature with 100µl per well WB with 2% (w/v) bovine serum albumin (Sigma A2153). The plates were washed again, and a standard curve formed by 1/3 dilutions from 2000 to 35ng/ml in WB of fibronectin obtained from human plasma (Sigma F0895). 50µl of each standard and each appropriately diluted samples were added in duplicate and the plate incubated overnight at +4°C. The plates were washed and a monoclonal mouse anti-human fibronectin antibody (Sigma F7387) diluted 1:500 in wash buffer was added for 2 hours. The plates washed and a horseradish peroxidase (HRP) conjugated anti-mouse immunoglobulin (Dako D260) diluted 1:1000 added for 2 hours. HRP was detected by adding 50µl of ELISA substrate (Appendix, chapter 8.1) and incubated for 10-20 minutes at room temperature until sufficient colour had developed. The reaction was then stopped using 75µl 1M sulphuric

acid per well and read using a plate scanner at 492nm. OD_{492} was plotted against the log fibronectin concentration. The resulting sigmoid curve, an example of which is shown in Figure 2.4. The steep linear section between 100 and 1000ng/ml defined the accepted limits of delectability of the assay. The inter-assay coefficient of variation was 9.1% at 395nm (data from 31 assays).

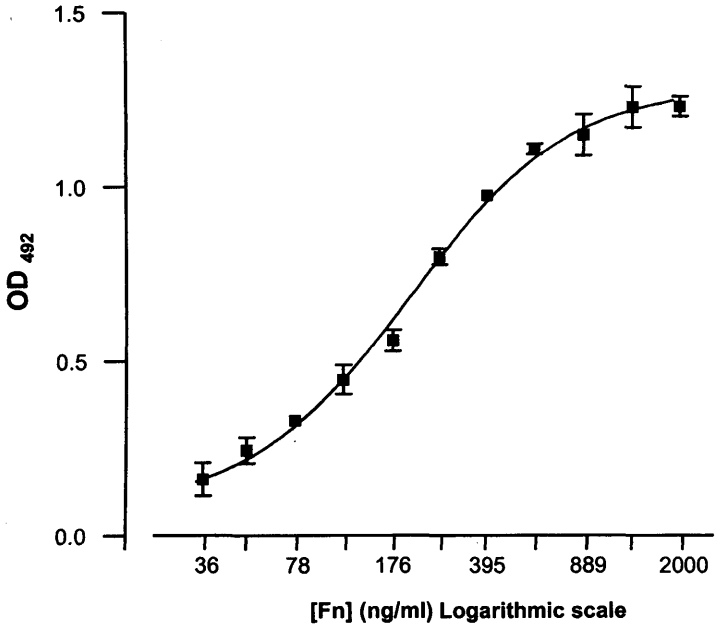


Figure 2.4 Representative standard curve for fibronectin ELISA

2.4.2 TGF-β ELISA

TGF-β was detected in the cell culture supernatants using a commercially available sandwich ELISA (Promega Ltd G1230). The assay was reliably able to detect a minimum TGF-β_1 concentration of 31pg/ml. TGF-β was activated in the samples prior to addition to the plate by incubating with 4µl 1M HCl per 200µl diluted sample for 15 minutes at room temperature, and the pH restored by adding a further 4µl 1M NaOH.

Immunoplates (Nunc) were coated using 100µl per well of the supplied TGF-β coating mAb at 1:1000 dilution in carbonate coating buffer (Appendix, chapter 8.1). Plates were incubated overnight at +4°C. The excess solution was cleared from the wells by inverting the plate and shaking it onto paper towels. The non-specific protein binding sites were blocked using 270µl per well of the supplied blocking buffer (X5) diluted in deionised water. The plate was incubated for 35 minutes at 37°C without shaking, and the wells cleared again as above. A standard curve was formed by halving dilutions of supplied TGF-β_1 standard in supplied sample buffer from 1000 to 15.6pg/ml. 100µl of each standard or appropriately diluted sample was added in duplicate to the plate. The plate was incubated at 37°C for 90 minutes with shaking (500±100rpm). The plate was washed using a mechanical plate washer 4 times with TBST (Appendix, chapter 8.1), and 100µl per well of anti-TGF-β_1 pAb at 1:1000 dilution in sample buffer added. The plate was incubated for 2 hours at room temperature with shaking, the plate washed as above and the wells refilled with 100µl antibody conjugate diluted 1:1000 in sample buffer. The plate was again incubated at room temperature for 2 hours, the plate washed, and TMB solution added at 100µl per well. Once sufficient blue colour had developed the reaction was stopped using 1M phosphoric acid. Absorbances were measured at 450nm, and a linear standard curve formed from the absorbance versus TGF-β concentration on linear axes. Samples containing unknown TGF-β concentrations were quantified from the linear regression equation.

2.5 Preparation of cell lysates

Cultured cells were lysed by scraping in 200µl 0.5M sodium hydroxide using the plunger from a 1ml plastic syringe and the suspension placed in 1.5ml plastic tubes. The plates

were then rinsed again with 100µl 0.5M sodium hydroxide, and this added to the previous 200µl sample. Cells were dispersed by sonication using a 50W sonicator (Jencons) for 5 seconds. Insoluble material was pelleted by centrifugation at 13,000rpm in a microcentrifuge. The sample was then sonicated and centrifuged again before the supernatant assayed for total protein estimation.

2.6 Protein assay

Total cell protein was measured using a commercial assay (BioRad DC protein assay) based on a modified Lowrie technique [89] following the manufacturers instructions. A standard curve was formed from halving dilutions of supplied BSA standard from 2 to 0.03mg/ml in 0.5M sodium hydroxide. 5µl of either sample or standard were applied in duplicate to a 96well microtitre plate. 25µl of reagent A (alkaline copper tartrate solution) was added to each well followed by 200µl reagent B (Folin reagent). The plate was incubated for 15 minutes at room temperature before absorbances were read at 760nm. A linear standard curve was formed from the absorbance versus protein concentration, and unknown protein samples were quantified from the linear regression equation.

2.7 Measurement of test medium pH

The pH of different test medium was measured at the end of the experimental time-course. Care was taken to ensure that the door of the tissue culture incubator had not been opened for at-least 1 hour before removing the supernatants. Culture plates were removed from the incubator and culture supernatants swiftly drawn up into a 1ml plastic syringes which were capped and placed on ice. The pH and pCO_2 were measured using a blood gas analyser (Corning Ltd). Any minor variation in the measured CO_2 concentration compared

to the original CO_2 concentration in the incubator (5%) would affect the measured pH. A correction was made for this by using a simplified Henderson-Hasselbach equation.

$$\text{pH at 5\% } pCO_2 = \text{pH at measure } pCO_2 + \log \left[\frac{\text{measured } pCO_2}{5} \right]$$

2.8 Proliferation (^{3}H-thymidine incorporation)

Proliferation of confluent quiescent cells exposed to different test medium was measured by the incorporation of ^{3}H-thymidine using a modified method of Greenberg et al [90]. Two different experimental procedures were followed. In method 1 HPMC were exposed to test medium for 12 hours, which was then replaced by fresh test medium containing ^{3}H-thymidine (Amersham, TRK61) for 6 hours. In method 2 HPMC were exposed to test medium for 48 hours, and the ^{3}H-thymidine was added to the test medium for the final 16 hours of the experiment. In both methods 0.5μCi ^{3}H-thymidine was added to each well. At the end of the experiment the supernatant was discarded and the cells incubated for 20 minutes at 37°C with fresh test medium containing 0.1mM non radio-labelled thymidine. The supernatant was again discarded and the cells rinsed once with ice-cold PBS, 3 times with 10% trichloroacetic acid, and once with PBS. Cells were dissolved in 300μl 10% percloric acid using the plunger from a 1ml syringe. The cell suspension was added to a 1.5ml plastic tube and incubated in a waterbath at 70°C for 20 minutes to dissolve the cellular DNA. Cell wall proteins that were not solubised in this solution were removed by precipitation at +4°C, and pelleted by centrifugation at 5000rpm for 5 minutes +4°C. 100μl of the resulting solution was added to 4ml of Ecoscint-A scintillation fluid (National Diagnostics) and centrifuged before counting using a LKB 1219 liquid scintillation counter with a ^{226}Ra external standard and quench correction.

2.9 Cell Viability (lactate dehydrogenase assay)

Cell viability was assessed by measurement of lactate dehydrogenase (LDH) activity in the cell supernatant and cell lysate using a commercial available method based on the reduction of pyruvate to lactate (Sigma DG1340-K). This method has previously been used to assess cell viability in other culture systems [59,91]. The rate of decrease in absorbance at 340nm due to formation of NAD is directly proportional to the LDH activity in the sample. LDH activity was measured in the cell culture supernatant and the cell lysate. The cell lysate was prepared as described above, but cells suspended in deionised water not sodium hydroxide. The spectrophotometer (Cecil CE 2040) was set to 340nm wavelength and zeroed using deionised water. 700µl of reagent A (NADH in phosphate buffer) was placed in a cuvette and 30µl of sample added and mixed. After 60 seconds 30µl reagent B (pyruvate) was added and mixed. After a further 30 seconds to equilibrate the absorbance at 340nm was measured, and the absorbance measurement repeated every minute for five minutes.

Rate of change of absorbance (ΔA) per minute was calculated by linear regression and LDH activity calculated using the following equation:

$$\text{LDH activity (U/L)} = \frac{\Delta A \text{ per min} \times TV \times 1000}{6.22 \times LP \times SV}$$

where TV = total volume of original sample (ml)

SV = sample volume (0.030ml)

6.22 = millimolar absorbency of NADH at 340nm

LP = light path (1cm)

1000 = conversion of units per ml to units per L

Results were expressed as the percentage of supernatant to total LDH activity (supernatant plus cell lysate).

2.10 Northern Analysis

All molecular biology was conducted in strict RNAse free conditions. Glassware was baked in an oven for 4 hours at for 200°C prior to use, and all other receptacles unable to withstand this treatment were rinsed thoroughly with RNase-away® (Life technologies 10328-011). All reagents were of "molecular biology" grade unless otherwise stated. All water used was nanopure deionised water pre-treated by adding 0.1% vol:vol DEPC (Sigma D5758), incubating overnight and then autoclaved at 131°C for 20 minutes. Gloves were worn throughout all procedures.

2.10.1 RNA extraction

RNA was extracted using a modified guanidine thiocyanate extraction method using Trizol® reagent (Life Technologies 15596) as previously described [92]. After removal of the test medium the cells were washed three times with PBS and the cells dispersed in 1.5ml Trizol® reagent. In this form the flasks could be stored at -20°C to await further processing. The cell suspension in Trizol was transferred to a 2ml plastic centrifuge tube and 300µl chloroform (200µl per ml Trizol) was added to each tube and vortexed for 15 seconds before standing at room temperature for 10 minutes. The sample was then centrifuged at 13000rpm for 15 minutes at 4°C and the upper aqueous phase removed to a

second 2ml plastic centrifuge tube. The RNA was precipitated by adding 750µl propan-2-ol (500µl per ml Trizol), vortexing, and standing for ten minutes at room temperature before again centrifuging at 4°C, 10000rpm for 10 minutes. The RNA pellet was washed by resuspending in 750µl 70% ethanol at -20°C and centrifuged again at 10000rpm for 5 minutes at 4°C. The RNA was then re-suspended in 20µl of DEPC water, heated in a waterbath at 65°C for ten minutes and stored at –70°C.

2.10.2 Quantification of RNA amount

2µl of RNA suspension was diluted in 400µl DEPC water. The optical density (OD) of the sample was measured using a Cecil spectrophotometer (CE 2040) at 260 and 280nm. The RNA purity was assessed by the ratio of OD_{260} to OD_{280}, and a value of 1.4-1.6 considered desirable. RNA concentration was calculated using the formula

$$\text{RNA concentration } (\mu g/\mu l) = \frac{OD_{260} \times \text{dilution factor} \times \text{extinction coefficient RNA}}{1000}$$

Dilution factor = 200

Extinction coefficient RNA = 40

2.10.3 Gel electrophoresis

Aliquots (30µg) of RNA were electrophoresed on a 1% agarose gel (Sigma A9539) containing 1.9% formaldehyde in MOPS buffer. 1.5g agarose was dissolved in 150ml MOPS buffer by heating in a domestic microwave at 750W for 2 minutes. This was allowed to cool before 7.8ml formaldehyde was added and the gel poured into a

electrophoretic cell and allowed to set. Sample buffer was prepared (appendix, Chapter 8.1) and frozen at –70°C in 300μg aliquots, and then 1.2μl 10mg/ml ethidium bromide (Sigma E1510) added before use. RNA samples were prepared by adding 25ml sample buffer to each 30μg aliquot of RNA and heated for 15 minutes at 65°C in a waterbath. Samples were loaded into the agarose gel, surrounded by an excess of MOPS buffer and electrophoresed for 4 hours at 80V. A representative example of resolved RNA showing the integrity of the ribosomal subunits is shown in Figure 2.5

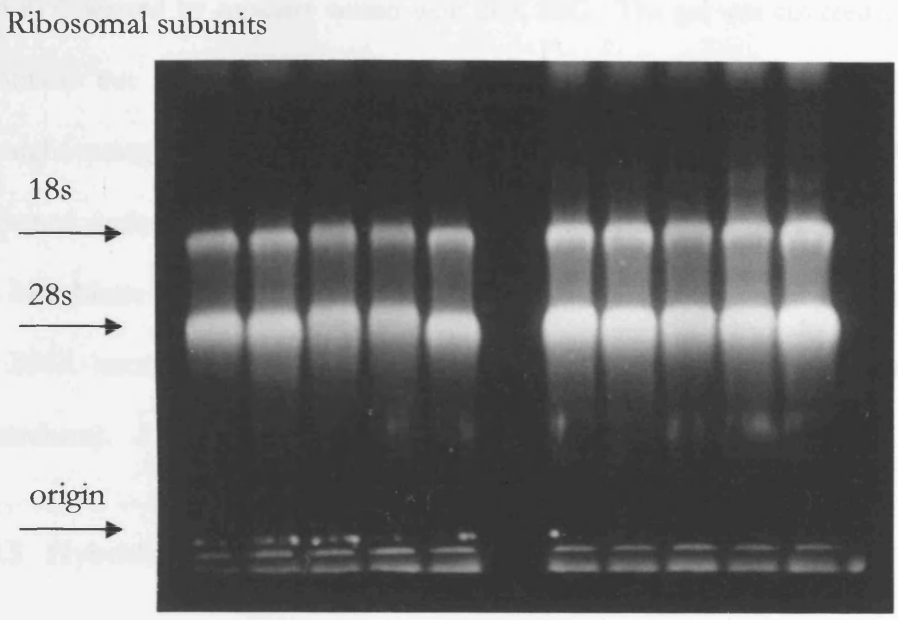


Figure 2.5 Resolved HPMC RNA showing ribosomal sub-units

2.10.4 Blotting

The resolved RNA was transferred onto Hybond™-N nylon membranes (Amersham, RPN 203N) by capillary action using 20X SSC. The agarose gel was washed for fifteen minutes three times in DEPC water before transfer to Whatman 3M filter paper (Whatman 1003 917) wetted by capillary action with 20X SSC. The gel was covered with Hybond-N membrane cut to size, filter paper, Quick-Draw™ paper (Sigma P6803), and pressed overnight using the weight of a glass plate. The following day RNA transfer was confirmed under UV light and the location of the RNA lanes marked on the membrane. The Membrane was rinsed in DEPC water, dried for 10 minutes in an oven at 80°C and the RNA cross-linked using a UV cross-linker designed for Hybond membranes (Amersham).

2.10.5 Hybridization of membrane with ^{32}P labeled cDNA probes

2.10.5.1 Source of cDNA probes

All cDNA probes were supplied contained in DNA plasmids transformed into viable E.Coli (see section 2.11). The E.Coli were stored at –70°C in 850µl of Luria Broth (Millers LB broth base, 25g/L, Life Technologies) with 150µl glycerol. In this state they could be stored for extended periods of time without loss of cell viability. All plasmids contained an ampicillin resistance gene to allow selection of E.Coli containing the cDNA of interest from wild-type E.Coli.

Fibronectin

The human fibronectin cDNA probe was generously supplied by the UK HGMP Resource Centre, Cambridge, U.K. The clone is a 2000bp cDNA insert in a pAT153/PvuII plasmid.

TGF-β

The TGF-β cDNA probe was supplied by the American Tissue culture collection (ATCC) and consisted of a 1050bp insert in a pSP64 plasmid.

Cyclophilin

Cyclophilins are a group of ubiquitous, highly conserved, abundant proteins believed to be the target for cyclosporin-A [93]. The cyclophilin probe was a gift from SmithKline Beecham Pharmaceuticals and consisted of a cDNA clone of human liver cyclophilin (pBHLCP11), and was used to correct for differences in RNA loading [94]. Cyclophilin is ideally suited as a comparator for extra-cellular matrix protein mRNA amount as it is a conserved, non-structural protein.

2.10.5.2 Labeling of cDNA with ^{32}P (Prime-a-gene®) and hybridization

Membranes were pre-hybridised in a hybridisation oven for 4 hours at 42°C with 200μg/ml denatured salmon sperm DNA in 50% formamide (Sigma F9037), 1% SDS (Sigma L4390), 5X Denhardt's (appendix, chapter 8.1), and 5X SSPE (Sigma S2015). cDNA probes were klenow DNA polymerase-labelled with, using a random primer labelling system (Prime-a-Gene®, Promega Ltd) as follows. Samples were all prepared on ice and the klenow returned swiftly to storage at –20°C. 5μl diluent buffer was added to 25ng cDNA and was heated for 10 minutes at 65°C in a waterbath. To a separate 1.5ml epindorph was added 10μl of supplied buffer, 2μl random nucleotides, and water to make a total final volume of 50μl. The two tubes were combined, and then 2μl BSA, 5μl [^{32}P]dCTP and 1μl klenow fragments were added. After one hour the labelled cDNA probe was separated from unincorporated ^{32}P-labelled nucleotides using a NICK™ column

(Pharmacia Biotech, 52-2076). The membranes were then hybridised overnight with cDNA probe in fresh buffer as above. After hybridisation the membranes were washed twice with 1% SDS, 2X SSPE at room temperature for ten minutes, and twice with 1% SDS, 0.2X SSPE at 65°C.

2.10.5.3 Detection of labeled membrane

Autoradiography was carried out in a specifically designated darkroom with red safety light. Washed membranes wrapped in cellophane were exposed to X-Omat LS film (Kodak) with intensifier screens at –70°C. After an appropriate exposure time judged by the radioactivity of the membrane using a Geiger counter the film was removed from the cassette and developed. The film was incubated in a 500ml Kodak GBX developer (Sigma P7042) for one minute with gentle agitation. The film was washed in tap-water and then incubated in Kodak GBX fixer and replenisher (Sigma P7167) for one minute. The film was rinsed and allowed to dry. Densitometric analysis of the transcripts was carried out on a BioRad GS 700 flatbed scanner and molecular analyst software.

2.10.6 Removal of hybridised probe

Following detection of the labelled probe membranes were wrapped in cling-film and stored at –20°C. 1L of boiling stripping solution (appendix, chapter 8.1) was prepared and was poured over the membranes in a plastic tray and allowed to cool with agitation. The membrane was then allowed to dry on Whatman 3MM filter paper and stored at +4°C wrapped in cling-film.

2.11 cDNA preparation

2.11.1 Storage and culture of E.Coli

Luria Broth (Millers LB broth base, Life Technologies) was prepared by dissolving 25g per litre DEPC water and autoclaving at 131°C for 20 minutes, and ampicillin was added at 50μg/ml. A small scraping of the E.Coli containing the cDNA of interest was added to a 30ml universal plastic container containing 20ml of the pre-prepared Luria broth. This was incubated at 37°C for 16hrs with gentle agitation until cloudy, and then added to 400ml of Luria broth in a 1000ml conical flask. This was then incubated for a further 24 hours at 37°C with agitation until cloudy before further processing.

2.11.2 Extraction and purification of plasmid (Maxipreps®)

Extraction of plasmid DNA from intact E.Coli was performed using a commercial kit (Wizard™ *Plus* Maxipreps DNA Purification system, Promega Ltd) following the manufacturers instructions. The 400mls of E.Coli in Luria broth was divided equally between two 250ml centrifuge bottles and pelleted by centrifuging at 5000g for 10 minutes in a room temperature SLA-1500 fixed angle rotor (Sorvall) in a RC 5B Plus high speed centrifuge (Sorvall). The supernatant was discarded and replaced by 15ml cell re-suspension solution, and the pellet re-suspended by pipetting up and down until completely re-suspended. 15ml Cell lysis solution was added and the suspension gently mixed by inversion until it became clear and viscous. 15ml neutralising solution was then added and mixed before centrifuging again at 14,000g for 15 minutes. The resulting supernatant was filtered using Whatman #1 filter paper, the volume measured and split between two 50ml centrifuge bottles. The DNA was precipitated from the solution by addition of half the volume of room temperature propan-2-ol and mixing by inversion.

The DNA was pelleted by centrifugation at 14,000g for 15 minutes in a SS-34 fixed angle rotor (Sorvall). The supernatant was discarded and the DNA re-suspended in 2ml of Tris-EDTA (TE).

10ml DNA purification resin was added and the resulting suspension was drawn through a Maxicolumn using a vacuum source. 25ml of column wash was then drawn through the column, followed by 5ml of 80% ethanol. The vacuum was allowed to draw for a further minute before the Maxicolumn was transferred to a 50ml screw cap tube and centrifuged in a swinging bucket rotor at 1,300g (2,500rpm) for 5 minutes. The Maxicolumn was connected again to the vacuum source and allowed to draw for a further five minutes. The DNA was then eluted from the column by adding 1.5ml of TE preheated to 65°C, and centrifuging again in a swinging bucket rotor in a screw top tube at 1,300g for 5 minutes. The resulting eluate was filtered by passing through a 0.22μm syringe filter into a 1.5ml tube.

The DNA concentration was quantified by mixing 4μl of this solution in 400μl DEPC water (1:100 dilution) and measuring the absorbance at 260 and 280nm. The DNA purity is assessed by measuring the ratio of the two absorbances (A_{260}/A_{280}) and should be greater than 1.6.

A DNA concentration of 50μg/ml has a A_{260} of 1.

Therefore the DNA concentration (μg/ml) = A_{260} x 100 (dilution) x 50.

The remaining plasmid was stored at –70°C.

2.11.3 Excision of cDNA from plasmid

Excision of the cDNA of interest from the plasmid was performed using the appropriate restriction endonucleases. These were incubated with their specified buffers for 90 minutes at 37°C with agitation. All restriction endonucleases and buffers were supplied by Sigma, U.K. Details of the enzymes used, concentration and the buffers are summarised in Table 2-3.

	Fibronectin	Cyclophilin	TGF-β
Plasmid (30μg)	75μl	29.5μl	119μl
Buffer I	Black Palette 15μl	Black Palette 10μl	Black Palette 20μl
Buffer II	-	-	-
Enzyme I	BamH III 15μl	EcoR1, 4μl	EcoR1, 10μl
Enzyme II	Hind III 22.5μl	Hind III, 10μl	-
BSA (B8894)	15μl	-	20μl
Water	7.5μl	46.5μl	31μl
Final volume	150μl	100μl	200μl

Table 2-3 cDNA restriction endonuclease summary

2.11.4 cDNA purification

A 1% agarose gel was prepared in Tris-Acetate-EDTA (TAE, Sigma T9650) by dissolving 1.5g agarose (Sigma A9539) in 150ml TAE in a 500ml conical flask and microwaving on full power in a 750W microwave for 2 minutes. The gel was allowed to cool to approximately 50°C, and 0.5μg/ml ethidium bromide (Sigma) was added before pouring into a gel tray (Flowgen). An eight well comb (11 x 2 x 10mm) was hung into the gel and the gel allowed to set for 30 minutes.

In order to identify the relevant size cDNA from the digested plasmid a λDNA/Hind III fragment ladder was prepared and electrophoresed with the plasmid DNA. 2μg of λDNA/Hind III fragments (Life Technologies, 15612-013) were heated in a 1.5ml tube with 5μl diluent buffer (appendix, chapter 8.1) at 65°C in a waterbath for 10 minutes. 5μl DNA loading buffer (appendix, chapter 8.1) was added to the ladder just prior to loading into the gel. The same volume of DNA loading buffer was added to the plasmid DNA as its original volume (i.e. 150μl in the case of the fibronectin cDNA). 450ml of TAE was poured over the gel tray in-order to completely cover the gel, the plasmid DNA in loading buffer was divided between the 3-4 wells, and the DNA ladder added to the final well of the gel. A constant voltage of 100V was applied across the gel for two hours to resolve the DNA. A typical gel is shown in Figure 2.6

2.11.5 Extraction of cDNA from agarose gel

The cDNA of interest was extracted from the agarose gel using a commercial kit (Sephaglas™ BandPrep Kit, Pharmacia Biotech) following the manufacturers instructions. cDNA of the correct size was excised from the gel using a scalpel in the minimum of gel possible. This was divided into 1.5ml plastic microcentrifuge tubes and weighed. The

volume of subsequent reagents required that less than 750mg of gel was added per tube. 1µl per mg gel of gel solubilizer was added to each tube and heated in a waterbath at 65°C until dissolved (5 minutes). 5µl of Sephaglas BP was added to each tube per estimated µg of DNA, and incubated at room temperature for 5 minutes, vortexing lightly every minute to re-suspend the Sephaglas. The tubes were then centrifuged in a bench-top microcentrifuge at 13,000rpm for 30 seconds and the supernatant discarded. The pellet was centrifuged again and any residual liquid removed. The pellet was then washed 3 times in Wash buffer, using 16X the volume of Sephaglas added each time, the pellet dispersed by vortexing and then re-pelleting by centrifugation. After 3 washed the pellet was allowed to air dry in the inverted tube for 10 minutes. Elution buffer (4X the volume of Sephaglas) was then added to each tube and the pellet re-suspended by gentle vortexing every minute for five minutes. The Sephaglas was then re-pelleted and the supernatant stored in a clean 1.5ml tube at –70°C.

2.11.6 cDNA quantification

A 1% agarose gel in TAE was prepared as previously described, and wells formed using a 16 well comb (5 x 1 x 10mm). 4µg, 2µg, 1µg, 0.5µg, 0.25µg of λDNA/Hind III fragments were mixed with 5µl diluent buffer (appendix1) and heated in a waterbath at 65°C for 10 minutes. 5µl of DNA loading buffer was added to each, and each added to a well in the gel, along with 5µl of the cDNA prepared above. These were electrophoresed at 100V for 2 hours, and the cDNA quantified by comparison with the fluorescence of each standard assessed using a video camera above a UV light source and the BioRad Gel-Doc software. A representative gel and an example of the calculation is shown in Figure 2.7.

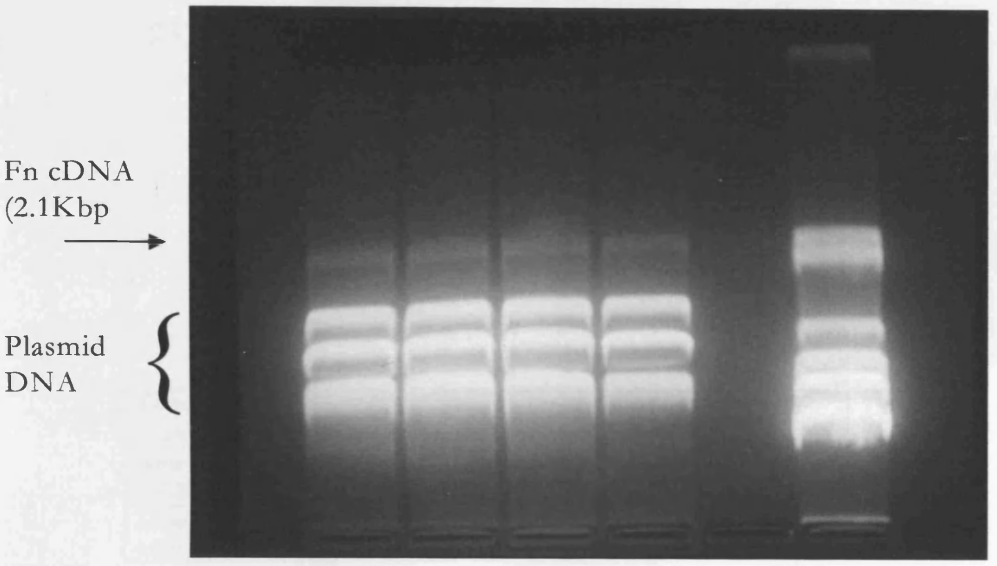
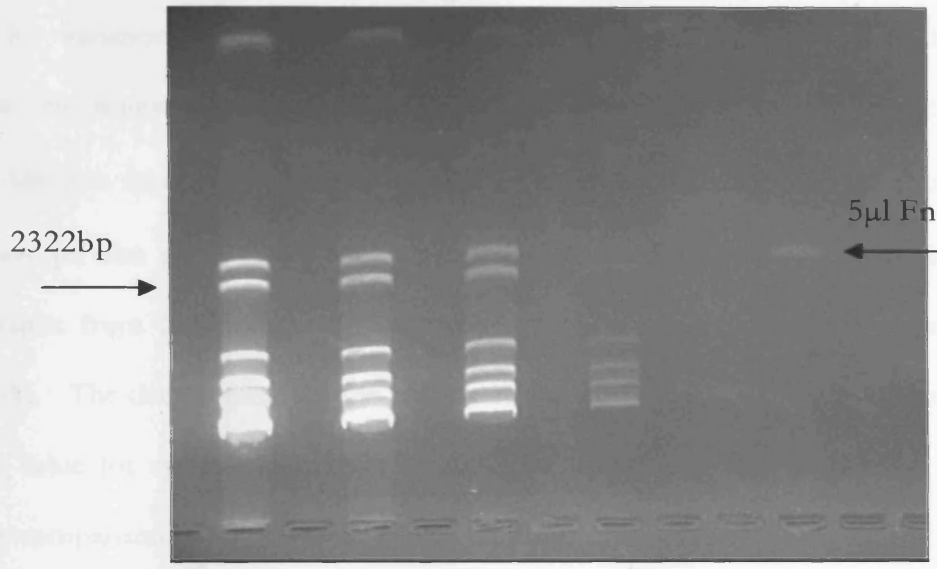


Figure 2.6 Resolved plasmid DNA showing Fibronectin cDNA of interest



Total amt λDNA (ng)	2	1	0.5	0.25	Unknown Fn cDNA
Amt DNA of 2322bp (ng)	95.7	47.8	23.9	12.0	-
Densitometric units	1435	576	322	100	108

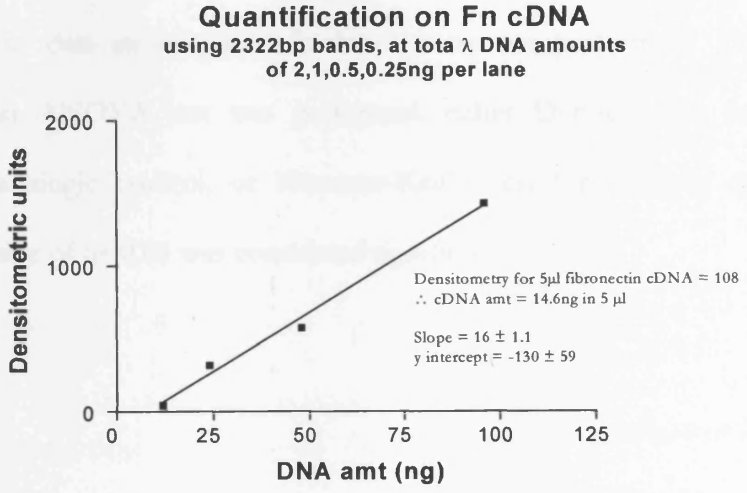


Figure 2.7 Quantification of fibronectin cDNA

2.12 Statistical analysis

To allow for variation in mesothelial cell fibronectin production between multiple experiments, the results are expressed as percentage increase over control (5mmol/L glucose). Absolute levels of fibronectin and TGF-β protein corrected for cell protein concentration are also given in the text where appropriate. Densitometric analysis of autoradiographs from Northern blots were analysed using Molecular analyst software (BioRad Ltd.). The density value for the mRNA of the protein of interest was divided by the density value for cyclophilin mRNA to allow for variation in mRNA loading, and hence allow comparison of mRNA amount between different test conditions.

All data are expressed as mean ± standard error. Comparison between two or more groups was performed using a one-way analysis of variance (ANOVA). If a statistically significant result was apparent by ANOVA, and inspection of the data suggested that a dose response existed, then a post-test analysis for linear trend was performed. For single comparisons between two sets of data an un-paired Student's t-test was performed. For multiple comparisons a post ANOVA test was performed, either Dunnet's test for repeated comparison with a single control, or Newman-Keul's test for multiple comparisons between data. A value of $p<0.05$ was considered significant.

3 Effects of Glucose on mesothelial cells

3.1 Introduction

Long-term exposure to peritoneal dialysis solutions has been associated with changes in the peritoneum, including the deposition of excess matrix protein [29]. Peritoneal mesothelial cells are the most abundant cell type in the peritoneum and as previously discussed are known to produce the extra-cellular matrix proteins fibronectin and collagens I and III [79]. It is a reasonable assumption therefore that the peritoneal mesothelial cell may contribute to the production or regulation of extra-cellular matrix in the peritoneum.

The most common peritoneal dialysate (1.36% PD4) contains 76mmol/L D-glucose. In many other cell culture systems, high glucose concentration has been associated with an increase in ECM. This has been shown *in-vitro* with endothelial cells [95], human proximal tubular cells (HPTC) [96], fibroblasts [97] and mesangial cells [98].

Previous work has shown that HPMC are influenced by hyperosmolar glucose in culture medium [99,100]. These studies suggested that hyperosmolar glucose inhibited mesothelial cell proliferation and cause an increase in extra-cellular matrix. However, the experiments were conducted over 3 to 6 days; a situation that does not exist on long-term CAPD with the dialysis solution replaced every 6-12 hours. In addition they were conducted using culture medium; quite different in constituents to dialysis solution which also contains a high lactate concentration and low pH. Culture medium is rich in growth factors, and hence will not reflect the peritoneal microenvironment in CAPD.

High glucose concentrations are known to form part of the pathogenesis of diabetes mellitus, where the serum (and urinary) glucose concentration is elevated for prolonged periods. The pathophysiological mechanism by which hyperosmolar glucose influences cell functions has therefore been the subject of previous investigation. The polyol and the

hexosamine glycolytic pathways have both been implicated in the pathogenesis of diabetes mellitus and have been studied separately to investigate the differential effect on cell function [96,101]. In the polyol pathway (Figure 3.1), D-glucose is converted to sorbitol by the rate limiting enzyme aldose reductase utilising NADPH. Sorbitol is then converted in a non-rate limiting manner to D-fructose by sorbitol dehydrogenase. Increased levels of sorbitol have been detected in HPMC exposed to high glucose conditions [102] In addition, inhibiting the polyol pathway in proximal tubular cells can prevent glucose induced fibronectin accumulation [103], suggesting that the polyol pathway has a pathogenic role in ECM deposition in response to hyperosmolar glucose.

The hexosamine pathway is illustrated in Figure 3.1, and is a method of generating amino sugars for the synthesis of glycoproteins, glycolipids, and proteoglycans. Glucose enters the cell through the glucose transporter and is metabolised to glucose-6-phosphate by hexokinase and then on to fructose-6-phosphate. The hexosamine biosynthetic pathway then separates from the glycolytic pathway by using fructose-6-phosphate to form glucosamine-6-phosphate. Glutamine is utilised as the donor of the amino group. In the hexosamine pathway the rate-limiting enzyme is glutamine: fructose-6-phosphate-amidotransferase. D-glucosamine (2-amino-2-deoxy-D-glucose) has been widely used in the investigation of the hexosamine pathway. It can also enter the cell through the glucose transporter and is rapidly phosphorylated by hexokinase yielding glucosamine-6-phosphate. Unlike glucose, glucosamine thereby bypasses the first, rate-limiting step of the hexosamine biosynthetic pathway, and hence offers a method of investigating the effect of increased flux through this pathway.

Unlike the polyol pathway there is a less established link between increased flux in the hexosamine pathway and complications in diabetes mellitus. However, work in mesangial

cell culture has shown that adding glucosamine at 12mmol/L resulted in significant translocation of protein kinase C (PKC) [104], and in both mesangial cells and also proximal tubular cells stimulate an increase in the mRNA for the pro-fibrotic growth factor TGF-β [105]. During CAPD the peritoneum is also exposed to high glucose concentration for prolonged periods, and the environment could be considered similar to that present in poorly controlled diabetes mellitus. It is possible to speculate therefore that either of these two glucose metabolic pathways, both of which have been associated with pro-fibrotic events, could be key to any effect of hyperosmolar glucose on HPMC.

The aim of the following experiments was to investigate the effect of hyperosmolar glucose on fibronectin production, and any differential effect of glucose metabolism via the different glycolytic pathways. The glucose concentration range studied was between 5 and 40mmol/L to allow assessment over a patho-physiological concentration range. A 40mmol/L glucose solution represents the effect of diluting 1.36% glucose dialysate (76mmol/L glucose) with culture medium.

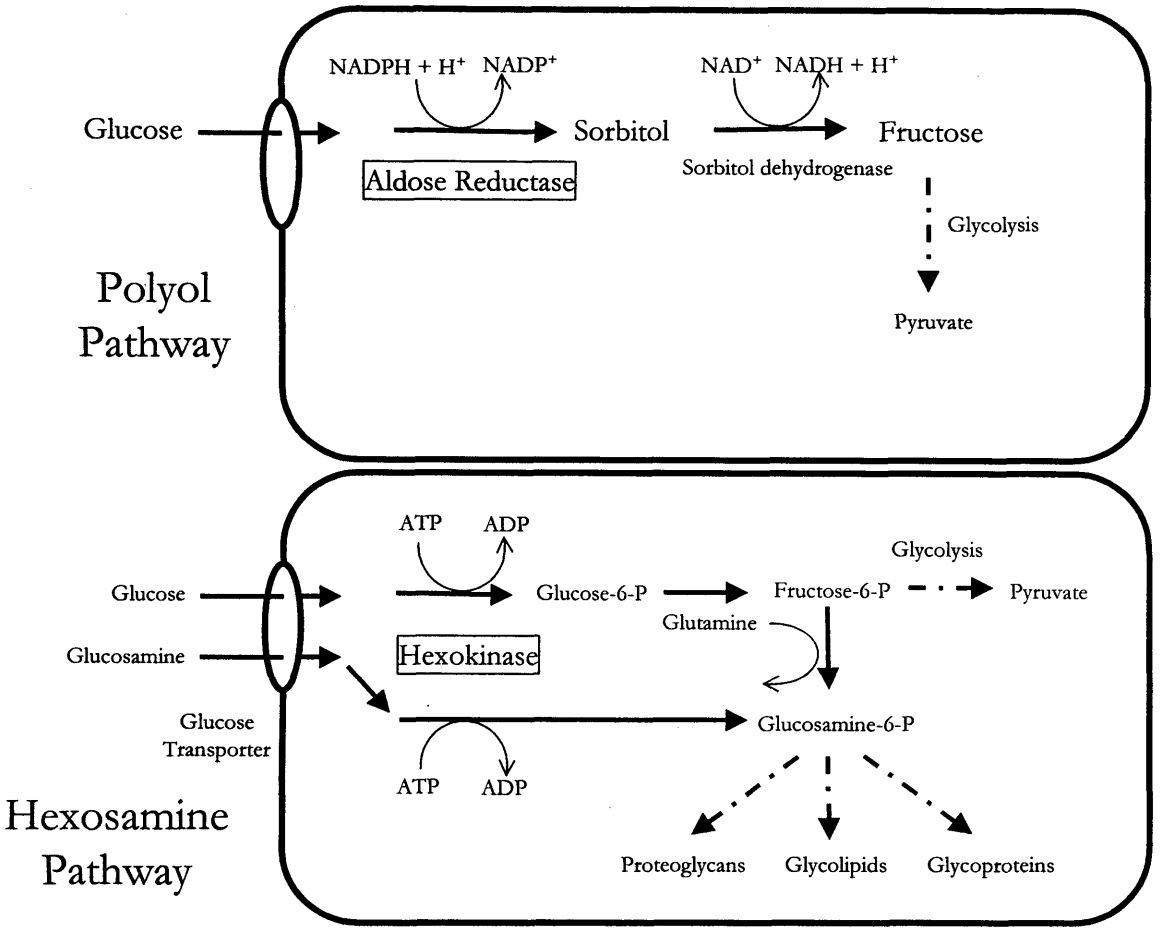


Figure 3.1 Polyol and Hexosamine pathways

3.2 Cell viability

3.2.1.1 Materials and Methods

Quiescent HPMC on twelve well plates were exposed to a 50:50 mixture of dialysate and M199 with 0.1%FCS. The dialysate used was a laboratory manufactured, filter sterilised dialysate of identical electrolyte composition and pH to the widely used commercial dialysate PD4 (Baxter Healthcare Ltd). The use of this laboratory manufactured dialysate enabled adjustment of the final test medium added to mesothelial cells to a D-glucose concentration of between 5-40mmol/L. Mannitol at 40mmol/L was used as an osmotic control. Experiments were also performed using commercial 1.36% glucose dialysate diluted 50:50 with medium to allow comparison with the laboratory manufactured dialysate. A 1.36% glucose solution contains 76mmol/L D-glucose, and hence the 40mmol/L final glucose concentration in the laboratory manufactured test medium corresponds approximately with a 50:50 dilution of this commercial solution with culture medium. Further experiments were performed using 3.86% commercial dialysate to investigate cell viability at higher glucose concentration. Cells were cultured in test medium for 12 hours and for 72 hours. LDH activity was measured in the cell culture supernatant and cell digest as described in section 2.9.

3.2.1.2 Results

Increasing osmolality resulted in an incremental rise in LDH release and hence decrease in cell viability. This was effect present both at 12 hours (Figure 3.2) and 72 hours (Figure 3.3). The effect was independent of glucose concentration, with both mannitol and D-glucose producing the same decrease in cell viability. LDH release was comparable between laboratory manufactured, and commercial dialysate at the same final glucose

concentration of 40mmol/L (6.04±1.33% vs. 5.12 ± 0.62% LDH release respectively). The 50:50 mixture of commercial 3.86% PD4 resulted in significant cytotoxicity (10.6±1.71% LDH release vs. 2.63±0.69% control), and for this reason was not used in subsequent experiments.

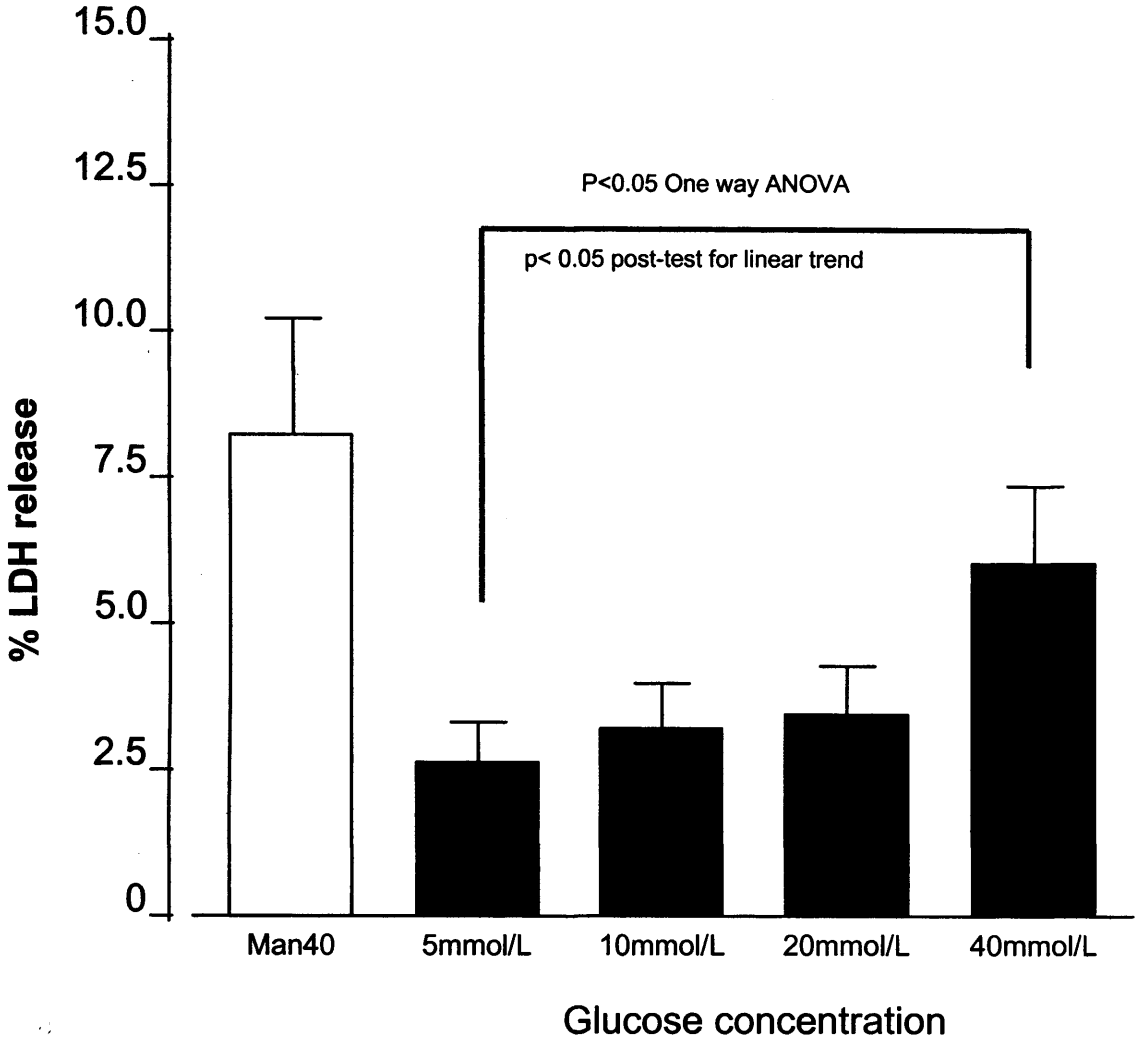


Figure 3.2 Effect of glucose on HPMC LDH release at 12 hours

Laboratory manufactured dialysate was diluted 50:50 with M199. The final glucose concentration was as shown. Mannitol 40mmol/L osmotic control shown in open bars. Increasing osmolality, results in a decrease in cell viability independent of glucose concentration. Results are percentage LDH activity in culture supernatant / total LDH activity expressed as means ± SEM of 3 experiments in duplicate. P<0.05 post ANOVA test for linear trend

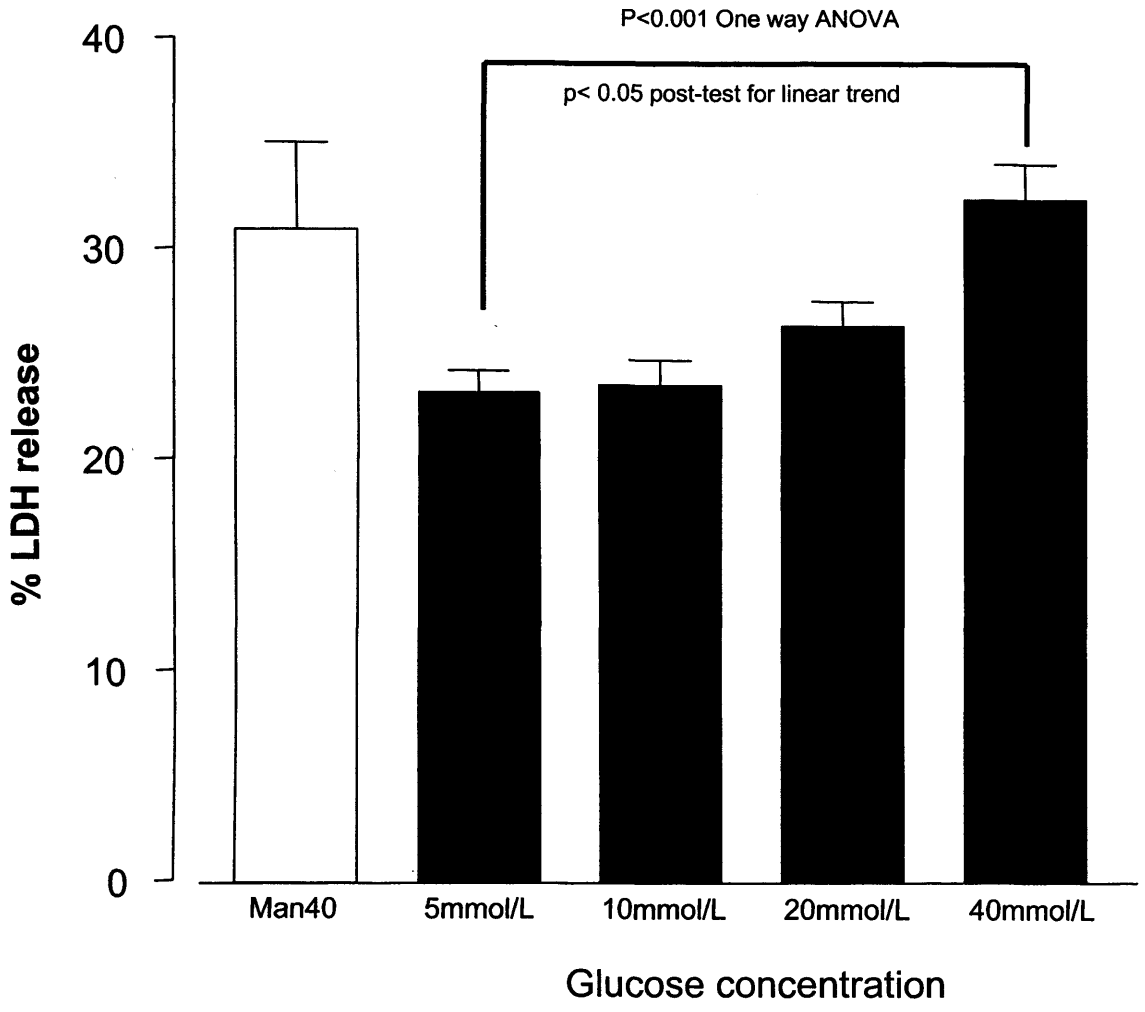


Figure 3.3 Effect of glucose on HPMC LDH release at 72 hours

Laboratory manufactured dialysate was diluted 50:50 with M199. The final glucose concentration was as shown. Mannitol 40mmol/L osmotic control shown in open bars. Increasing osmolality, results in a decrease in cell viability independent of glucose concentration. Results are percentage LDH activity in culture supernatant / total LDH activity expressed as means ± SEM of 3 experiments in duplicate. $P<0.05$ post ANOVA test for linear trend

3.3 Proliferation

3.3.1.1 Materials and methods

Quiescent HPMC in collagen coated 12 well plates were exposed to a 50:50 mixture of dialysate and M199 with 0.1% FBS. The dialysate used was a laboratory manufactured dialysate of identical composition to PD4 but with no D-glucose. The final concentration of glucose was adjust to either 5mmol/L or 40mmol/L by addition of D-glucose. Two different protocols were used.

A HPMC were exposed to test medium for 12 hours before this was removed and replaced with fresh test medium containing 1μCi/ml ^{3}H-thymidine for a further 6 hours. 3H-thymidine incorporation was then measured as described in section 2.8.

B HPMC were exposed to test medium for 32 hours before 1μCi/ml ^{3}H-thymidine was added to each well and allowed to incubate for a further 16 hours. ^{3}H-thymidine incorporation was then measured as described in section 2.8.

3.3.1.2 Results

The results of both experiments are shown in Table 3-1. During the longer 48 hour protocol hyper-osmolar glucose test medium caused a significant decrease in HPMC proliferation rate, although there was no significant effect on ^{3}H-thymidine incorporation using the 12 hour protocol.

	12 hour protocol	48 hour protocol
5mmol/L glucose	16700 ± 2270	60500 ± 4270
40mmol/L glucose	14700 ± 3250	37400 ± 4570

Table 3-1 HPMC 3H-thymidine incorporation. Effect of glucose

Laboratory manufactured dialysate was diluted 50:50 with M199. Final glucose concentration was as shown. 40mmol/L glucose test medium caused a decrease in proliferation rate in the 48 hour protocol ($p<0.01$ unpaired t-test). No significant effect was observed in the 12 hour experiment. Results are expressed as DPM ± SEM of four experiments in triplicate.

3.4 Fibronectin protein production and gene expression

3.4.1 Effect of glucose on fibronectin protein production

3.4.1.1 Materials and methods

HPMC in 12 well plates were rendered quiescent before exposure to a 50:50 mixture of dialysate and M199 with 0.1% FBS. Time and dose response on fibronectin production was considered.

A Time course of fibronectin production

Glucose concentration in the test medium was adjusted to 5mmol/L or 40mmol/L by addition of D-glucose. 40mmol/L mannitol was used as an osmotic control. Test medium was applied to the quiescent cells and incubated for a total of 72 hours. Small samples of the supernatant were removed at 12, 24,48 and72 hours and assayed for fibronectin by ELISA as described in section 2.4.1. Results are expressed correct for total cell protein as described in section 2.6.

B Glucose dose response on fibronectin production

Glucose concentration test medium was adjusted to 5, 10, 20 or 40 mmol/L by addition of D-glucose. 40mmol/L mannitol was used as an osmotic control. Test medium was applied to the cells for 12 hours and the supernatant fibronectin measured by ELISA as described in section 2.4.1. Results were expressed corrected for total cell protein as described in section 2.6.

3.4.1.2 Results

The effect and time course of hyper-osmolar glucose on fibronectin production by HPMC is shown in Figure 3.4. Fibronectin amount increased in test medium with time. 40mmol/L glucose caused an increase in fibronectin production compared with 5mmol/L glucose at the 12 and 48 hour time points. In addition, there was a trend toward higher fibronectin amount at 40mmol/L glucose at the other two time points, but this did not reach statistical significance. 40mmol/L mannitol did not result in an increase in fibronectin amount suggesting that the effect of glucose concentration is specific and not the effect of the osmolality of the test solution. Although the effect of high glucose concentration is apparent at time points longer than 12 hours the majority of subsequent experiments were conducted at this time point. Twelve hours was chosen to best mimic the overnight CAPD dwell, as longer dwells without replacement of dialysate do not occur in clinical practice.

The effect of different glucose concentration on fibronectin production by HPMC is shown in Figure 3.4. Results are expressed corrected for total cell protein which did not vary between test conditions (Table 3-2). Basal fibronectin production (5mmol/L glucose concentration) was 2.03±0.21μg/mg cell protein. Increasing glucose concentration resulted in a dose dependant rise in fibronectin production over the concentration range 5-40mmol/L with a 15.4±4.6% increase at 40mmol/L D-glucose. Fibronectin production with 40mmol/L mannitol was no different to that with 5mmol/L glucose control, suggesting that the effect is due to increasing glucose concentration, and not the result of increasing osmolality.

	Protein amount (μg)
5mmol/L glucose	60.3 ± 8.85
10mmol/L glucose	65.6 ± 8.80
20mmol/L glucose	63.3 ± 9.06
40mmol/L glucose	58.9 ±8.35
40mmol/L mannitol osmotic control	66.2 ± 10.7

Table 3-2 Protein amount with increasing glucose concentration

Increasing glucose concentration had no effect on mean total protein content despite the previously demonstrated decrease in viability and proliferation in the hyperosmolar glucose condition. Results are mean total cell protein content of the culture wells ± SEM for 4 experiments in triplicate

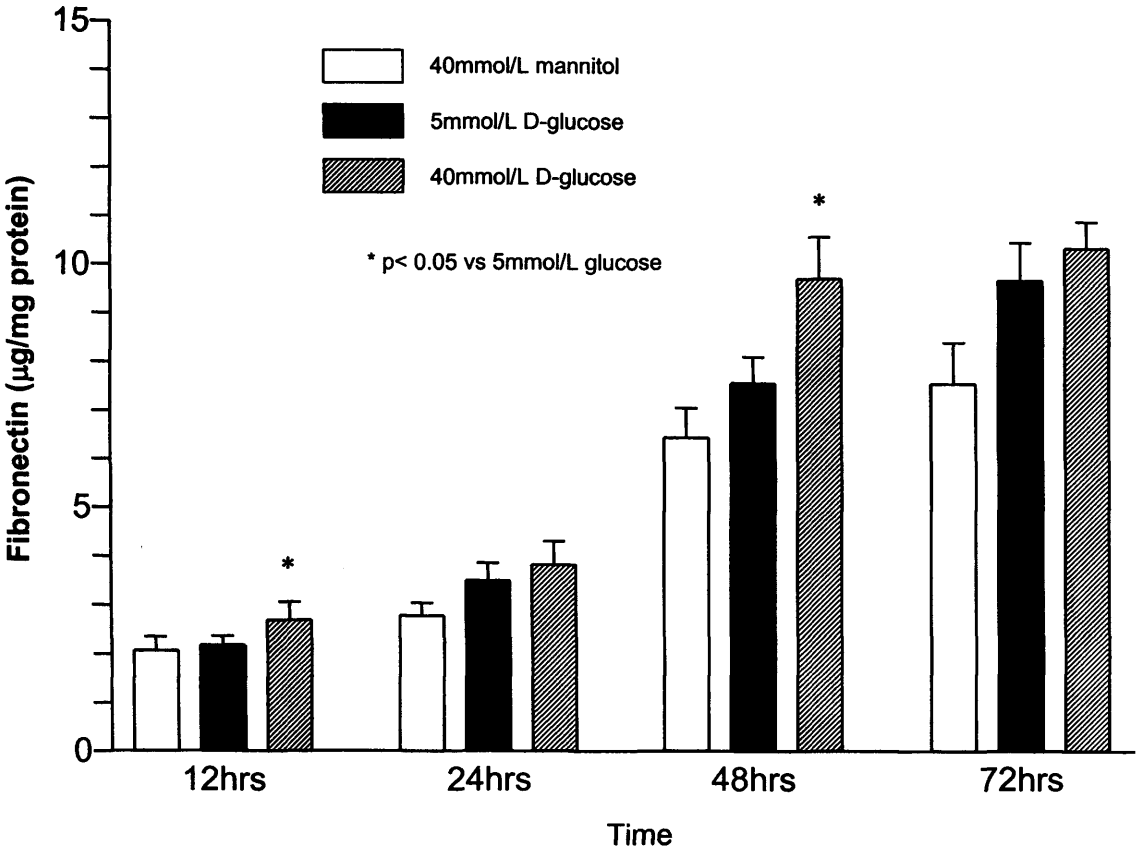


Figure 3.4 Effect of glucose on the time course of fibronectin protein production

40mmol/L Laboratory manufactured dialysate was diluted 50:50 with M199. The final glucose concentration was as shown. Fibronectin amount in test medium increased with time in all test conditions ($p<0.05$ one way ANOVA with post test for linear trend). 40mmol/L glucose resulted in an increase in fibronectin protein production at 12 and 48 hours. . Results shown as percentage increase in fibronectin expressed as means ± SEM of 3 experiments in triplicate. $p<0.05$ unpaired t-test at 12 and 48 hours.

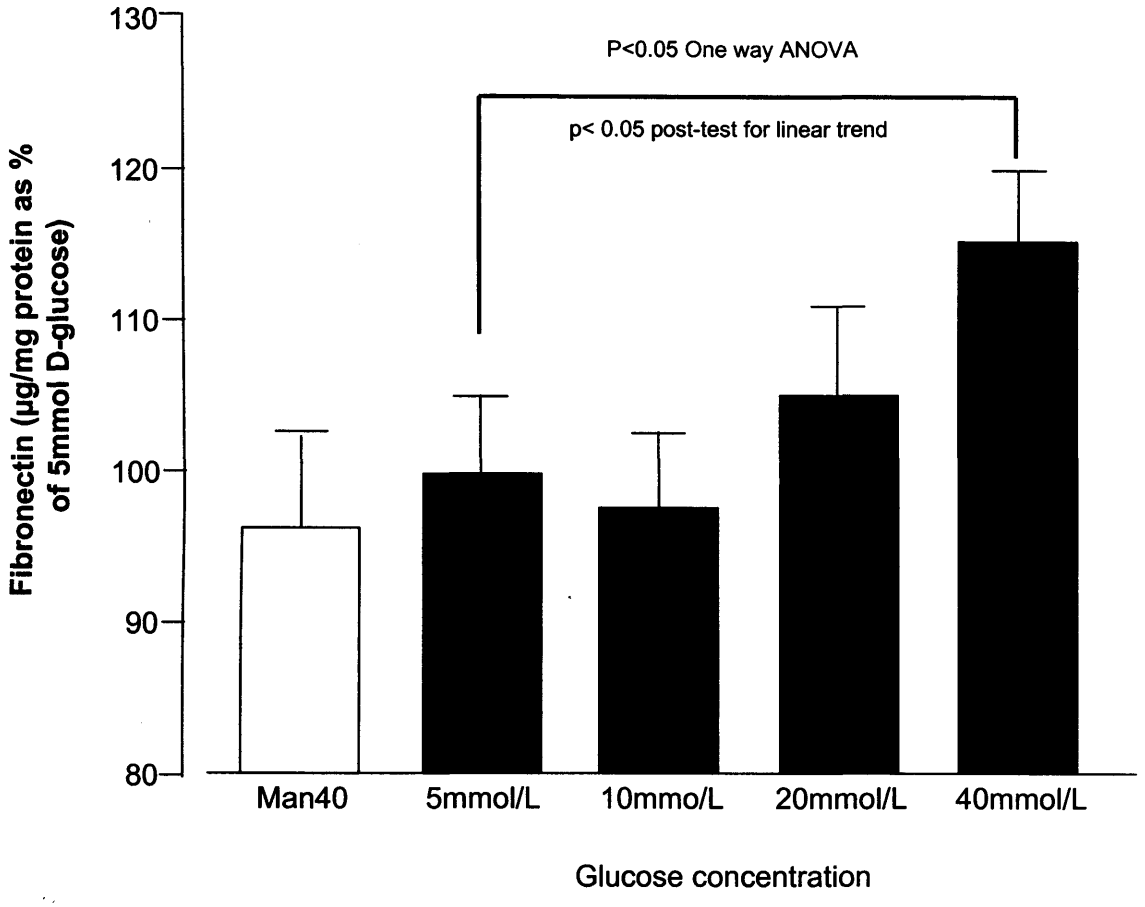


Figure 3.5 Effect of glucose on fibronectin protein production at 12 hours

Laboratory manufactured dialysate was diluted 50:50 with M199. The final glucose concentration was as shown. Mannitol 40mmol/L osmotic control shown in open bars. Increasing glucose concentration results in an increase in fibronectin production by HPMC. Results shown as percentage increase in fibronectin corrected for total cell protein expressed as means ± SEM of 4 experiments in triplicate. $p<0.05$, post ANOVA test for linear trend

3.4.2 Effect of glucose on fibronectin mRNA

3.4.2.1 Materials and Methods

Confluent HPMC cultured in 25cm^2 flasks were rendered quiescent by 48hours exposure to M199 with 0.1% FBS. The medium was then replaced by test medium for 12 hours. Test medium was a 50:50 mixture of dialysate and M199 with 0.1% FBS. The dialysate used was a laboratory manufactured dialysate of identical composition to PD4 but without D-glucose. The final glucose concentration of the test medium was adjusted to either 5mmol/L or 40mmol/L by addition of D-glucose. Total messenger RNA was extracted as described in section 2.10.1, and quantified in the two test conditions by Northern analysis.

3.4.2.2 Results

Figure 3.6 shows a representative Northern blot for fibronectin mRNA levels in the 5mmol/L and 40mmol/L glucose conditions, and a graphical representation of the differences. Results are normalised for cyclophilin mRNA levels to account for variations in RNA loading. Hyperosmolar glucose test medium stimulated a 1.4±0.09 fold increase in fibronectin mRNA levels.

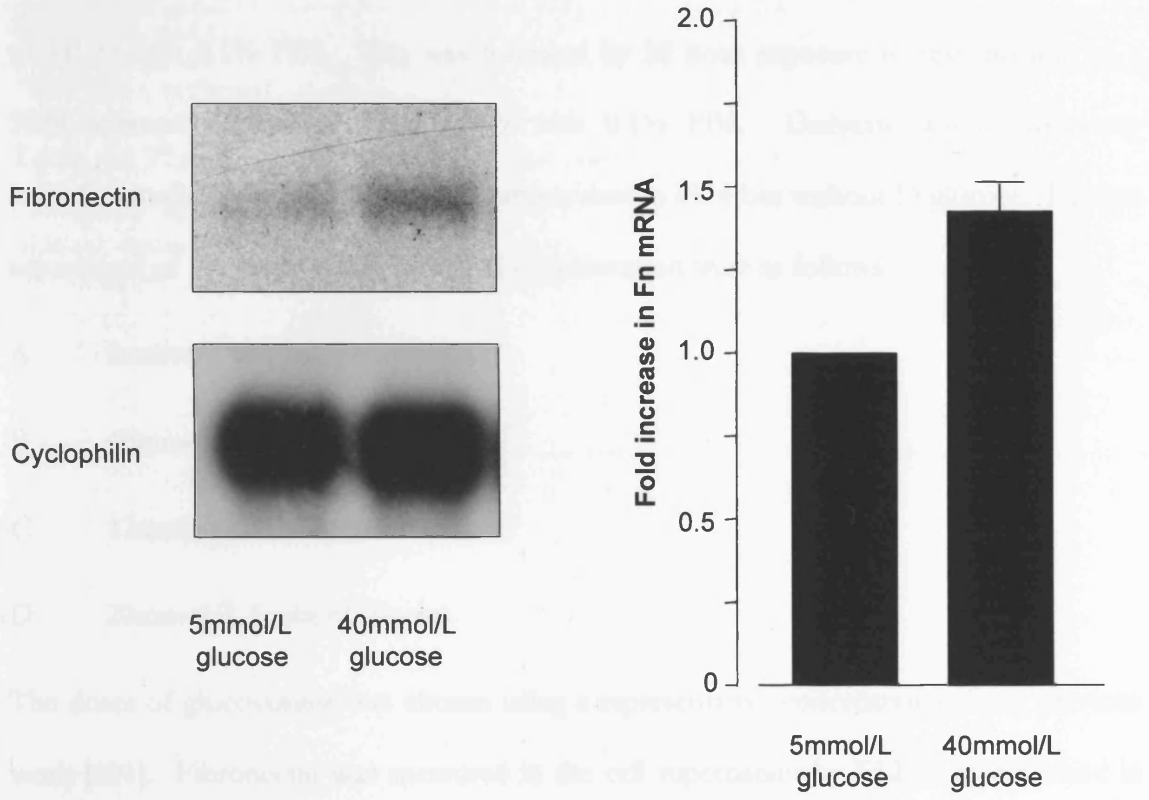


Figure 3.6 Effect of glucose concentration on fibronectin gene expression

Laboratory manufactured (LAB) was diluted 50:50 with M199. The final glucose concentration was as shown. Increased glucose concentration results in a significant increase in fibronectin gene expression. Results shown as fold increase in densitometric units for three experiments.

3.5 Sorbitol and hexosamine glycolytic pathways

3.5.1.1 Materials and methods

Confluent HPMC cultured in 12 well plates were rendered quiescent by 48 hours exposure to M199 with 0.1% FBS. This was followed by 12 hour exposure to test medium of a 50:50 mixture of dialysate and M199 with 0.1% FBS. Dialysate was a laboratory manufactured dialysate with identical composition to PD4 but without D-glucose. Further adjustment of the test medium to a final concentration were as follows

A 5mmol/L D-glucose

B 40mmol/L D-glucose

C 12mmol/L Glucosamine (Sigma)

D 20mmol/L Sorbitol (Sigma)

The doses of glucosamine was chosen using a representative concentration from previous work [101]. Fibronectin was measured in the cell supernatant by ELISA as described in section 2.4.1, and total protein measured in the cell digest (section 2.5 and 2.6).

3.5.1.2 Results

The results of adding either hyperosmolar glucose or the intermediates from the two glycolytic pathways are shown in Table 3-3. As previously described in section 3.4.1 hyperosmolar glucose dialysate caused an increase in HPMC fibronectin production. Sorbitol reproduced the increase in fibronectin seen with 40mmol/L glucose with a 120 ± 12.3% increase in fibronectin. Glucosamine at 12mmol/L produced amounts of fibronectin similar to those under control conditions.

	Fibronectin (ng/mg protein as percentage of 5mmol/L glucose condition)
5mmol/L D-glucose	100 ± 3.31
40mmol/L D-glucose	112 ± 4.47
12mmol/L glucosamine	98.9 ± 6.49
20 mmol/L sorbitol	120 ± 12.3

Table 3-3 The effect of glucosamine and sorbitol on fibronectin production

Laboratory dialysate was diluted 50:50 with M199. Both hyperosmolar glucose and sorbitol caused an increase in HPMC fibronectin production ($p<0.05$ unpaired t-test). Glucosamine did not produce this effect. Results shown as percentage increase in fibronectin corrected for total cell protein expressed as means ± SEM of 3 experiments, with 3-6 wells per experimental condition.

3.6 Summary

These experiments considered the effect on peritoneal mesothelial cells of altering glucose concentration. The test medium was a lactate-based dialysate modified by mixing 50:50 with M199 and the addition of 0.1% FBS. In this manner the experiments have avoided the direct cytotoxicity of neat dialysate, whilst maintaining a similar composition to the peritoneal dialysate on CAPD. The following effects have been shown

1. Increasing osmolality of the test medium resulted in a decrease in cell viability measured by LDH release. The effect was not the effect of increasing glucose concentration of the test medium, and present at both a short (12 hour) and prolonged (72 hour) time point.

2. 40mmol/L D-glucose inhibited HPMC proliferation compared with 5mmol/L D-glucose after 48hours. No effect was observed at a 12 hour time point

3. Increasing glucose concentration resulted in an rise in fibronectin amount and an increase in mRNA levels in HPMC. The increase in fibronectin was independent of the osmolality of the solution with no increase in fibronectin seen with 40mmol/L osmotic control.

4. Sorbitol, an intermediary in the polyol pathway stimulated a similar increase in fibronectin as found with 40mmol/L glucose. Glucosamine, an intermediary of the hexosamine pathway did not have this effect.

3.7 Conclusion

Hyperosmolar glucose, lactate based dialysate is the most widely used dialysis solution for patients on CAPD, and hence it is important to understand the effects of this solution on the resident peritoneal cells. These data suggests that the high glucose concentration of the solution may have a crucial role in promoting extra-cellular matrix (ECM) production by the mesothelial cells lining the peritoneal cavity.

Increasing glucose concentration resulted in an increase in both fibronectin protein production and gene expression by HPMC. This effect was independent of the increase in osmolality of the dialysis solution. In contrast the increase in LDH release and hence decrease in cell viability of mesothelial cells appears to be purely the result of increasing osmolality of the solution, with no specific effect of high glucose concentration. The results show that above 40mmol/L final glucose concentration cell viability falls dramatically. In clinical practice these glucose concentrations are not sustained as there is a progressive decline in glucose concentration over the duration of a CAPD dwell, with a mean decrease to only 38% initial glucose concentration in four hours during a PET [87]. For these reasons further experiments were restricted to the pathophysiological concentration range of 5-40mmol/L glucose final concentration. Results of fibronectin protein production were expressed corrected for total cell protein, and although cell viability decreased there was no detectable change in total cell protein. The changes in fibronectin protein production cannot therefore be accounted for simply by changes in either cell viability or total cell protein.

In the twelve-hour protocol a 15% increase in fibronectin protein production, and 1.4 fold increase in gene expression was observed. Although this represents a relatively small change it must be remembered that patients on CAPD will be repeatedly replacing dialysis bags 4 to 5 times per day, and hence any small change in synthesis over twelve hours

potentially would result in considerable increase in extra-cellular matrix accumulation over many years of CAPD. Although no *in-vitro* cell culture protocol can truly mimic the condition *in-vivo,* the twelve-hour time point was chosen to mimic the long overnight CAPD dwell, after which *in-vivo,* a patient would change the dialysate for a fresh solution. Longer dwells without replacement of dialysate do not occur in clinical practice. The same effects to high glucose concentration are observed with longer time-courses indicating that there is little adaptation to the effect of high glucose even over longer dwells.

In addition to promoting extra-cellular matrix protein production, hyperosmolar glucose caused inhibition of HPMC proliferation. This effect was only seen after 48 hours exposure to test medium, with no difference detected after 12 hours exposure. This may reflect an adaptive process that has to occur before proliferation is inhibited, or may reflect a relatively slow rate of basal rate proliferation making it difficult to detect after a short time period. A further possible explanation may lie in the difference in experimental design. In the 12 hour protocol the medium was changed and replaced with fresh test medium containing ^{3}H-thymidine. In the following chapter, the role of autocrine production of growth factors will be considered. It is possible that replacing the medium resulted in removal of growth factors which had been stimulated by the hyperosmolar glucose and hence the inhibition of proliferation. Other authors have subsequently published data supporting this finding with 48 hour incubation [106], although they required much greater glucose concentration (75 to 222mM) to demonstrate the effect.

It is now recognised that the regulation of ECM turnover is the product of both alterations in protein synthesis, and protein degradation. The key elements in ECM protein degradation are the metalloproteinases and the tissue inhibitors of metalloproteinases (TIMPs). Fibronectin, investigated here, is a known substrate for MMP3 (stromelysin), and this is inhibited by TIMP I. The increase in fibronectin mRNA levels, coupled with

the increase in protein levels seen in this study is evidence, at least in part, for an increase in extra-cellular matrix synthesis. However a contribution from a decrease in degradation cannot be excluded on the basis of these results. HMPC have been shown to constitutively produce MMP2 and MMP3 [107]. Hyperosmolality (either with D-glucose or mannitol) has been shown to produce a decrease in MMP9 activity *in-vitro* [108]. In addition used peritoneal dialysate has been shown to increase MMP9 secretion by HPMC in culture and this could be inhibited by co-incubation with an IL-1 receptor antagonist [107]. In the cell culture system here, cells were exposed to a high glucose concentration (and hence osmolality), which has previously been associated with a decrease in metalloproteinase activity [108]. Although it is impossible to comment on the extra-cellular matrix degradation in these experiments it is clear from our results that the balance of production and degradation in this model of CAPD favours an increase in extra-cellular matrix.

In mesangial cell culture glucosamine has been shown to increase TGF-β production. TGF-β is known to stimulate ECM production in other cultured cells, and it was expected therefore that glucosamine might stimulate mesothelial cell ECM protein production. In this culture system however, glucosamine does not appear to stimulate fibronectin protein production. Therefore, it is unlikely that the increase in fibronectin observed with high glucose concentration is the result of an increase in flux through the hexosamine pathway.

Sorbitol, an intermediary in the metabolism of glucose by the polyol pathway, has been shown to accumulate in both in cultured mesangial [109] and mesothelial cells [102] exposed to high glucose concentrations. Aldose-reductase is a key regulatory enzyme in the polyol pathway. In human proximal tubular cells, inhibition of aldose-reductase prevented the glucose induced increase in TIMP production thought to be responsible for

the net increase in collagen and fibronectin observed. Consistent with this, the experiments described here show that sorbitol stimulates mesothelial cell production of fibronectin. In the mesangial cell it has been proposed that increased polyol pathway activity facilitates *de novo* formation of diacylglycerol (DAG) from glucose by donating NADH formed during the conversion of sorbitol to fructose [110]. DAG has previously been shown to activate protein kinase C, and in turn promote the deposition of ECM. Further work in Leicester has shown that the increase in fibronectin seen in HPMC exposed to high glucose concentration can be inhibited by the aldose reductase inhibitor zopolrestat (generous gift from Pfizer Pharmaceutics Ltd) [111]. It appears likely therefore that the polyol pathway has a pivotal role in the regulation of HPMC ECM exposed to elevated glucose concentration.

In conclusion therefore increasing glucose concentration causes HPMC to undergo a phenotypic change from proliferation with little ECM production, to a cell that undergoes little proliferation, but is stimulated to produce ECM. Metabolism of glucose through the polyol pathway, with accumulation of sorbitol appears to be partly responsible. This adaptation may contribute to the peritoneal fibrosis seen in long-term CAPD with glucose containing dialysate.

In the following chapter the role of the known pro-fibrotic growth factor TGF-β in orchestrating this alteration in phenotype will be explored.

4 The role of TGF-β in glucose induced ECM regulation

4.1 Introduction

Transforming growth factor beta (TGF-β) is a well characterised cytokine growth factor implicated in the regulation of tissue repair and remodelling. It is synthesised as a 391 amino acid precursor which is cleaved to a 112 amino acid subunit and associated peptide fragments. TGF-β is secreted from cells in a latent form in which TGF-β1 is non covalently bound to a "latency associated peptide" consisting of the peptide fragments from its earlier cleavage. Latent TGF-β is stored at the cell surface and in the ECM before conversion to an active 25kD dimeric protein by an unknown mechanism [112].

TGF-β has been shown to have many different cell functions, and is present in three isoforms (TGF-β1,2 and 3) with similar biological properties. TGF-β1 has been most studied, and therefore the most strongly implicated regulating tissue fibrosis. In addition to having three isoforms, TGF-β can bind to at least three different membrane receptors (types I,II and III) which are present in almost all cells. The type I and type II receptors are transmembrane serine-threonine kinases that interact with one another and facilitate each other's signalling [113]. The type III receptor, also called betaglycan, is a membrane-anchored proteoglycan that has no signalling structure but acts to present TGF-beta to the other receptors [114]. The effects of TGF-beta on the synthesis and deposition of extracellular matrix are mediated by the type I receptor whilst the effects on cell growth and proliferation are mediated by the type II receptor [112].

In-vitro TGF-β has been shown to promote ECM deposition, by a combination of causing increased ECM production [98], and decreased ECM degradation [108]. I*n-vivo* it has also been shown to increase wound healing [115], but in pathological settings also to promote fibrosis. In otherwise health rats, two weeks intravenous administration of TGF-β causes

widespread systemic fibrosis [116], demonstrating that in excess TGF-β can be pro-fibrotic alone. In addition, excess exogenous TGF-β has been shown to disrupt the normal process of tissue repair. Rats given exogenous intraperitoneal TGF-β following surgical injury to their uterine horns had an increase in adhesion formation compared to untreated controls [117].

In human disease TGF-β activation is implicated in fibrotic conditions of the kidney, liver and lung amongst many others. In addition it is thought to contribute to the complications of long-term diabetes mellitus, most extensively studied in the kidney. High glucose concentrations have been shown to increase production of TGF-β1 in rat glomeruli, and this in turn stimulated synthesis of fibronectin, collagens and proteoglycans [118,119]. It is not unreasonable to speculate that this process may be important in other situations where cells are exposed to high glucose concentration such as the peritoneum during CAPD.

The mechanism by which hyperosmolar glucose stimulates increased TGF-β activity are not fully elucidated. The regulation of TGF-beta1 secretion and action involves complex post-transcriptional events, including messenger RNA (mRNA) stabilisation, the assembly and activation of the latent TGF-beta1 complex, and the modulation of receptor expression [120]. High intra-cellular glucose concentrations have been shown to upregulate protein kinase C activity, which is a potent positive promoter of TGF-β gene transcription in the rat glomerulus [121]. In mesangial cells hyperosmolar glucose has also been shown to increase *de novo* synthesis of diacylgylcerol leading to stimulation of protein kinase C (PKC) [122]. In the kidney at least this mechanism appears important in increasing TGF-β amount, and subsequently ECM.

The mechanism by which TGF-β stimulates ECM has been considered. TGF-β has been shown to act on the transmembrane glucose transporter GLUT-1 in mouse fibroblasts to increase transport of glucose into the cell [123]. It is unknown if this increase in glucose uptake occurs in other cell types, but accumulation of intracellular glucose, and glucose metabolites are known to occur with cells in a high extracellular glucose environment, and these metabolites are themselves implicated in the formation of ECM [103], possibly again through activation of PKC [124].

The role of TGF-β in HPMC ECM regulation has not been well characterised, despite the parallels that the high glucose extracellular environment of CAPD with diabetes. There is evidence however supporting the local production of TGF-β in the peritoneal cavity during CAPD, even accounting for the filtration of TGF-β across the semi-permeable membrane [125]. *In vitro* increasing glucose concentration has been shown to increase HPMC TGF-β mRNA amount [126], and exogenous TGF-β has a suppressive effect on HPMC MMP9 activity [108], suggesting that TGF-β could indeed be important in HPMC ECM regulation. The aim of the current study therefore was to further investigate the role of TGF-β in the control of glucose stimulated extra-cellular matrix (ECM) protein production by peritoneal mesothelial cells.

4.2 Effect of glucose on TGF-β protein and mRNA amount

4.2.1 Effect of glucose on TGF-β protein production

4.2.1.1 Materials and methods

Confluent HPMC cultured in 12 well plates were rendered quiescent by 48 hours exposure to M199 with 0.1% FBS. This was followed by 12 hour exposure to test medium of a 50:50 mixture of dialysate and M199 with 0.1% FBS. Dialysate was a laboratory manufactured dialysate with identical composition to PD4 but without D-glucose. Final D-glucose concentration was adjusted to 5,10,20 or 40mmol/L glucose by addition of D-glucose. Mannitol at 40mmol/L was used as an osmotic control. Experiments were also performed using commercial 1.36% glucose dialysate diluted 50:50 with medium to allow comparison with the laboratory manufactured dialysate. Supernatant TGF-β concentration was measured by ELISA as described in section 2.4.2, and cell protein measured as described in section 2.6.

4.2.1.2 Results

The effect of increasing glucose concentration on HMPC TGF-β protein production is shown in Figure 4.1. Increasing glucose concentration resulted in an increase in TGF-β protein amount, reaching a 46.5±18.3% increase in the 40mmol/L glucose condition. This represents a mean TGF-β concentration in the culture supernatant of 150±24pg/ml in the 40mmol/L D-glucose condition. 40mmol/L mannitol did not have this effect, suggesting that the effect was due to an increase in glucose concentration, not the rise in osmolality. TGF-β concentration was comparable between laboratory manufactured, and commercial

dialysate at 40mmol/L final glucose concentration (147±12.3% vs. 144 ±26.5% respectively)

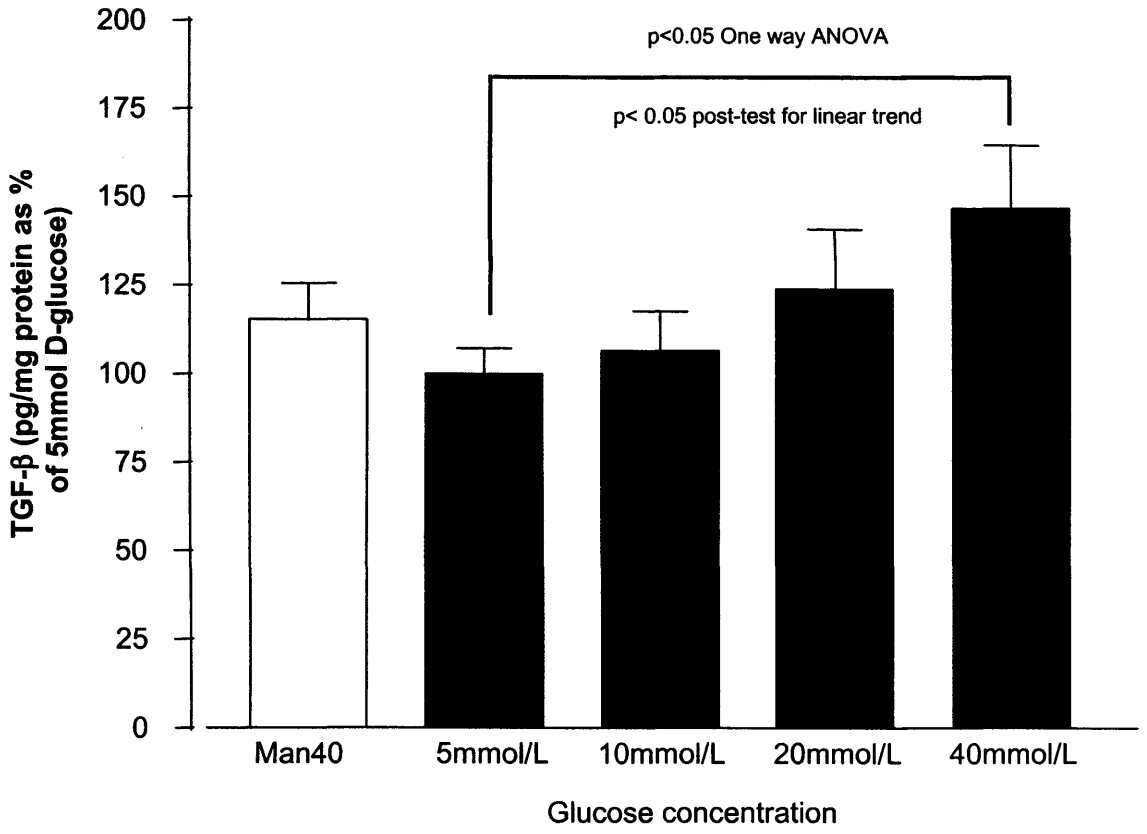


Figure 4.1 Effect of glucose concentration on TGF-β protein production

Laboratory manufactured dialysate was diluted 50:50 with M199. The final glucose concentration was as shown. Increasing glucose concentration results in an incremental rise in TGF-β production. Results shown as percentage increase in TGF-β corrected for total cell protein expressed as means ± SEM of 4 experiments in triplicate. $P<0.05$, post ANOVA test for linear trend.

4.2.2 The effect of glucose on TGF-β gene expression

4.2.2.1 Materials and methods

Confluent HPMC cultured in 25cm^2 flasks were rendered quiescent by 48hours exposure to M199 with 0.1% FBS. The medium was then replaced by test medium for 12 hours. Test medium was a 50:50 mixture of dialysate and M199 with 0.1% FBS. The dialysate used was a laboratory manufactured dialysate of identical composition to PD4 but without D-glucose. The final glucose concentration of the test medium was adjusted to either 5mmol/L or 40mmol/L by addition of D-glucose. Total messenger RNA was extracted as described in section 2.10.1, and quantified by Northern analysis.

4.2.2.2 Results

No difference was detectable between TGF-β mRNA levels in the 5mmol/L or 40mmol/L glucose conditions (Table 4-1) because of considerable variability in TGF-β mRNA levels in response to glucose.

	TGF-β mRNA amount
5mmol/L glucose	1.00
40mmol/L glucose	1.28 ± 0.53

Table 4-1 Effect of glucose on TGF-β mRNA amount

Laboratory manufactured (LAB) was diluted 50:50 with M199. The final glucose concentration was as shown. Increased glucose concentration had no effect on TGF-β mRNA amount measured by Northern analysis Results shown as fold increase in densitometric units for four experiments.

4.3 Effect of exogenous TGF-β on HPMC proliferation

4.3.1.1 Materials and methods

Quiescent HPMC in collagen coated 12 well plates were exposed to a 50:50 mixture of dialysate and M199 with 0.1% FBS. The dialysate used was a laboratory-manufactured dialysate of identical composition to PD4 but with no D-glucose. The final concentration of glucose was adjusted to 5mmol/L. Using this as the control medium, the test medium was of identical composition, but with the addition on 1ng/ml human platelet derived TGF-β_1 (R&D 100-B-001). Two different protocols were used.

A HPMC were exposed to test medium for 12 hours before this was removed and replaced with fresh test medium containing 1μCi/ml ^{3}H-thymidine for a further 6 hours. 3H-thymidine incorporation was then measured as described in section 2.8.

B HPMC were exposed to test medium for 32 hours before 1μCi/ml ^{3}H-thymidine was added to each well and allowed to incubate for a further 16 hours. 3H-thymidine incorporation was then measured as described in section 2.8.

4.3.1.2 Results

The effect of TGF-β on HPMC proliferation rate is shown in Table 4-2. TGF-β at 1ng/ml resulted in a profound inhibition of HMPC proliferation rate. It is important to note that this experiment was conducted in parallel with the experiments in section 3.3, and that this was accounted for in the statistical analysis (ANOVA $p<0.0001$, Dunnet's multiple comparison test $p<0.01$).

	12 hour protocol	48 hour protocol
5mmol/L glucose	16700 ± 2270	60500 ± 4270
5mmol/L glucose + 1ng/ml TGF-β	12900 ± 2770	14000 ± 2500

Table 4-2 Effect of TGF-β on HPMC proliferation

Laboratory dialysate was dilute 50:50 with M199 at 5mmol/L glucose. 1ng/ml TGF-β profoundly inhibited HMPC proliferation in the 48hr experiment ($p<0.01$ un-paired t-test). There was an apparent reduction in proliferation during the 12hr protocol, but this did not reach statistical significance. Results are expressed as DPM ± SEM of four experiments in triplicate

4.4 Effect of exogenous TGF-β on fibronectin protein production and gene expression

4.4.1 Effect of TGF-β on fibronectin protein production in HPMC

4.4.1.1 Materials and methods

Quiescent HPMC in collagen coated 12 well plates were exposed for 12 hours to a 50:50 mixture of dialysate and M199 with 0.1% FBS. The dialysate used was a laboratory manufactured dialysate of identical composition to PD4 but with no D-glucose. The final concentration of glucose was adjusted to 5mmol/L. Human platelet derived TGF-β_1 (R&D 100-B-001) was added at concentration between 0-5ng/ml. Fibronectin was measured in the supernatant by ELISA (section 2.4.1), and expressed corrected for total cell protein (section 2.6).

4.4.1.2 Results

Increasing exogenous TGF-β concentration resulted in a dose dependant increase in fibronectin protein production over the concentration range 0-1ng/ml (Figure 4.2), reaching a plateau of 280±45% increase at 1ng/ml TGF-β concentration. At higher concentrations (upto 5ng/ml) there was no further increase in fibronectin suggesting that maximal stimulation of fibronectin production had been attained.

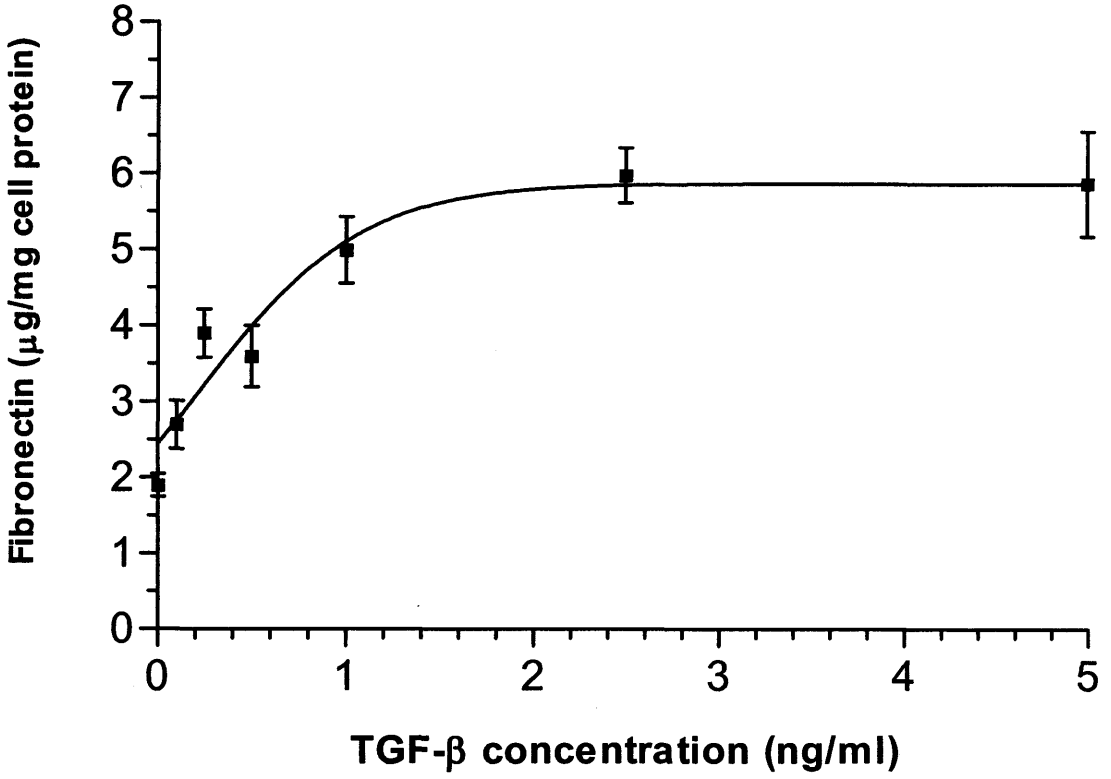


Figure 4.2 Effect of TGF-β on fibronectin protein

Laboratory dialysate was diluted 50:50 with M199 at 5mmol/L glucose. Increasing TGF-β concentration resulted in a dose dependant increase in fibronectin production over the concentration range 0-1ng/ml. Results shown as fibronectin amount corrected for total cell protein expressed as means ± SEM of 4 experiments in triplicate.

4.4.2 Effect of TGF-β on fibronectin and TGF-β mRNA

4.4.2.1 Materials and methods

Quiescent HPMC in collagen coated 25cm^2 flasks were exposed to a 50:50 mixture of dialysate and M199 with 0.1% FBS. The dialysate used was a laboratory-manufactured dialysate of identical composition to PD4 but with no D-glucose. The final concentration of glucose was adjusted to 5mmol/L. Using this as the control medium, the test medium was of identical composition, but with the addition on 1ng/ml human platelet derived TGF-β_1 (R&D 100-B-001). Total messenger RNA was extracted as described in section 2.10.1, and mRNA levels of fibronectin and TGF-β were quantified by Northern analysis.

4.4.2.2 Results

Northern analysis comparing a 50:50 mixture of 5mmol glucose dialysate:M199 with and without 1ng/ml TGF-β showed a significant increase in fibronectin (10.5±1.43 fold) and TGF-β (5.81±0.28 fold) mRNA amount in the presence of exogenous TGF-β (Figure 4.3).

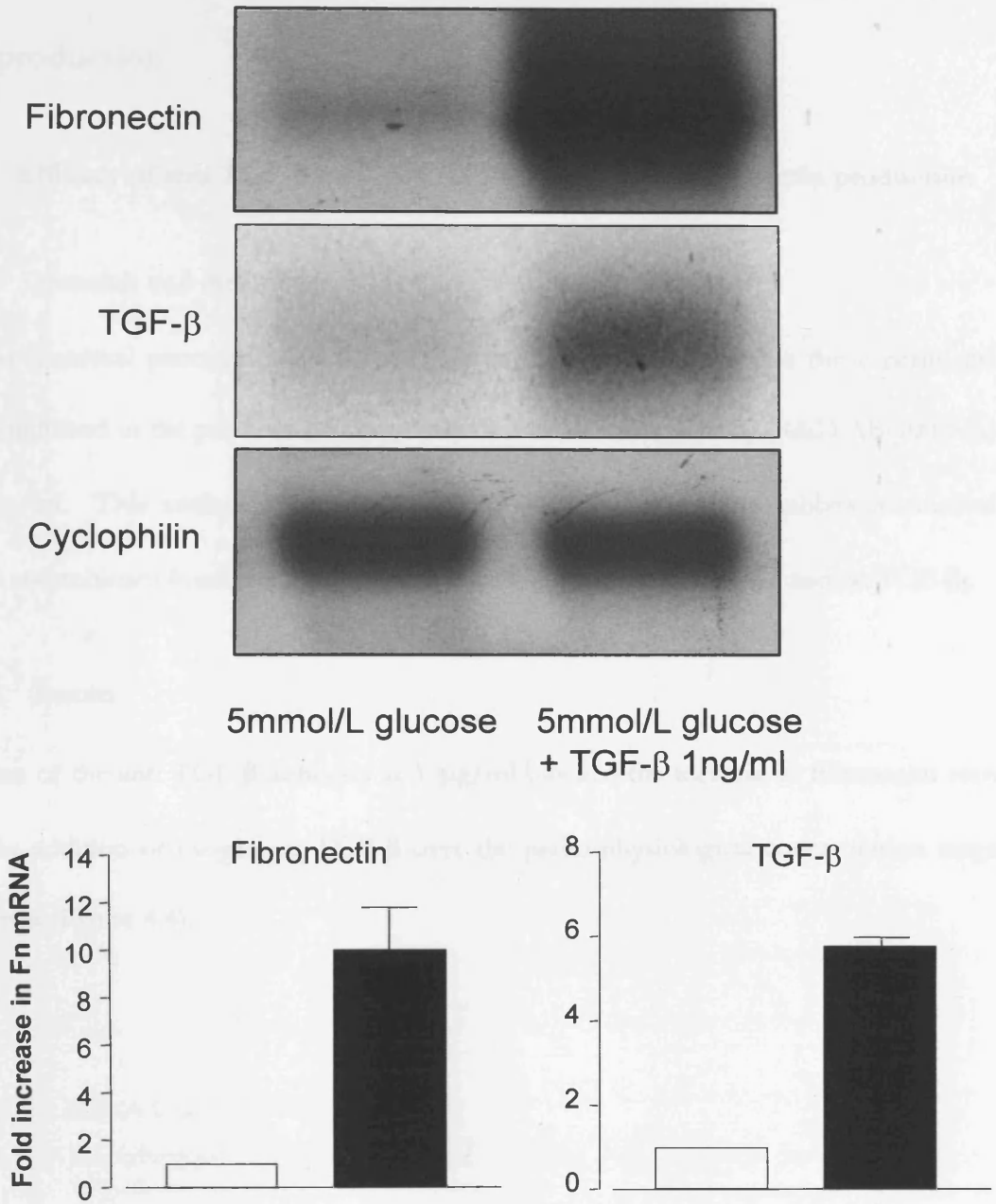


Figure 4.3 Effect of TGF-β on fibronectin and TGF-β mRNA

Representative Northern blots for fibronectin and TGF-β mRNA. Laboratory dialysate was diluted 50:50 with M199 at 5mmol/L glucose. The presence of TGF-β at 1ng/ml resulted in a significant increase in the fibronectin and TGF-β mRNA. Results shown with 1ng/ml TGF-β condition as solid boxes and as fold increase in densitometric units ± SEM for three experiments.

4.5 Effect of anti-TGF-β antibody on glucose induced fibronectin production

4.5.1 Efficacy of anti TGF-β antibody on TGF-β induced fibronectin production

4.5.1.1 Materials and methods

The experimental procedure was identical to section 4.4.1, except that the experiments were conducted in the presence of a pan-specific anti TGF-β antibody (R&D AB-100-NA) at 10μg/ml. This antibody is a mixture of total IgG derived from rabbits immunised against recombinant human TGF-β_1, porcine TGF-$\beta_{1.2}$, porcine TGF-β2 and rat TGF-β_5.

4.5.1.2 Results

Addition of the anti TGF-β antibody at 10μg/ml blocked the increase in fibronectin seen with the addition of exogenous TGF-β over the patho-physiological concentration range of interest (Figure 4.4).

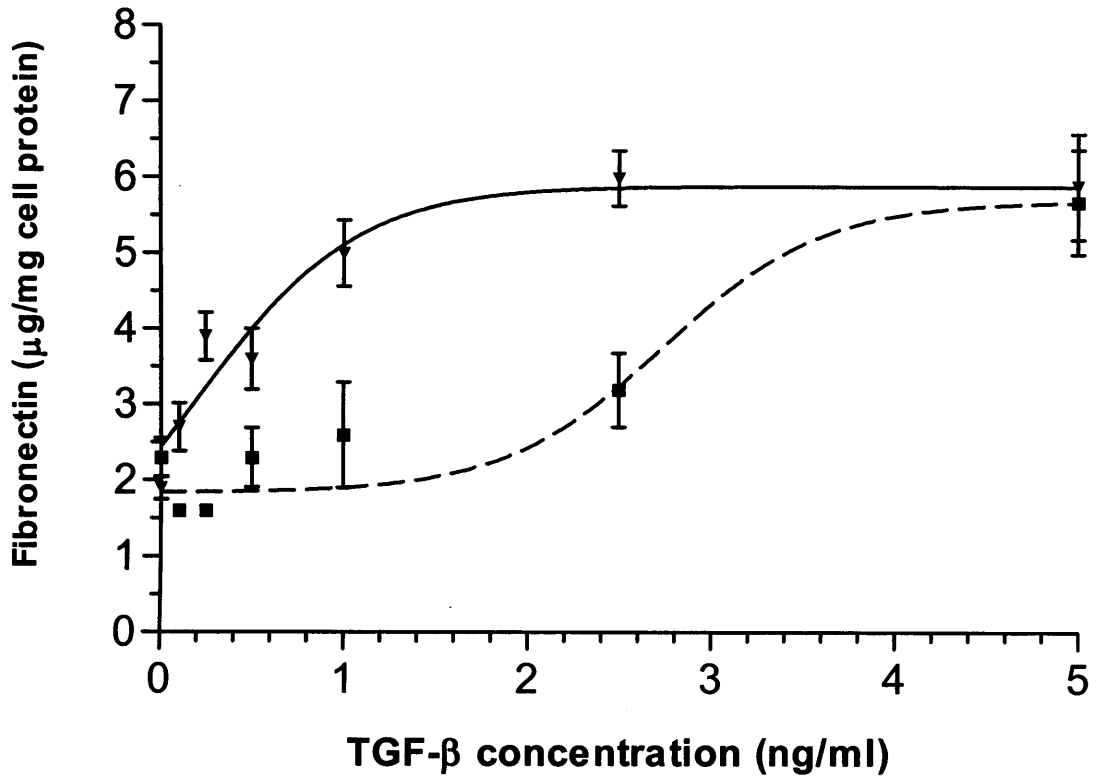


Figure 4.4 Efficacy of anti TGF- antibody

Laboratory manufactured dialysate was diluted 50:50 with M199 at 5mmol/L glucose. Increasing TGF-β concentration (—τ—) results in a dose dependant increase in fibronectin production over the concentration range 0-1ng/ml. Effect of anti TGF-β antibody at 10µg/ml (--v--) demonstrated that this effect could be blocked over the range of interest. Results shown as fibronectin amount corrected for total cell protein expressed as means ± SEM of 4 experiments in triplicate.

4.5.2 Effect of anti TGF- antibody on glucose induced fibronectin production

4.5.2.1 Materials and methods

Quiescent HPMC in collagen coated 12 well plates were exposed for 12 hours to a 50:50 mixture of dialysate and M199 with 0.1% FBS. The dialysate used was a laboratory-manufactured dialysate of identical composition to PD4 but with no D-glucose. The final concentration of glucose was adjusted to either 5mmol/L or 40mmol/L by addition of further D-glucose. Experiments were conducted in the presence and absence of a pan-specific anti TGF-β antibody at 1, 5 and 10 μg/ml. Under identical conditions a pan specific anti-PDGF antibody (R&D) was used at 10 μg/ml concentration as a further negative control. Fibronectin was measured in the supernatant by ELISA (section 2.4.1) and expressed corrected for total cell protein (section 2.6).

4.5.2.2 Results

The effect of anti TGF-β antibody on mesothelial cell fibronectin production in response to glucose is shown in Figure 4.5. As before 40mmol/L D-glucose caused an increase (30.6±11.2%) in fibronectin production. Anti TGF-β antibody had no effect on fibronectin release under control conditions (LAB at 5mmol/l D-glucose). However, the increase in fibronectin in response to 40mmol/L glucose was significantly reduced by anti TGF-β antibody to levels not different from control (93.6±6.6% at 10 μg/ml concentration, $p<0.05$ vs. no Ab). Lower concentrations of anti TGF-β antibody also reduced the increase in fibronectin seen with increased glucose concentration but to a

lesser degree. Anti-PDGF antibody had no effect on the production of fibronectin by HPMC.

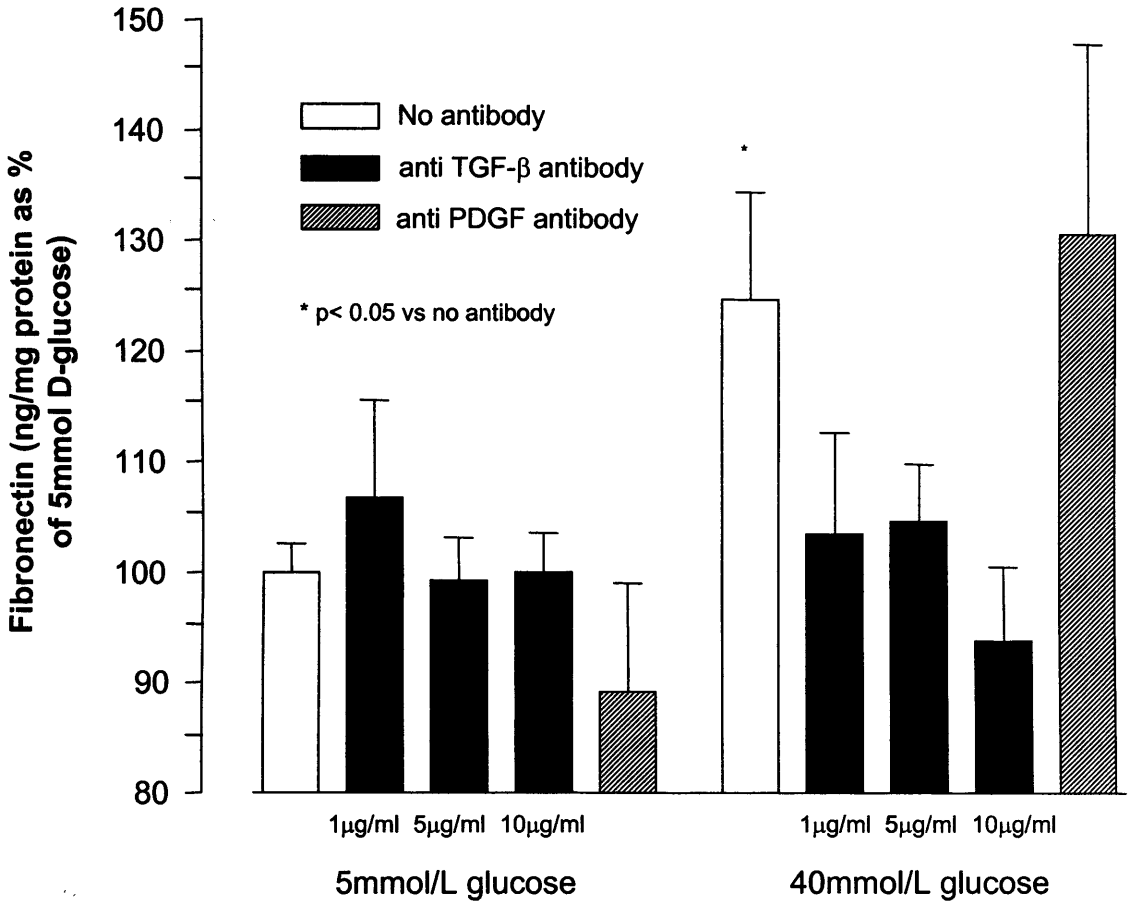


Figure 4.5 Effect of anti TGF-β on glucose induced fibronectin production

Laboratory manufactured dialysate was diluted 50:50 with M199 at 5mmol/L and 40mmol/L glucose with and without blocking antibody. The increase in fibronectin seen with 40mmol/L glucose concentration is blocked by the presence of an anti TGF-β antibody. An irrelevant antibody (anti PDGF) had no effect. Results shown as percentage increase in fibronectin amount corrected for total cell protein expressed as means ± SEM of 6 experiments in triplicate.

4.6 Effect of the putative TGF-β receptor antagonist FK-506

Tacrolimus (FK506) is a macrolide immunosuppressant drug licensed for use in transplantation as a maintenance anti-rejection therapy. Tacrolimus binds to the immunophilin FK506 binding protein (FKBP-12) which has peptidyl-prolyl isomerase activity. The immunosuppressive action is mediated via blockade of calaineurin mediated T cell receptor signal transduction and inhibition of IL-2 [127]. The FKBP is known to lie in close proximity to the TGF-β type I receptor on the cell surface, and it has been speculated that the FKBP may regulate signalling through the TGF-β receptor [128], possibly through competitive binding at the TGF-β type I receptor [129]. The TGF-β type I receptor is thought to be responsible for regulating the synthesis of ECM, and the type II receptor the TGF-β mediated effects on proliferation. Work in mesangial cells has confirmed that high concentrations of FK506 (100ng/ml) can inhibit the increase in fibronectin seen by adding TGF-β at 10ng/ml to the culture medium [130]. Because of the binding to plasma proteins this represents approximately ten times a therapeutic plasma concentration in renal transplantation (5-15ng/ml). Despite this FK506 had no effect on basal fibronectin production, nor was it cytotoxic at these concentrations. In contrast FK506 has been shown to increase, rather than decrease hepatic fibrosis in rats given carbon tetrachloride [131], and indeed FK506, like cyclosporin stimulates interstitial fibrosis in the kidney when given to patients in therapeutic amounts [132]. Intriguingly, other groups could found no discernible connection between FKBP and TGF-β signalling at all [133].

The aim of these experiments therefore were to investigate a potential therapeutic role for FK506 in preventing TGF-β induced ECM production by HPMC.

4.6.1 Effect of FK506 on TGF-β stimulated fibronectin production

4.6.1.1 Materials and methods

Quiescent HPMC in collagen coated 12 well plates were exposed to a 50:50 mixture of dialysate and M199 with 0.1% FBS. The dialysate used was a laboratory-manufactured dialysate of identical composition to PD4 but with no D-glucose. The final concentration of glucose was adjusted to 5mmol/L. The following test medium were then manufactured.

A Diluent for FK506 only (control medium)

B 10ng/ml FK506 (Fujisawa Pharmaceuticals Ltd)

C 1ng/ml human platelet derived TGF-β_1 (R&D 100-B-001)

D 1ng/ml TGF-β_1 + 10ng/ml FK506

FK506 was a generous gift from Fujisawa Pharmaceuticals Ltd, and supplied as a white powder, only poorly soluble in water (maximum concentration 2μg/ml). The initial dilution was therefore performed using absolute ethanol as the diluent. Absolute ethanol was added at the same concentration to the control medium (10^{-6} fold dilution in culture medium). The concentration of 10ng/ml was chosen to represent a concentration approximately ten times a therapeutic level in plasma (5-15ng/ml), accounting for the binding of FK506 to plasma proteins previously discussed. Fibronectin was measured in the supernatant by ELISA (section 2.4.1) and expressed corrected for total cell protein (section 2.6).

4.6.1.2 Results

The effect of FK506 on TGF-β induced fibronectin production are shown in Table 4-3. As previously described in section 4.4.1, TGF-β at 1ng/ml resulted in a significant increase

in fibronectin protein production (262 ± 21.7%). FK506 had no effect on fibronectin production either under control conditions, or on TGF-β stimulated fibronectin production.

	% Fibronectin production
5mmol/L glucose	100 ± 2.52
5mmol/L glucose + FK506 100ng/ml	98.1 ± 7.68
5mmol/L glucose + TGF-β 1ng/ml	262 ± 21.7
5mmol/L glucose + TGF-β + FK506	262 ± 27.5

Table 4-3 Effect of FK506 on TGF-β induced fibronectin production

Laboratory manufactured dialysate was diluted 50:50 with M199 at 5mmol/L glucose. As before TGF-β at 1ng/ml induced a significant increase in fibronectin amount. FK506 had no effect on fibronectin amount under either control or TGF-β 1ng/ml conditions. Results shown as percentage increase in fibronectin corrected for total cell protein expressed as means ± SEM of 4 experiments in triplicate.

4.6.2 Effect of FK506 on TGF-β induced inhibition of proliferation

4.6.2.1 Materials and methods

The test media used were identical to section 4.6.1. HMPC were exposed to test medium for 12 hours before this was removed and replaced with fresh test medium containing 1μCi/ml ^{3}H-thymidine for a further 6 hours. 3H-thymidine incorporation was then measured as described in section 2.8.

4.6.2.2 Results

The effect of FK506 on TGF-β induced proliferation is shown in Table 4-4. FK506 had no effect on HPMC proliferation rate under control conditions, but showed an additive inhibition when added in combination with TGF-β at 1ng/ml (45% decrease in proliferation rate, $p<0.05$). As discussed in section 3.3, over 12 hours there was a trend toward a decrease in proliferation rate with 1ng/ml TGF-β, but this did not reach statistical significance. This experiment was conducted in parallel with the experiment in section 4.3, and this was accounted for in the statistical analysis (Dunnet's post ANOVA test for multiple comparisons with control value, $p<0.05$).

	Proliferation rate
5mmol/L glucose	16700 ± 2270
5mmol/L glucose + FK506 100ng/ml	14100 ± 3220
5mmol/L glucose + TGF-β 1ng/ml	12900 ± 2770
5mmol/L glucose + TGF-β + FK506	7660 ± 1220

Table 4-4 Effect of TGF-β on HPMC proliferation

Laboratory manufactured dialysate was diluted 50:50 with M199 at 5mmol/L glucose. TGF-β at 1ng/ml induced a significant decrease in HPMC proliferation rate. FK506 at 100ng/ml has no effect on HPMC proliferation under control conditions, but has an additive effect with TGF-β causing an inhibition of proliferation rate ($p<0.05$). Results are expressed as DPM ± SEM of four experiments in triplicate.

4.7 Summary

In this series of experiments the role of TGF-β in HMPC ECM production has been investigated. In summary

1. Increasing glucose concentration resulted in an increase in TGF-β protein, but no statistically significant increase in TGF-β mRNA amount.

2. Exogenous TGF-β caused a dose dependant increase in fibronectin protein production over a pathophysiological concentration range. In addition TGF-β at 1ng/ml caused a significant increase in fibronectin mRNA amount.

3. TGF-β at 1ng/ml caused a significant reduction in HPMC proliferation rate.

4. A pan-specific anti TGF-β antibody blocked the hyper-osmolar glucose induced increase in fibronectin production to a level not different to control glucose conditions.

5. FK506 appeared to have no effect on TGF-β induced fibronectin production, but was able to accentuate TGF-β induced inhibition of proliferation by HPMC.

4.8 Conclusion

These experiments provide convincing evidence for the role of autocrine TGF-β production by HPMC to regulate extra-cellular matrix production in response to glucose. Hyperosmolar glucose produced an increase in TGF-β protein and fibronectin protein, and the latter could be inhibited by the addition of an anti TGF-β antibody. This suggests that TGF-β plays a key regulatory role in promoting extra-cellular matrix production by HPMC. A high glucose concentration has been shown to promote an increase in extra-cellular matrix in other cell types [95,96]. Furthermore exposure of mesangial cells to high glucose concentration resulted in an increase in collagen III deposition, which could be prevented by the addition of an anti TGF-β antibody [98]. These data on the effect of TGF-β on mesothelial cells is therefore consistent with this previous work in other cell culture systems, and have been subsequently confirmed by others to occur in a dose dependant fashion over TGF-β concentrations of 0.1-1ng/ml [106].

Exogenous TGF-β has been shown to increase the mRNA amount of fibronectin and collagen III, and cause a substantial increase in fibronectin protein. This supports the hypotheses that increase in matrix protein seen in HPMC is at least in part the consequence of increased ECM production. As discussed in the previous chapter, the amount of extra-cellular matrix is controlled by the rate of degradation in addition to the rate of production. TGF-β has previously been shown to increase HPMC production of the metalloproteinase MMP2 [107], biologically active MMP9 [108], PAI-1 [134], and the message for TIMP III [107]. The role of ECM degradation cannot be established from these experiments, but it is clear that exogenous TGF-β stimulates considerable accumulation of ECM by HPMC.

Exogenous TGF-β also stimulated an increase in TGF-β mRNA. This would appear counter-intuitive as a positive feedback on its own production, but has been observed in other cell systems [135]. Although the increase in mRNA may not translate into the production further active TGF-β because of other post transcriptional controls, the exact physiological reason for this amplification in response is not clear.

Tacrolimus (FK506) is an immunosuppressive drug whose receptor (FK binding protein) has a putative role in controlling signalling at the TGF-β type I receptor because of close spatial proximity. The type I receptor is believed to regulate ECM, whilst the type II receptor controls cell proliferation. Addition of FK506 to HMPC in culture would therefore be predicted to inhibit TGF-β induced ECM production, whilst having no effect on the decrease in proliferation previously demonstrated. In the HPMC however the effect of FK506 on ECM and proliferation, does not support the hypothesis that the FKBP and TGF-β receptor are linked. The addition of FK506 appeared to have no effect on TGF-β fibronectin production, and accentuated the inhibition of cell proliferation.

It would therefore appear unlikely therefore that FK506 has a therapeutic role in preventing peritoneal fibrosis in CAPD. The previous study using mesangial cells [130] used a ten fold higher concentration of both TGF-β and FK506. The concentration of FK506 chosen for the experiments here is ten times greater than a therapeutic plasma concentration. As FK506 is thought to act competitively with the TGF-β type I receptor, it is unlikely that this negative result is the result of an insufficiently high concentration of FK506. In addition, the aim of this experiment was to investigate a potential therapeutic role of FK506. The dose of FK506 was therefore chosen to be representative of a potential therapeutic concentration *in-vivo*, and even this modest FK506 concentration, if used in the peritoneum, might well result in an unacceptably high plasma concentration in

practice. Finally, the additive effect on inhibition of HPMC proliferation would appear to be disadvantageous in the clinical context of mesothelial cell loss in long-term CAPD.

The mechanism by which glucose stimulates TGF-β production has not been considered. Previous work has implicated protein kinase C (PKC) in this process [121], and in the rat glomerulus PKC acts to increase TGF-β gene transcription [121]. In the work presented here however, no difference was detectable in TGF-β mRNA amount with hyperosmolar glucose. Either the change in mRNA was too small to detect at the 12 hour time point, or the process of increased TGF-β protein is the result of post transcriptional changes. It is well recognised that TGF-β activity can be regulated by alterations in assembly and activation of TGF-β complex, as well as mRNA stabilisation, or changes in receptor expression [120].

In conclusion therefore, hyperosmolar glucose has been shown to increase HPMC TGF-β amount, and the use of a pan-specific anti TGF-β antibody prevented the glucose induced increase in ECM protein. Exogenous TGF-β has been shown to increase HPMC ECM protein and mRNA amount. This provides strong evidence for the role of TGF-β in HMPC ECM production. The putative TGF-β receptor inhibitor FK506 had no therapeutic effect in this *in-vitro* culture system.

5 A different osmotic agent: effects of amino acid dialysate

5.1 Introduction

Patients on long-term dialysis are well known to be at risk of malnutrition from a loss appetite and a state of persistent mild metabolic acidosis [136]. Additionally, patients on CAPD are at risk of protein malnutrition in particular because of the high protein loss in the peritoneal dialysate (5-15g/day). Patients on CAPD may therefore benefit from supplements of essential amino acids. This can be achieved by adding amino acids to the peritoneal dialysate to act as an alternative osmotic agent. Although larger in molecular size than glucose they can be absorbed across the semi-permeable peritoneal membrane during peritoneal dialysis. In addition, unlike glucose they could stimulate protein synthesis rather forming a metabolic fuel for gluconeogenesis and fat production.

Commercial amino acid dialysate consists of a 1.1% solution of mixed amino acids (Table 5-1) in a lactate-buffered solution of otherwise similar composition to PD4. As amino acids form the osmotic agent, the solution only contains 3.5mmol/L D-glucose. The standard dialysis prescription is for one amino acid bag per day, with other exchanges of the standard hyperosmolar glucose dialysate. Used in this way amino acid dialysate has been shown to improve serum albumin and transferrin in a three month study of 15 malnourished patients on CAPD [36], although this has not been borne-out in longer-term studies [137].

Technique survival with amino acid dialysate has not been compared to that with glucose dialysate alone. However, *in-vitro* a 1.1% amino acid solution has previously been shown to inhibit proliferation of non-confluent human peritoneal mesothelial cells in culture [72]. As previously discussed a study of individual amino acid supplements to complete culture medium has suggested that different amino acids may have a differential effect on cell function [70]. Any differential effect on extra-cellular matrix metabolism is not known.

The aim of this study therefore was to investigate the effect of a 1.1% mixed amino acid solution on cell function. By using a test system previously validated in the investigation of the effects of hyperosmolar dialysate the effects could be compared to previous data with this solution. L-arginine is present at supra-physiological concentration in amino acid dialysate and high concentrations of L-arginine have been associated with ECM production in other cell culture systems [138]. The role of nitric oxide will therefore also be considered.

Amino acid	Concentration (g/L)
L-Valine	1.39
L-Leucine	1.02
L-Isoleucine	0.85
L-Methionine	0.85
L-Lysine	0.76
L-Threonine	0.65
L-Phenylalanine	0.57
L-Tryptophan	0.27
L-Histidine	0.71
L-Arginine	1.07
L-Alanine	0.95
L-Proline	0.60
Glycine	0.51
L-Serine	0.51
L-Tyrosine	0.30

Table 5-1 Amino acid composition of Nutrineal® dialysate

5.2 Cell viability

5.2.1 Effect of amino acid dialysate on HPMC viability

5.2.1.1 Materials and methods

Confluent HPMC cultured in 12 well plates were rendered quiescent by 48 hours exposure to M199 with 0.1% FBS. This was followed by 12 hour exposure to test medium of a 50:50 mixture of dialysate and M199 with 0.1% FBS. The dialysate used was either:

A. Laboratory manufactured dialysate of identical composition to PD4 (Final glucose concentration 5mmol/L)

B. Commercial 1.36% glucose dialysate (PD4) (Final glucose concentration 38mmol/L)

C. 1.1% amino acid dialysate (Nutrineal®).

The final pH of the test medium was measured using a blood gas analyser as described in section 2.7. LDH activity was measured in the cell culture supernatant and cell digest as described in section 2.9.

5.2.1.2 Results

The pH of the 50:50 mixture of dialysate and M199 5mmol/L glucose after 12 hours at 5% CO_2 = 7.07 ± 0.04, and was unaffected by glucose concentration. The pH of the 50:50 mixture of amino acid dialysate and M199 after 12 hours at 5% CO_2 = 7.05 ± 0.001. As before, hyperosmolar glucose dialysate resulted in a increase in HPMC LDH release and hence decrease in viability (Table 5-2). Amino acid dialysate has an osmolality of 365mOsm/L, greater even than 1.36% glucose PD4 dialysate. Despite this amino acid

dialysate caused no increase in LDH release and represented comparable cell viability to control conditions (5mmol/L glucose dialysate).

	LDH release
LAB - 5mmol/L glucose	2.6 ± 0.7
Commercial 1.36% - 38mmol/L glucose	5.1 ± 0.6
Amino acid dialysate	3.6 ± 1.4

Table 5-2 Effect of amino acid dialysate on LDH release

Laboratory manufactured (LAB) and commercial dialysate was diluted 50:50 with M199. Hyperosmolar glucose dialysate causes an increase in LDH release. Amino acid dialysate does not have this effect, with LDH release comparable to control conditions. Results are percentage LDH activity in culture supernatant / total LDH activity expressed as means ± SEM of 3 experiments in triplicate

5.3 Proliferation

5.3.1 Effect of amino acid dialysate on proliferation

5.3.1.1 Materials and methods

Confluent HPMC cultured in 12 well plates were rendered quiescent by 48 hours exposure to M199 with 0.1% FBS. Test medium consisted of a 50:50 mixture of dialysate and M199 with 0.1% FBS. The dialysate used was either:

A. Laboratory manufactured dialysate of identical composition to PD4 but with glucose concentration of the final test mixture adjusted to 5mmol/L

B. 1.1% amino acid dialysate (Nutrineal®).

HPMC were exposed to test medium for 32 hours before 1μCi/ml ^{3}H-thymidine was added to each well and allowed to incubate for a further 16 hours. 3H-thymidine incorporation was then measured as described in section 2.8.

5.3.1.2 Results

The effect of amino acid dialysate on HPMC proliferation is shown in Table 5-3. Amino acid dialysate caused a significant inhibition of HPMC proliferation rate. It is important to note that this experiment was conducted in parallel with experiments in section 3.3, and that this was accounted for in the statistical analysis (ANOVA $p<0.0001$, Dunnet's multiple comparison test $p<0.01$).

	3H incorporation
LAB - 5mmol/L glucose	60511 ± 4266
Amino acid dialysate	24341 ± 6427

Table 5-3 Effect of amino acid dialysate on proliferation

Laboratory manufactured (LAB) and commercial dialysate was diluted 50:50 with M199. Amino acid dialysate caused an inhibition of HPMC proliferation ($p<0.05$ un-paired t-test) compared to 5mmol/L LAB control. Results are expressed as DPM ± SEM of four experiments in triplicate.

5.4 Fibronectin protein production and mRNA amount

5.4.1 Effect of amino acid dialysate on fibronectin protein production

5.4.1.1 Materials and methods

Confluent HPMC cultured in 12 well plates were rendered quiescent by 48 hours exposure to M199 with 0.1% FBS. Test medium consisted of a 50:50 mixture of dialysate and M199 with 0.1% FBS. The dialysate used was either:

A. Laboratory manufactured dialysate of identical composition to PD4 but with glucose concentration of the final test mixture adjusted to 5mmol/L.

B. 1.1% amino acid dialysate (Nutrineal®).

Supernatant fibronectin concentration was measured by ELISA as described in section 2.4.1, and cell protein measured as described in section 2.6.

5.4.1.2 Results

The effect of amino acid dialysate on HMPC fibronectin protein production is shown in Figure 5.1. Amino acid dialysate caused a similar increase in fibronectin (20.5 ± 6.22%) to that seen with hyperosmolar glucose dialysate despite containing only 3.5mmol/L D-glucose.

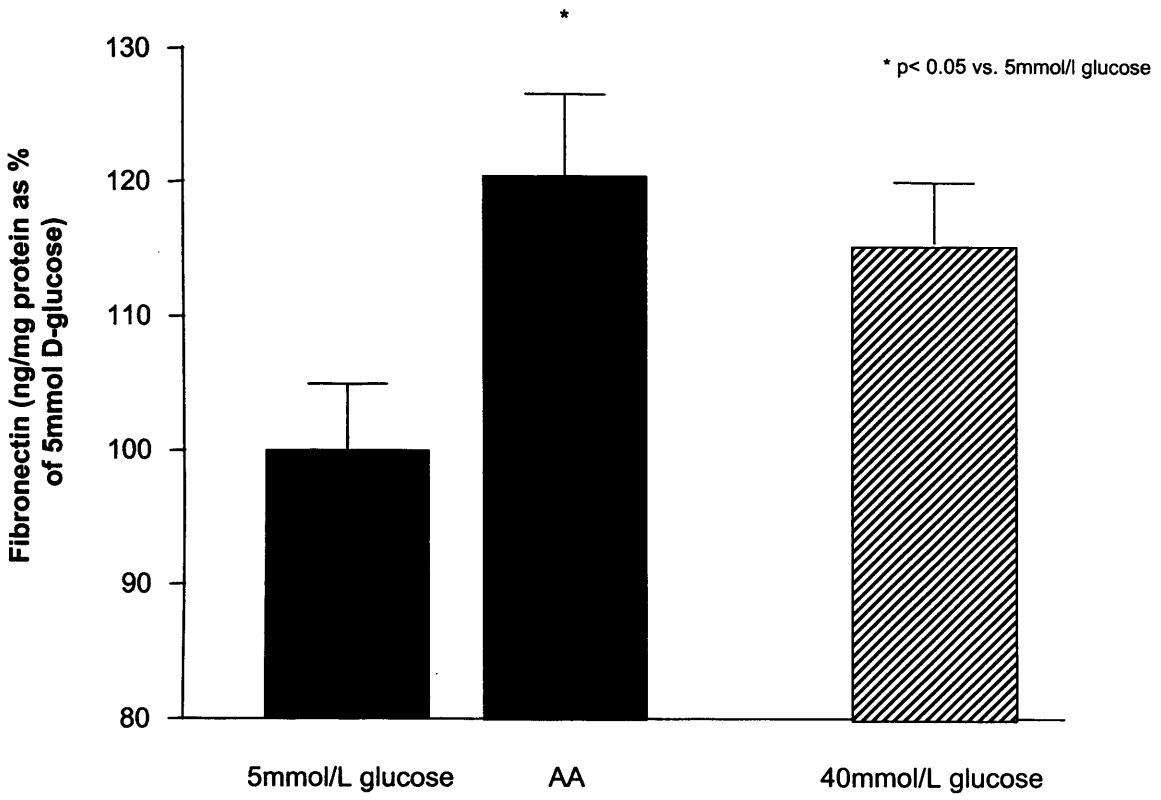


Figure 5.1 Effect of amino acid dialysate on fibronectin protein production

Laboratory manufactured (LAB) dialysate (final glucose concentration 5mmol/L) and amino acid dialysate were mixed 50:50 with M199. Effect of LAB 40mmol/L glucose (section 4.2) is shown in as a hatched bar for comparison. Amino acid dialysate stimulated a comparable increase in fibronectin to hyperosmolar glucose dialysate despite containing only 3.5mmol/L glucose. Results are from four experiments in triplicate. $p<0.05$ un-paired t-test

5.4.2 The effect of amino acid dialysate on fibronectin mRNA

5.4.2.1 Materials and methods

Confluent HPMC cultured in 25cm^2 flasks were rendered quiescent by 48hours exposure to M199 with 0.1% FBS. The medium was then replaced by test medium for 12 hours. Test medium was a 50:50 mixture of dialysate and M199 with 0.1% FBS. Test medium consisted of a 50:50 mixture of dialysate and M199 with 0.1% FBS. The dialysate used was either:

A. Laboratory manufactured dialysate of identical composition to PD4 but with glucose concentration of the final test mixture adjusted to 5mmol/L.

B. 1.1% amino acid dialysate (Nutrineal®).

Total messenger RNA was extracted as described in section 2.10.1, and fibronectin mRNA quantified by Northern analysis.

5.4.2.2 Results

Figure 5.2 shows a representative Northern blot for fibronectin mRNA with amino acid dialysate. In three experiments there was a 5.1±2.1 fold increase in fibronectin mRNA in the amino acid dialysate condition.

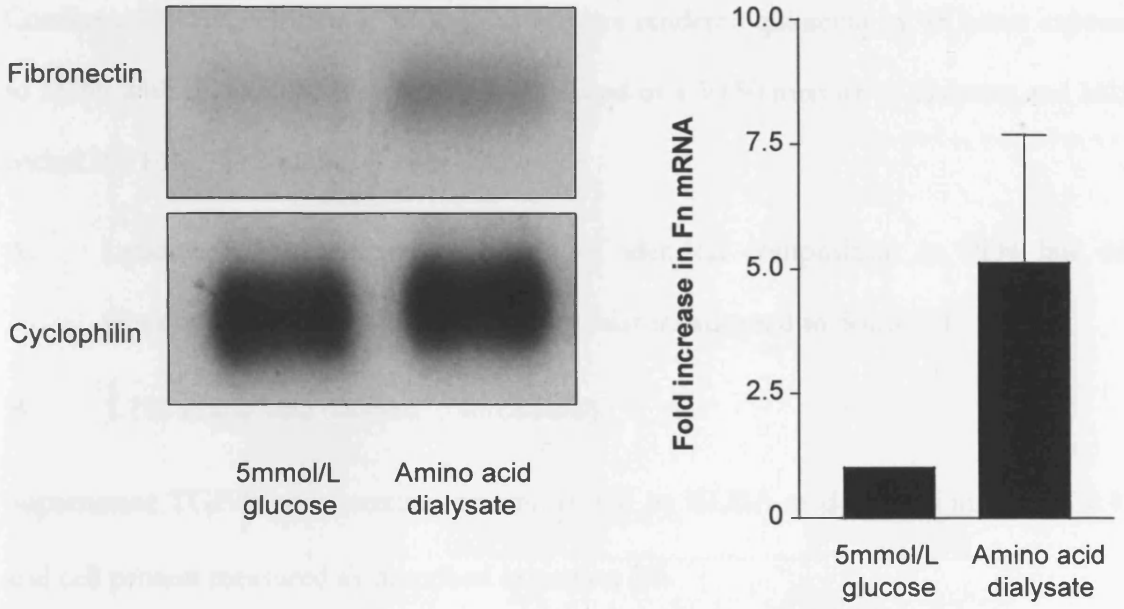


Figure 5.2 Effect of amino acid dialysate on fibronectin mRNA

Laboratory manufactured (LAB) dialysate (final glucose concentration 5mmol/L) and amino acid dialysate were mixed 50:50 with M199. Amino acid dialysate caused an increase in fibronectin mRNA in comparison to 5mmol/L glucose LAB control. Results shown as fold increase in densitometric units for three experiments.

5.5 TGF-β protein production and mRNA amount

5.5.1 Effect of amino acid dialysate on TGF-β protein

5.5.1.1 Materials and methods

Confluent HPMC cultured in 12 well plates were rendered quiescent by 48 hours exposure to M199 with 0.1% FBS. Test medium consisted of a 50:50 mixture of dialysate and M199 with 0.1% FBS. The dialysate used was either:

A. Laboratory manufactured dialysate of identical composition to PD4 but with glucose concentration of the final test mixture adjusted to 5mmol/L

B. 1.1% amino acid dialysate (Nutrineal®).

Supernatant TGF-β concentration was measured by ELISA as described in section 2.4.2, and cell protein measured as described in section 2.6.

5.5.1.2 Results

The effect of amino acid dialysate on TGF-β protein is shown in Figure 5.3. Amino acid dialysate caused an increase in TGF-β protein amount (320±58.8%) compared to 5mmol/L glucose dialysate control conditions. This represents a mean TGF-β concentration in the culture supernatant of 250±30pg/ml. Amino acid dialysate therefore causes a considerably greater increase in TGF-β protein amount than seen with 40mmol/L glucose test medium (approximately twice the final concentration).

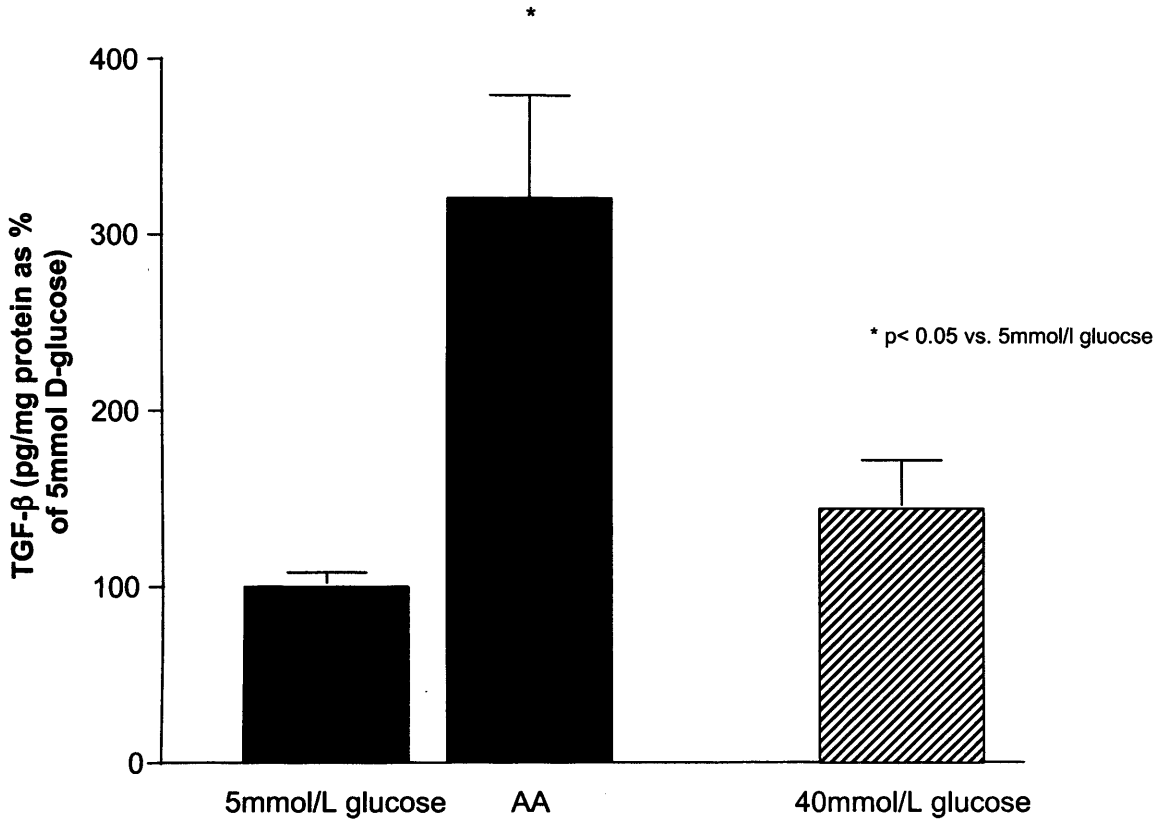


Figure 5.3 Effect of amino acid dialysate on TGF-β protein production

Laboratory manufactured (LAB) dialysate (final glucose concentration 5mmol/L) and amino acid dialysate were mixed 50:50 with M199. Effect of LAB 40mmol/L glucose. Effect of 40mmol/L glucose (section 4.2.1) is shown in as a hatched bar for comparison. Amino acid dialysate stimulated a significant increase in TGF-β, greater than hyperosmolar glucose dialysate despite containing only 3.5mmol/L glucose. Results are from four experiments in triplicate. $p<0.05$ un-paired t-test.

5.5.2 The effect of amino acid dialysate on TGF-β mRNA

5.5.2.1 Materials and methods

Confluent HPMC cultured in 25cm^2 flasks were rendered quiescent by 48hours exposure to M199 with 0.1% FBS. The medium was then replaced by test medium for 12 hours. Test medium was a 50:50 mixture of dialysate and M199 with 0.1% FBS. Test medium consisted of a 50:50 mixture of dialysate and M199 with 0.1% FBS. The dialysate used was either:

A. Laboratory manufactured dialysate of identical composition to PD4 but with glucose concentration of the final test mixture adjusted to 5mmol/L.

C. 1.1% amino acid dialysate (Nutrineal®).

Total messenger RNA was extracted as described in section 2.10.1, and TGF-β mRNA quantified by Northern analysis.

5.5.2.2 Results

Figure 5.4 shows a representative Northern blot for TGF-β mRNA with amino acid dialysate. In three experiments there was a 2.0±0.81 fold increase in TGF-β mRNA in the amino acid dialysate condition.

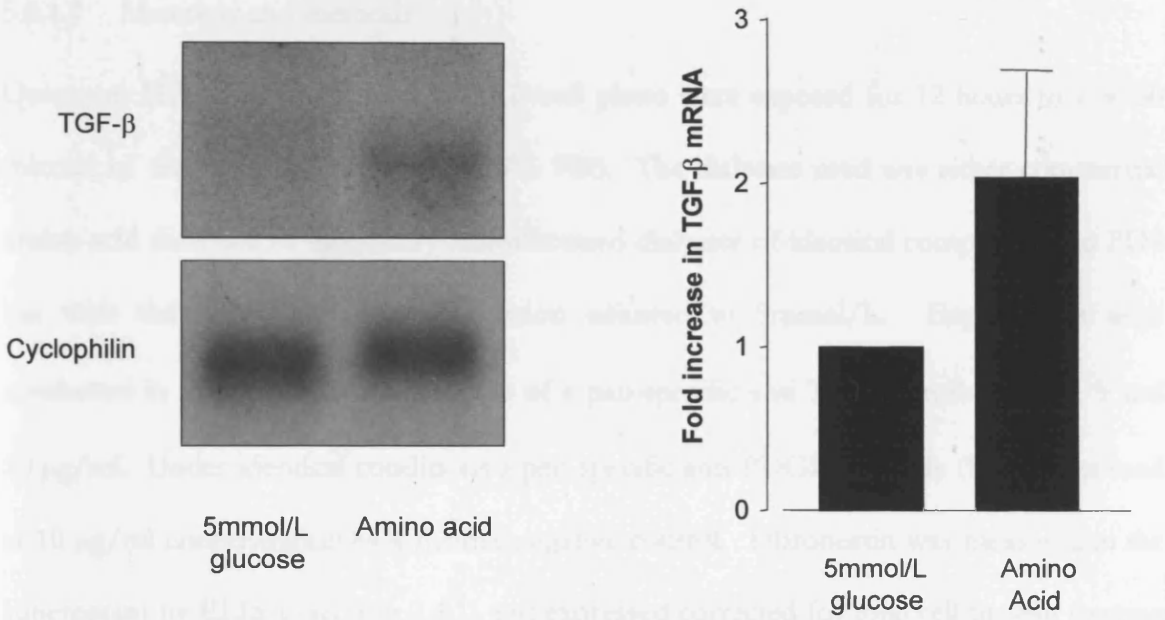


Figure 5.4 Effect of amino acid dialysate on TGF-β mRNA

Laboratory manufactured (LAB) dialysate (final glucose concentration 5mmol/L) and amino acid dialysate were mixed 50:50 with M199. Amino acid dialysate caused an increase in TGF-β mRNA in comparison to 5mmol/L glucose LAB control. Results shown as fold increase in densitometric units for five experiments.

5.6 Anti-TGF-β antibody strategy

5.6.1 Effect of anti TGF-β antibody on amino acid dialysate induced fibronectin production

5.6.1.1 Materials and methods

Quiescent HPMC in collagen coated 12 well plates were exposed for 12 hours to a 50:50 mixture of dialysate and M199 with 0.1% FBS. The dialysate used was either commercial amino acid dialysate or laboratory-manufactured dialysate of identical composition to PD4 but with the final glucose concentration adjusted to 5mmol/L. Experiments were conducted in the presence and absence of a pan-specific anti TGF-β antibody at 1, 5 and 10 μg/ml. Under identical conditions a pan specific anti-PDGF antibody (R&D) was used at 10 μg/ml concentration as a further negative control. Fibronectin was measured in the supernatant by ELISA (section 2.4.1) and expressed corrected for total cell protein (section 2.6).

5.6.1.2 Results

The effect of anti TGF-β antibody on HPMC fibronectin production in response to amino acid dialysate is shown in Figure 5.5. As previously amino acid dialysate caused an increase (27.3±10.7%) in fibronectin production. Anti-TGF-β antibody had no effect on fibronectin release under control conditions (LAB at 5mmol/l D-glucose). However, the increase in fibronectin in response to amino acid dialysate was significantly reduced by 10ng/ml anti TGF-β antibody to levels not different from control (108.8±6.3%, $p<0.05$ vs. no Ab). Lower concentrations of anti TGF-β antibody also reduced the increase in

fibronectin seen with amino acid dialysate but to a lesser degree. Anti-PDGF antibody had no effect on the production of fibronectin by HPMC.

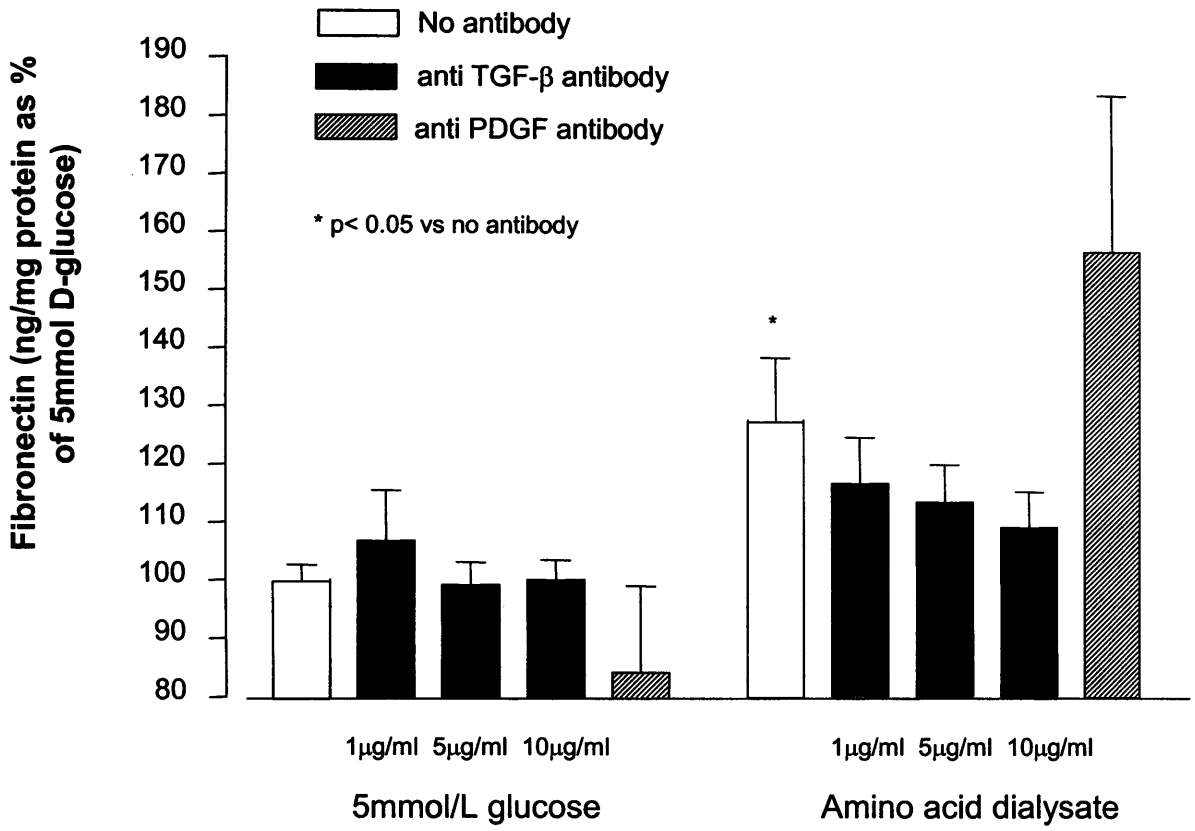


Figure 5.5 Effect of anti TGF-β antibody on amino acid dialysate induced fibronectin production

Laboratory manufactured (LAB) dialysate (final glucose concentration 5mmol/L) and amino acid dialysate was diluted 50:50 with M199 with and without blocking antibody. The increase in fibronectin seen with amino acid dialysate is blocked by the presence of an anti TGF-β antibody. Results shown as percentage increase in fibronectin amount corrected for total cell protein expressed as means ± SEM of 6 experiments in triplicate.

5.7 Investigation of the role of Nitric Oxide

5.7.1 The effect of L-NAME and L-arginine on HPMC fibronectin protein production

5.7.1.1 Materials and Methods

Quiescent HPMC in collagen coated 12 well plates were exposed for 12 hours to a 50:50 mixture of dialysate and M199 with 0.1% FBS. The dialysate used was either commercial amino acid dialysate or laboratory-manufactured dialysate of identical composition to PD4 but with the final glucose concentration adjusted to 5mmol/L (control). Experiments were conducted in the presence and absence of the nitric oxide inhibitor L-NAME at 5mM final concentration. In addition experiments were conducted comparing control medium with control medium supplemented with 8mmol/L L-arginine. Fibronectin was measured in the supernatant by ELISA (section 2.4.1) and expressed corrected for total cell protein (section 2.6).

5.7.1.2 Results

The results of the experiment are summarised in Figure 5.6. As shown previously amino acid dialysate caused a 25.2±16.8% increase in fibronectin compared with control medium. The presence of L-NAME inhibited fibronectin to similar levels to control conditions. However, L-NAME caused a significant unexpected increase in fibronectin protein under control conditions. L-arginine produced a statistically insignificant increase in fibronectin amount compared with control.

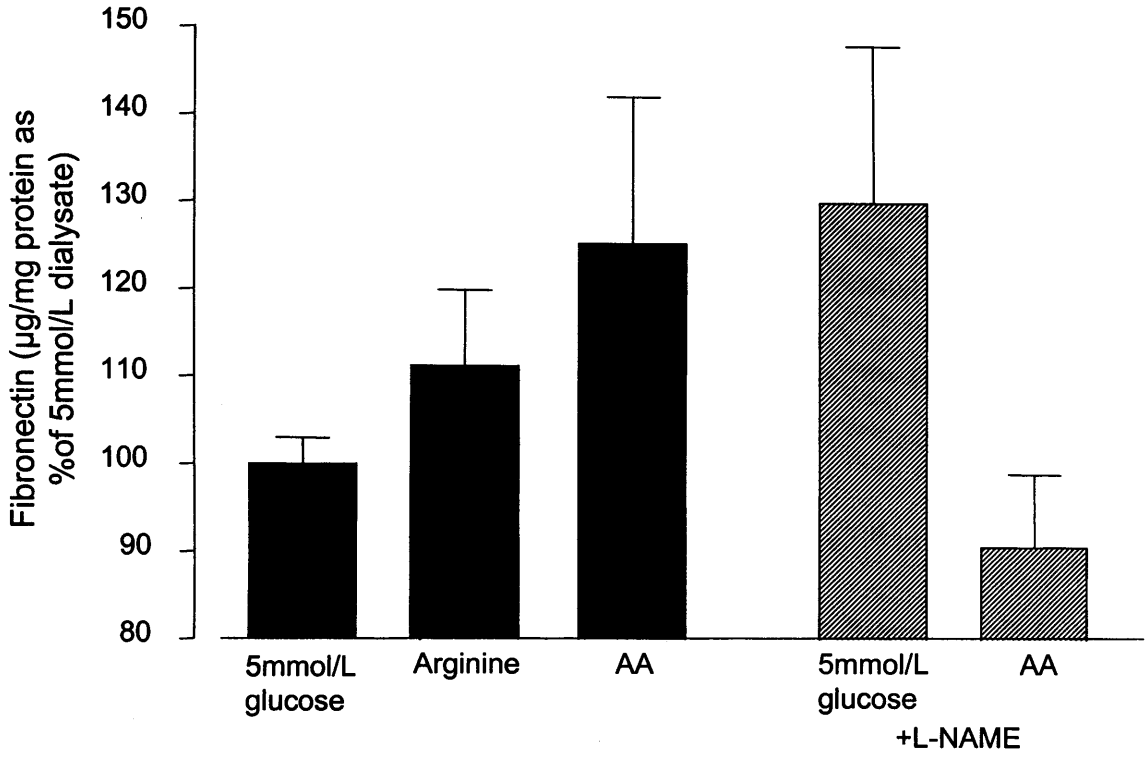


Figure 5.6 Effect of L-NAME and L-arginine on HPMC fibronectin amount

Laboratory manufactured (LAB) dialysate (final glucose concentration 5mmol/L) and amino acid dialysate was diluted 50:50 with M199 with and without L-NAME. Separately the effect of L-arginine 8mmol/L was considered. Amino acid dialysate increased fibronectin which was blocked by the presence of L-NAME. L-arginine had no statically significant effect. Results shown as percentage increase in fibronectin amount corrected for total cell protein expressed as means ± SEM of 4 experiments in triplicate.

5.8 Summary

These experiments investigated the effect of a 1.1% amino acid dialysate on mesothelial cell function. The follow results were found

1. Cell viability with amino acid dialysate was comparable to 5mmol/L glucose control dialysate.
2. Amino acid dialysate significantly inhibited HPMC proliferation.
3. Amino acid dialysate stimulated an increase in fibronectin protein and mRNA despite only containing 3.5mmol/L D-glucose.
4. Amino acid dialysate stimulated caused an increase in TGF-β protein.
5. The increase in fibronectin protein seen with amino acid dialysate could be blocked by addition of an anti TGF-β antibody to levels of fibronectin not different to those in control conditions.
6. The nitric oxide inhibitor L-NAME inhibited amino acid dialysate induced increases in fibronectin. However L-NAME also affected fibronectin production under control conditions. L-arginine was unable to reproduce the effect of amino acid dialysate on increasing HPMC fibronectin production.

5.9 Conclusions

Amino acids have many theoretical advantages over glucose as an osmotic agent in CAPD. As discussed patients are at risk of protein malnutrition, and amino acid dialysate has been shown to improve serum proteins in malnourished patients [36]. Amino acid dialysate is manufactured at a pH of 6.5 closer to physiological, an as a consequence it could be hypothesised to be less directly cytotoxic to peritoneal cells during CAPD. These data presented here do suggest that although amino acid dialysate is less cytotoxic to cultured cells, it does promote a similar increase in extra cellular matrix production to that seen with 40mmol/L glucose. It is unclear why amino acid dialysate is not associated with the decrease in cell viability previously seen in experiments of high osmolality test medium (section 3.2). It is only speculative to suggest that amino acid dialysate has an uncharacterised protective effect on HPMC hyperosmolar cytotoxicity.

In this study amino acid dialysate stimulated a 60% reduction in mesothelial cell proliferation rate; a similar reduction to that seen with 1ng/ml exogenous TGF-β (section 4.4.1). This is finding is consistent with the level of TGF-β protein production by human peritoneal mesothelial cells exposed to amino acid dialysate. After twelve hours exposure to the amino acid dialysate the mean TGF-β concentration in the culture medium was 0.25ng/ml. It is likely that the concentration would be higher still by 48 hours in the experiments on proliferation, although TGF-β concentration at 48 hours was not measured.

Amino acid dialysate caused a significant increase in fibronectin amount, with a 17.9% increase at 12 hours. This was associated with a 5.1 fold increase in fibronectin mRNA levels. As previously discussed in Chapter 3, it is not possible to exclude an effect from

difference in rate of matrix degradation. However, the data implies that on balance there is a net increase in fibronectin production by mesothelial cells when exposed to amino acid dialysate. This is consistent with the finding of increased TGF-β protein and mRNA, which has previously been shown to increase fibronectin protein and mRNA levels in mesothelial cells (section 4.4). Additionally, the increase in fibronectin seen by mesothelial cells exposed to amino acid dialysate was blocked by the presence of a pan specific anti TGF-β antibody. This is further supporting evidence for the hypothesis that the amino acid dialysate stimulates autocrine TGF-β production that in turn increases fibronectin protein amount.

The amino acid L-arginine is an intermediary in the formation of nitric oxide, which has been shown to inhibit TGF-β production in mesangial cells [139] and chondrocytes [140]. Nitric oxide is constitutively produced in most cells by a diverse spectrum of pathways, and is essential for many cell functions. Nitric oxide synthase (NOS) has at least three different forms and differential up-regulation of these NOS forms results in different down stream events [141]. Because of its widespread constitutive production it is reasonable to presume that HPMC are capable of generating NO, and HPMC functions are likely to be modulated by it.

What is unclear however, is whether an increase in concentration of L-arginine is capable of altering nitric oxide production in the peritoneum. This is important as L-arginine is in present in high concentration in amino acid dialysate (3.3mmol/L). The effect on L-arginine on NO production has been widely investigated in the context of diabetes mellitus. Although the evidence is incomplete, it is clear that L-arginine does influence NO production, at least in organs such as the kidney [138]. Conventionally the rate of the complex reaction involving oxidation of L-arginine to NO should not be dependant on the

concentration of the precursor. However in diabetes (a condition of high extra cellular glucose), plasma arginine concentration is reported to be low in both animals and human [142,143], and the postulated NO deficit seen in diabetes can be reversed by administration of L-arginine [144]. As clinically amino acid dialysate is used in conjunction with hyperosmolar glucose dialysate a possible role for L-arginine stimulated NO production in the peritoneum could be postulated.

An *in-vivo* study using rabbits doing 1 hour CAPD dwells of 3.86% glucose dialysate was able to show differences in peritoneal albumin transport which the authors attributed to increased NO. L-arginine was add to 3.86% glucose dialysate at 0.8mmol/L, and induced similar effects on peritoneal transport to nitroprusside. The NOS inhibitor L-NMMA had no effect however [145]. In humans a study using amino acid dialysate demonstrated an effective difference in peritoneal surface area and peritoneal blood flow whilst patients were using amino acid dialysate. Although this effect could have been explained by the known biological activity of NO, the mass transfer coefficient (MTAC) for nitrate and cGMP did not change with use of amino acid dialysate, effectively ruling out an increase local NO [146]. From the available data it appears uncertain whether L-arginine in amino acid dialysate has any effect on intraperitoneal NO.

In-vitro nitric oxide synthesis can be studied in by variety of different methods. One method is to measure the nitric oxide breakdown products nitrate and nitrite in the cell culture medium. Attempts were made to measure nitrite in this cell culture system (Cayman's Nitrite assay, Alexis Corporation 850-001-KI01), but nitrate levels were below the limits of detection of the assay (~5μM) in all test conditions. It is likely that the short time course of the experiments had allowed insufficient time for the concentration of

nitrite in the test medium to rise significantly even if alteration had occurred in cellular nitric oxide synthesis.

In addition attempts were made to investigate the effect of exposing HPMC to test medium supplemented with 8mmol/L L-arginine. However, the addition of L-arginine to test medium was unable to reproduce the increase in fibronectin seen in the presence of amino acid dialysate. The nitric oxide inhibitor L-NAME inhibited amino acid induced HPMC fibronectin production. However L-NAME also produced an unexpected increase in fibronectin under control 5mmol/L glucose conditions. The data is therefore difficult to interpret with such a pronounced effect of nitric oxide inhibition under control conditions. Although this data is limited and may suggest an alteration of nitric oxide synthesis in this culture system, in other culture systems an increase in NO has been associated with an inhibited rather than augmented production of TGF-β and ECM [139,140]. Alterations in NO would therefore be unlikely to account for the increase in HPMC TGF-β and fibronectin seen in this culture system with amino acid dialysate.

In summary amino acid dialysate offers improvement in cell viability in this culture system compared with hyperosmolar glucose dialysate. However, amino acid dialysate inhibits proliferation, and stimulates the production of extra-cellular matrix. This effect is mediated through autocrine release of TGF-β. However, what this means *in-vivo* is unclear.

6 A different buffering agent: effect of bicarbonate vs. lactate dialysate

6.1 Introduction

High lactate concentration has been implicated previously in the inhibitory effects of peritoneal dialysate on cultured cells [47] independent of glucose concentration. Recently bicarbonate has been developed as a practical alternative buffering agent in PD solutions. Bicarbonate buffered dialysate has the theoretical advantage of neutral pH at the point of instillation into the peritoneal cavity, but requires a more complicated "twin-bag" delivery system to prevent the precipitation of insoluble calcium salts. Dialysate containing bicarbonate are becoming available to selected patients for clinical use, but in general clinical experience with these solutions remains limited.

In vitro bicarbonate based dialysate does appear to be less cytotoxic to white cells (PMN, PBMC and peritoneal macrophages) [60,147-149] measured by a variety of techniques including cytosolic free-calcium mobilisation, superoxide production and endotoxin stimulated TNF-α production. However, other groups have shown bicarbonate dialysate to remain inhibitory to the migratory and phagocytic capacity of PMN [150]. It could be speculated that although lactate-bicarbonate dialysate has a physiological pH of 7.4, this is at the expense of a supra-physiological pCO_2, and that this could provide a possible mechanism for continued cytotoxicity.

Studies with HPMC show less inhibition of ATP production if cultured cells were exposed to 1.36% glucose, bicarbonate (38mmol/L) or bicarbonate:lactate (25:15mmol/L) buffered dialysate compared with conventional lactate dialysate (40mmol/L lactate) [66]. At 3.86% glucose all solutions suppressed ATP production, although suppression was greater with the lactate buffered dialysate. In all cases ATP production returned to normal after a further 30 minutes in rest medium. Similarly cell function expressed as IL-1 induced IL-6 production also showed less inhibition with bicarbonate buffered dialysate than

conventional lactate buffered dialysate with both HPMC and peritoneal fibroblasts [151]. These studies have investigated short exposure to neat dialysis solutions however, and are unable to draw any conclusion on the long-term effect throughout the CAPD dwell.

Using a 72 hour protocol of 50:50 dialysate with medium and 10% FBS one study has demonstrated that bicarbonate:lactate was less inhibitory to HPMC cell growth, but only at 3.86% glucose concentration [152]. Despite showing no differences in cell growth using 1.36% glucose the bicarbonate:lactate solution was associated with lower concentrations of glucose degradation products at either glucose concentration. This is may be clinically significant as GDP have previously been implicated in cytotoxicity in their own right [153].

Two *in vivo* studies have modelled CAPD in animals to investigate a differential effect of bicarbonate dialysate. A study in rabbits showed no difference between bicarbonate and lactate based dialysate when used for four weeks of CAPD, comparing a wide variety of histological and biochemical parameters [154]. A similar four week CAPD regimen in rats (two dialysis exchanges per day) however did show differences in the number of peritoneal cells shed in spent dialysate [155]. In addition, there were fewer neutrophils, and higher percentage of macrophages which led the authors to conclude that there was a reduction in non-specific dialysis related inflammation.

Conventional lactate buffered dialysate has been used in large numbers of patients, and it is generally accepted that the 40mmol/L lactate concentration in dialysate is sufficient to correct the acidosis of CRF. As previously discussed this requires a dialysate lactate concentration which is significantly greater than the physiological concentration in plasma. With bicarbonate buffered dialysate it is apparent that the bicarbonate concentration of dialysate needs to be similar to the previously validated lactate concentration to correct renal acidosis. An early study which changed patients to 33mol/L bicarbonate dialysate

resulted in a fall in plasma bicarbonate from 28.8 to 23mmol/L over two months of CAPD [156]. Using a 38mmol/L solution a subsequent study showed no such fall, and resulted in preservation of plasma bicarbonate when patients changed to bicarbonate from conventional lactate dialysate [157]. Effective correction of acidosis is of considerable importance as there is a weight of evidence in favour of high normal, or even supra-normal bicarbonates in patients on dialysis to prevent bone disease [158] or protein catabolism [159]. Because of this most subsequent studies have concentrated on either 38mmol/L bicarbonate solutions, or a bicarbonate:lactate mixture of 25:15mmol/L.

Two large clinical studies have been reported on the tolerability of bicarbonate dialysate. The largest study randomised 69 patients to receive either lactate (34mmol/L) or bicarbonate (34mmol/L) for six months [160]. The study showed no difference in acid-base status, peritoneal membrane characteristics (PET) or dialysis adequacy between the study groups. The second study compared lactate (40mmol/L) with bicarbonate:lactate (25:15mmol/L) and bicarbonate only buffer (38mmolL) for 2 months. Approximately 20 patients were included in each study group. This study too showed no difference in acid-base balance or any other biochemical parameter. Studies of this design would be unable to detect subtle differences in peritoneal membrane viability as these would be unlikely to manifest as changes in clinical parameters for months or years. With so many other confounding influences on technique survival on CAPD it is unlikely that differences would be apparent until large numbers of patients were using the solution in routine clinical practice.

The only human study to investigate the cellular effects of bicarbonate dialysate looked at CA125 concentration in PD effluent. The study showed an increased PD effluent CA125, thought by some to represent a surrogate marker of mesothelial cell mass, and a decrease

in hyaluronic acid [161]. In summary, these solutions do initially appear to be safe and well tolerated, and in a small study bicarbonate based PD solution has also been shown to reduce infusion pain [162], presumably because of the solution pH closer to a physiological one.

No studies have previously considered the effect of bicarbonate buffer composition on extra-cellular matrix production. In chapter 3 it has been shown that increasing glucose concentration resulted in a decrease in cell viability and proliferation, and an increase in fibronectin protein and mRNA. In these experiments a differential effect on dialysate buffer will be considered.

6.2 Cell viability

6.2.1 Bicarbonate / lactate effect on LDH release

6.2.1.1 Materials and Methods

Confluent HPMC cultured in 12 well plates were rendered quiescent by 48 hours exposure to M199 with 0.1% FBS. This was followed by 12 hour exposure to test medium of a 50:50 mixture of dialysate and M199 with 0.1% FBS. Two different comparisons were made:

A Commercial bicarbonate/lactate dialysate composition is summarised in Table 1-1, and contains 25mmol/L bicarbonate and 15mmol/L lactate buffers. Laboratory manufactured dialysate was therefore prepared with bicarbonate:lactate buffer proportions varied to 40:0, 10:30, 20:20 and 0:40mmol/L respectively. Final glucose concentration was adjusted to 40mmol/L glucose.

B Laboratory dialysate was manufactured to identical composition to commercial lactate dialysate (40mmol/L lactate) and bicarbonate/lactate dialysate (25:15mmol/L bicarb:lactate) but without glucose. Final glucose concentration was adjusted to either 5mmol/L or 40mmol/L with addition of D-glucose.

LDH activity was measured in the cell culture supernatant and cell digest as described in 2.9, and results expressed as percentage supernatant to total LDH activity.

6.2.1.2 Results

Results of experiment A are shown in Figure 6.1. Changes in dialysate buffer between 40:0 mmol/L and 0:40mmol/L bicarbonate:lactate at constant glucose concentration (40mmol/L) made no difference to the proportion of LDH release. LDH release in the

40mmol/L lactate condition was comparable to previous data on LDH release with this test medium (section 3.2).

Results of experiment B are shown in Figure 6.2. In the 40mmol/L lactate dialysate the increase in glucose concentration from 5 to 40mmol/L resulted in an increase in LDH release as previously demonstrated (section 3.2). With 25:15 bicarbonate:lactate dialysate the release of LDH was comparable to that with 40mmol/L lactate dialysate at both concentrations of glucose.

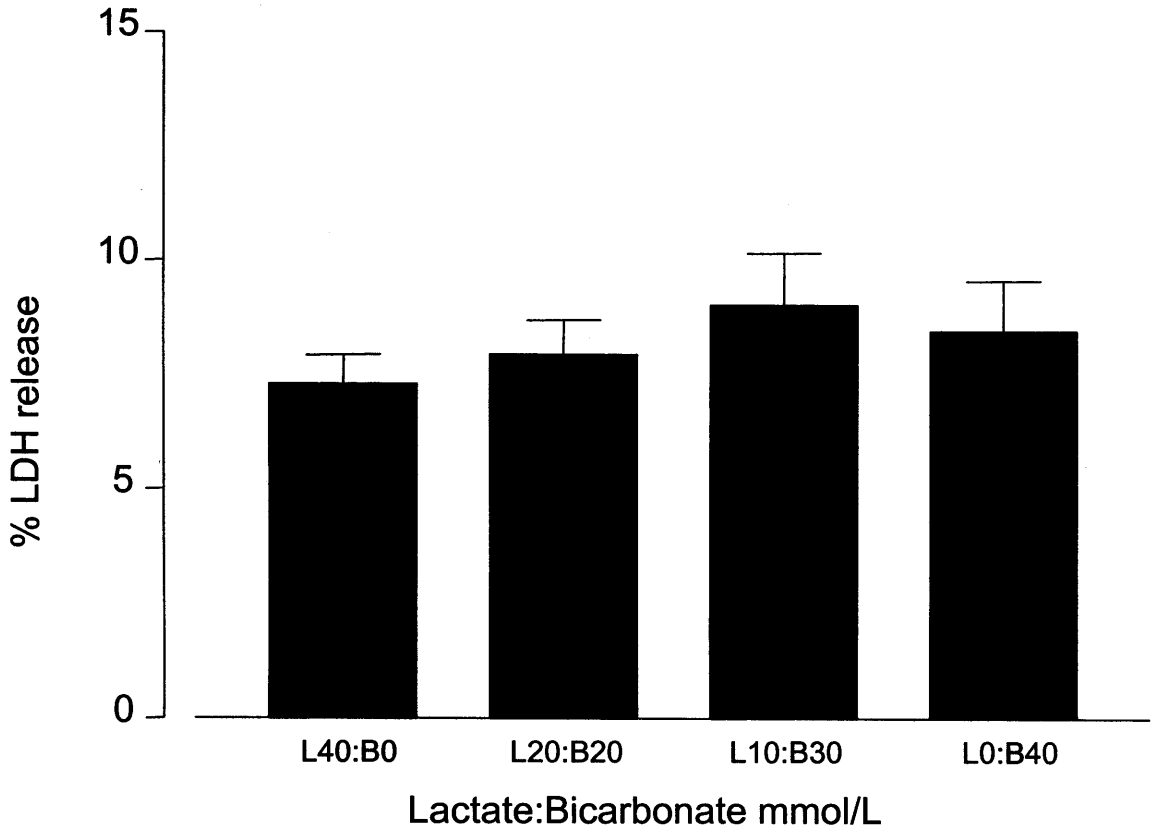


Figure 6.1 Effect of buffer on LDH release at 40mmol/L glucose

Laboratory manufactured dialysate was mixed 50:50 with M199 at 40mmol/L glucose. Dialysate buffer was adjusted between 40:0 mmol/L to 0:40mmol/L bicarbonate:lactate. Changes in the dialysate buffer had no effect on LDH release at 40mmol/L glucose. Results are mean ± SEM of three experiments in triplicate.

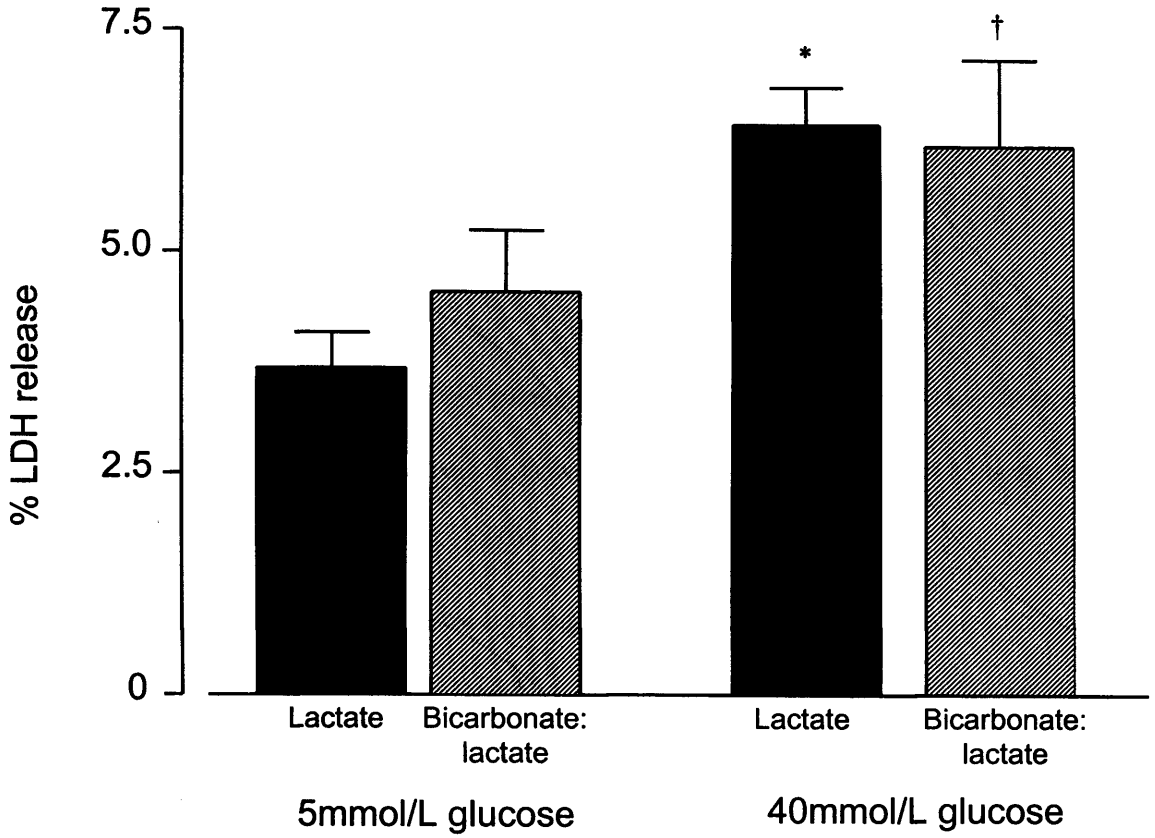


Figure 6.2 Effect of dialysate buffer on LDH release

Laboratory manufactured dialysate was mixed 50:50 with M199. Dialysate buffer was either 40mmol/L lactate or 25:25mmol/L bicarbonate:lactate. Final glucose concentration was 5 or 40mmol/L. Increase in glucose concentration resulted in an increase in LDH release independent of the buffer concentration. No difference was detected between LDH release with the two buffers at either glucose concentration. Results are mean ± SEM for three experiments in triplicate. * $p<0.05$ vs. 5mmol/L glucose lactate-dialysate, † $p<0.05$ vs. 5mmol/L glucose bicarb:lactate-dialysate

6.3 Proliferation

6.3.1 Bicarbonate / lactate effect on proliferation

6.3.1.1 Materials and Methods

Confluent HPMC cultured in 12 well plates were rendered quiescent by 48 hours exposure to M199 with 0.1% FBS. This was followed by 12 hour exposure to test medium of a 50:50 mixture of dialysate and M199 with 0.1% FBS. Dialysate was laboratory manufactured to identical composition to either commercial 1.36% glucose lactate dialysate (40mmol/L lactate) or commercial 1.36% glucose bicarbonate/lactate dialysate (25:15mmol/L bicarb:lactate). Final glucose concentration was varied to either 5 or 40 mmol/L. Proliferation was measured by ^{3}H-thymidine incorporation as described in section 2.8 using a 48 hour protocol as described in section 3.3. Cells were incubated for 48 hours with test medium, and 0.5 μCi ^{3}H-thymidine added for the final 16 hours of the experiment without changing the test medium.

6.3.1.2 Results

Effect of different buffers on proliferation is shown in Figure 6.3. With lactate buffer increasing glucose concentration resulted in a decrease in HPMC proliferation rate as previously seen. With bicarbonate:lactate dialysate the HPMC proliferation rate was higher at either glucose concentration than with lactate dialysate and not suppressed by higher glucose concentration.

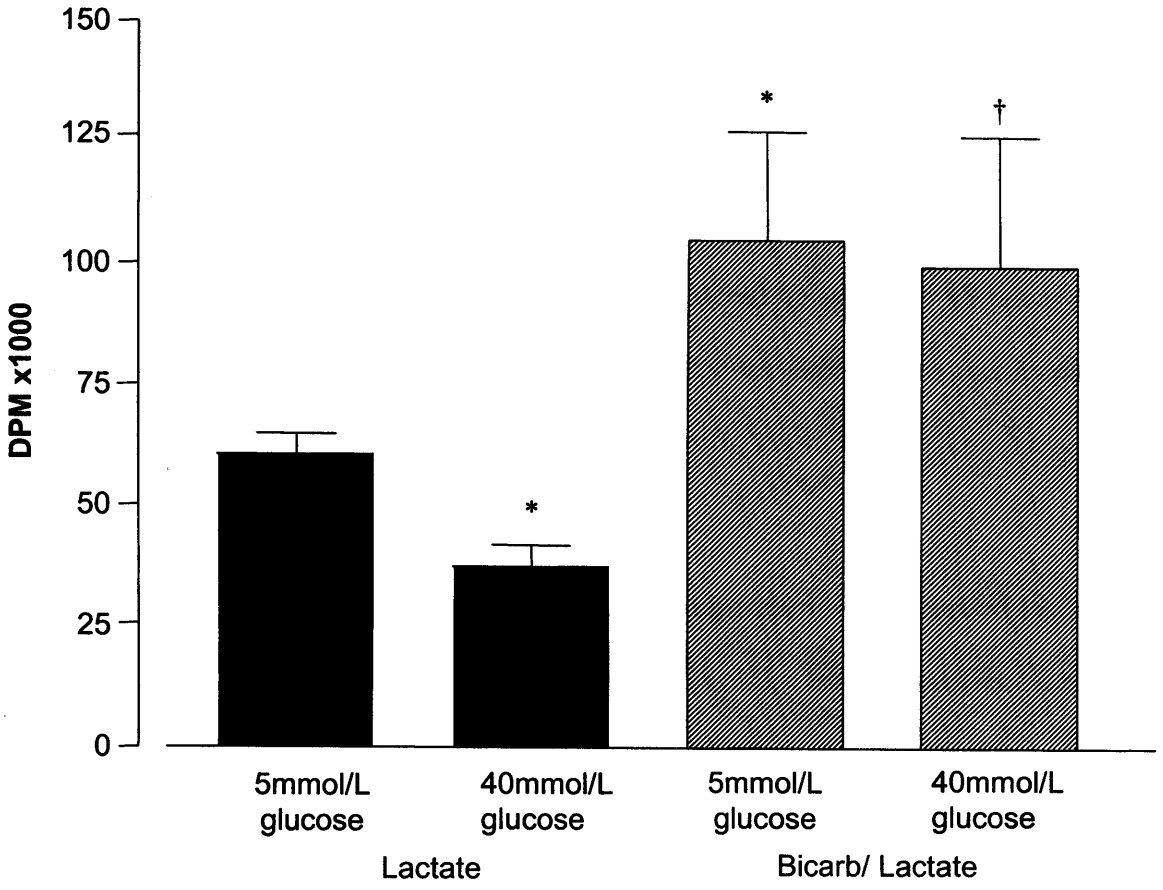


Figure 6.3 Effect of buffer on proliferation

Laboratory manufactured was mixed 50:50 with M199. Dialysate buffer was either 40mmol/L lactate or 25:25mmol/L bicarbonate:lactate. Final glucose concentration was either 5 or 40mmol/L. With lactate buffer increase in glucose concentration resulted in an significant inhibition of proliferation. Proliferation rate was higher with bicarbonate:lactate buffer at either glucose concentration, and not suppressed by an increase in glucose. Results are mean ± SEM for three experiments in triplicate. * $p<0.05$ vs. 5mmol/L glucose lactate-dialysate, † $p<0.05$ vs 40mmol/L glucose lactate-dialysate.

6.4 Fibronectin protein production

6.4.1 Effect of buffer on fibronectin protein production

6.4.1.1 Materials and methods

Confluent HPMC cultured in 12 well plates were rendered quiescent by 48 hours exposure to M199 with 0.1% FBS. This was followed by 12 hour exposure to test medium of a 50:50 mixture of dialysate and M199 with 0.1% FBS. Two different comparisons were made:

A Commercial bicarbonate/lactate dialysate composition is summarised in Table 1-1, and contains 25mmol/L bicarbonate and 15mmol/L lactate buffers. Laboratory manufactured dialysate was therefore prepared with bicarbonate:lactate buffer proportions varied to 40:0, 10:30, 20:20 and 0:40mmol/L respectively. Final glucose concentration was adjusted to 40mmol/L glucose.

B Laboratory dialysate was manufactured to identical composition to commercial lactate dialysate (40mmol/L lactate) and bicarbonate/lactate dialysate (25:15mmol/L bicarb:lactate) but without glucose. Final glucose concentration was adjusted to either 5mmol/L or 40mmol/L with addition of D-glucose.

Supernatant fibronectin measured by ELISA as described in section 2.4.1. Results were expressed corrected for total cell protein as described in section 2.6.

6.4.1.2 Results

Effect of different dialysate buffer concentration on fibronectin protein at 40mmol/L glucose are shown in Figure 6.4. Changes in dialysate buffer at constant glucose

concentration (40mmol/L) had no effect on fibronectin amount. Figure 6.5 shows the difference in fibronectin seen with 5 and 40mmol/L glucose concentration in either lactate or bicarbonate:lactate dialysate. Fibronectin production was no different between conditions at the same glucose concentration. Increase in glucose concentration resulted in an increase in fibronectin protein production that was comparable between solutions using the different buffers.

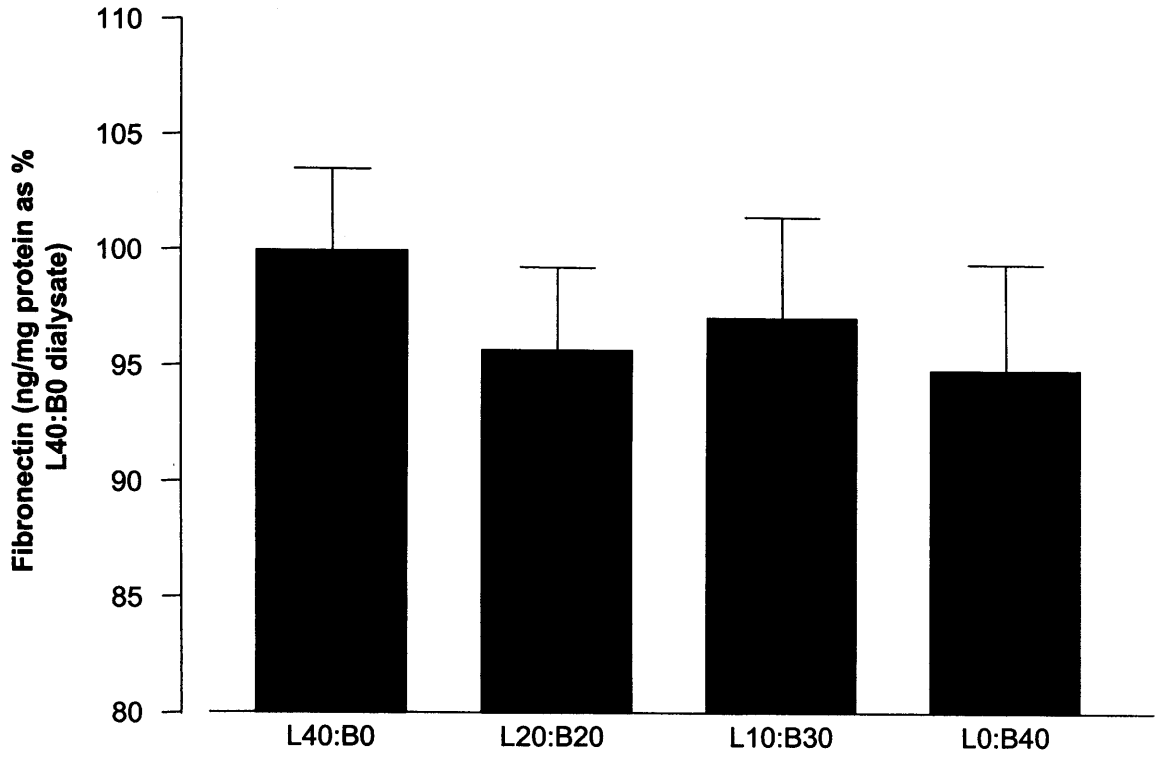


Figure 6.4 Effect of buffer on fibronectin production at 40mmol/L glucose

Laboratory manufactured dialysate was mixed 50:50 with M199 at 40mmol/L glucose. Dialysate buffer was adjusted between 40:0 mmol/L to 0:40mmol/L bicarbonate:lactate. Changes in the dialysate buffer had no effect on fibronectin production at 40mmol/L glucose. Results are mean ± SEM of three experiments in triplicate.

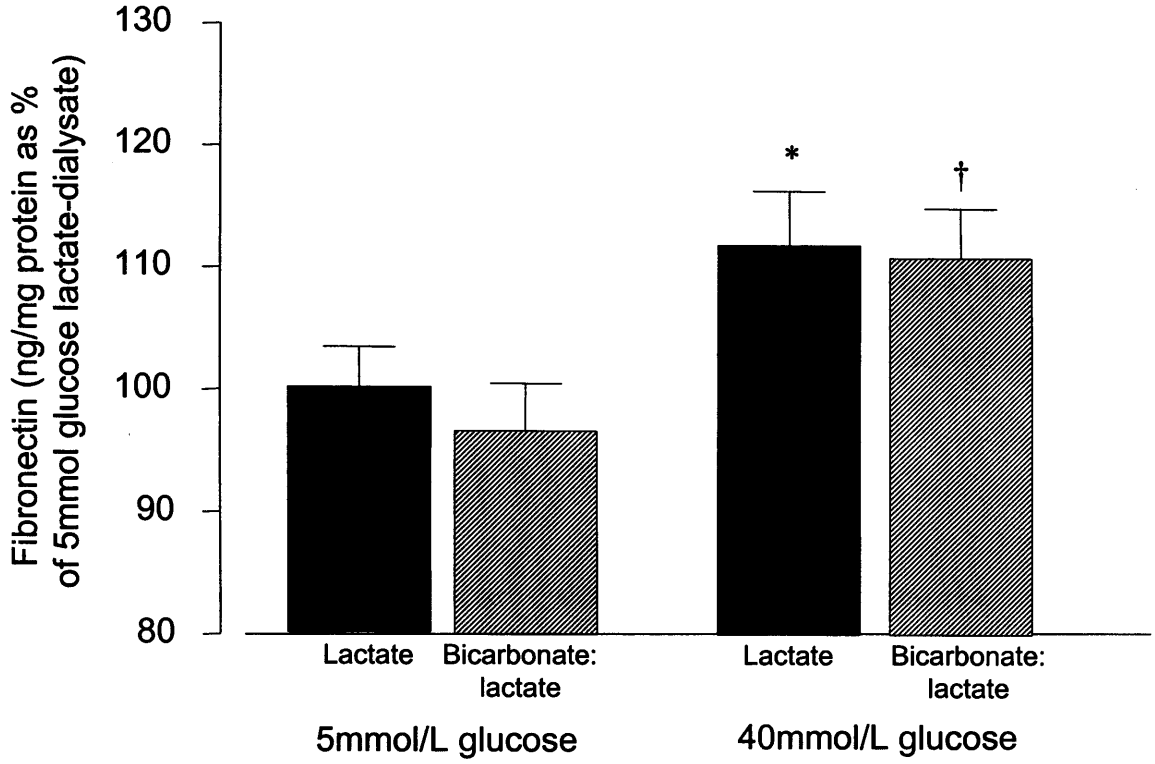


Figure 6.5 Effect of dialysate buffer on fibronectin production

Laboratory manufactured was mixed 50:50 with M199. Dialysate buffer was either 40mmol/L lactate or 25:25mmol/L bicarbonate:lactate. Final glucose concentration was either 5 or 40mmol/L. Increase in glucose concentration resulted in an increase in fibronectin production. No difference was observed between different buffers Results are mean ± SEM for three experiments in triplicate. * $p<0.05$ vs. 5mmol/L glucose lactate-dialysate, † $p<0.05$ vs. 5mmol/L glucose bicarb:lactate-dialysate.

6.5 TGF-β protein production

6.5.1 Effect of dialysate buffer on TGF-β production

6.5.1.1 Materials and methods

Confluent HPMC cultured in 12 well plates were rendered quiescent by 48 hours exposure to M199 with 0.1% FBS. This was followed by 12 hour exposure to test medium of a 50:50 mixture of dialysate and M199 with 0.1% FBS. Two different comparisons were made:

A Commercial bicarbonate/lactate dialysate composition is summarised in Table 1-1, and contains 25mmol/L bicarbonate and 15mmol/L lactate buffers. Laboratory manufactured dialysate was therefore prepared with bicarbonate:lactate buffer proportions varied to 40:0, 10:30, 20:20 and 0:40mmol/L respectively. Final glucose concentration was adjusted to 40mmol/L glucose.

B Laboratory dialysate was manufactured to identical composition to commercial lactate dialysate (40mmol/L lactate) and bicarbonate/lactate dialysate (25:15mmol/L bicarb:lactate) but without glucose. Final glucose concentration was adjusted to either 5mmol/L or 40mmol/L with addition of D-glucose.

Supernatant TGF-β was measured by ELISA as described in section 2.4.2. Results were expressed corrected for total cell protein as described in section 2.6.

6.5.1.2 Results

Figure 6.6 shows HPMC TGF-β production at 40mmol/L glucose with dialysate buffer varied between bicarbonate:lactate 0:40 and 40:0mmol/L. No difference was detectable with different buffer composition. Figure 6.7 shows the increase in TGF-β production by HPMC in lactate dialysate of similar magnitude to that previously described in section 4.2.1. Bicarbonate:lactate dialysate showed comparable results, but on this occasion neither difference with increasing glucose concentration reached statistical significance.

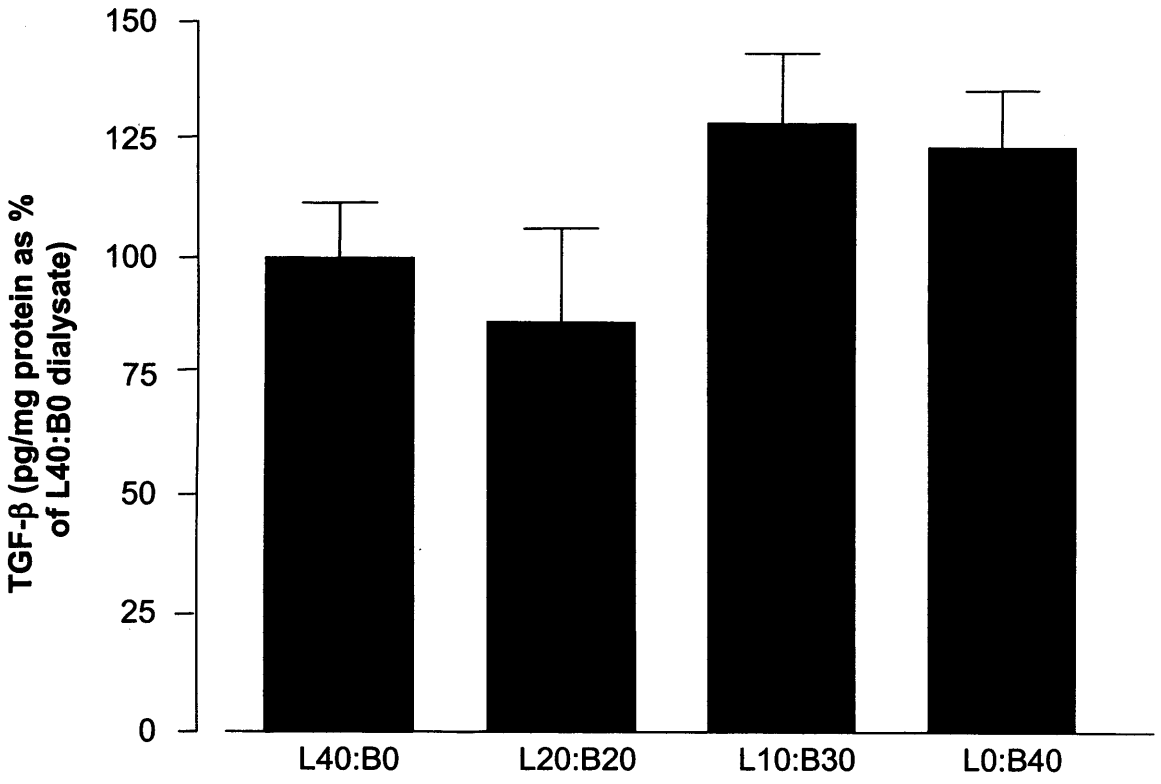


Figure 6.6 Effect of buffer on TGF-β production at 40mmol/L glucose

Laboratory manufactured dialysate was mixed 50:50 with M199 at 40mmol/L glucose. Dialysate buffer was adjusted between 40:0 mmol/L to 0:40mmol/L bicarbonate:lactate. Changes in dialysate buffer had no effect on TGF-β production at 40mmol/L glucose. Results are mean ± SEM of three experiments in triplicate.

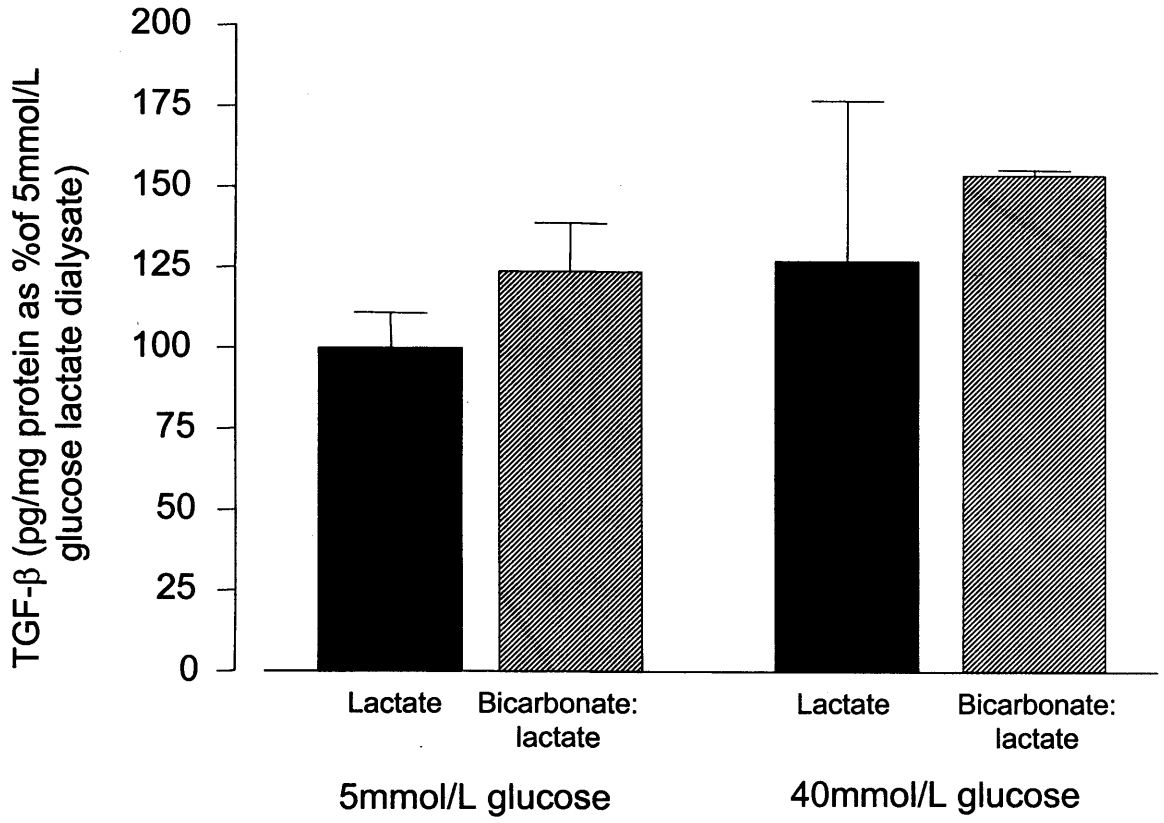


Figure 6.7 Effect of dialysate buffer on TGF-B production

Laboratory manufactured was mixed 50:50 with M199. Dialysate buffer was either 40mmol/L lactate or 25:25mmol/L bicarbonate:lactate. Final glucose concentration was either 5 or 40mmol/L. Increase in glucose concentration resulted in an increase in TGF-β production, although this did not reach statistical significance. No difference was observed between different buffers Results are mean ± SEM for three experiments in triplicate.

6.6 Summary

These experiments investigated the effect of the dialysate buffer on mesothelial cell function and compared lactate-buffered dialysate with bicarbonate and bicarbonate:lactate dialysate at different glucose concentrations. The following results were found

1. Increase in dialysate glucose produced a decrease in cell viability as previously described (Chapter 3). Changes in dialysate buffer between 0:40 bicarb:lactate and 40:0mmol/L made no difference to cell viability.

2. HPMC proliferation was greater in 25:15mmo/L bicarbonate:lactate dialysate at either 5mmol/L or 40mmol/L glucose concentration. In addition, hyperosmolar glucose did not suppress proliferation in bicarbonate:lactate dialysate as shown with conventional lactate-buffered dialysate

3. Increase in glucose concentration produced an increase in fibronectin protein production independent of dialysate buffer, and was unaffected by changes in dialysate buffer proportions.

4. A trend toward an increase in TGF-β amount was seen with increased glucose concentration with both lactate and bicarb:lactate buffered dialysate. Changes in dialysate buffer had no effect on TGF-β production at 40mol/L glucose.

6.7 Conclusions

These data enable comparison of the different effects of dialysate buffer on HPMC viability, proliferation, and fibronectin production. Unlike the effect of glucose or TGF-β on cell function there are few other settings in which cells are exposed to high concentrations of different buffers except in the peritoneum during peritoneal dialysis. As a consequence there is far less data from other systems with which to draw comparisons or support findings.

In these experiments high glucose concentration has again been shown to decrease cell viability and inhibit proliferation in the presence of lactate buffered dialysate. The same effect on cell viability was seen with bicarbonate or bicarbonate:lactate dialysate, with no detectable effect of the different buffers. This is in contrast to previous studies in which neat bicarbonate dialysate has been shown to be less inhibitory to cells in short exposure [66]. As discussed in chapter 3 this type of culture system is unable to enlighten about the effects of chronic exposure during a 8-12 hour CAPD dwell. In addition, in our culture system, the use of a 50:50 mixture of dialysate with M199 partially corrects the initial low pH of neat lactate dialysate to levels seen later in the CAPD dwell [86]. Consequently, it avoids differences in cytotoxicity that are the result of low initial pH.

Bicarbonate:lactate dialysate does however appear to be less inhibitory to mesothelial cell proliferation. In addition these results suggest that an increase in glucose concentration has no adverse effect on proliferation previously noted with lactate-dialysate. This may well be the result of differences in test medium pH, as even 50:50 lactate-dialysate:M199 has an initial pH of 7.07±0.02 in a 5% pCO_2 atmosphere (paragraph 2.3.2), whilst for a

50:50 mixture of 25 mmol/L bicarbonate: 15mmol/L lactate dialysate with M199 the pH was 7.38±0.02. The greater proliferation is not a consequence of decreased TGF-β production, in part the explanation of hyperosmolar glucose suppression of proliferation, as supernatant TGF-β concentration was no different between dialysate conditions. Although it is difficult to extrapolate data from an *in-vitro* cell culture system, it could be speculated that the increase in proliferation seen with bicarbonate:lactate dialysate *in-vitro* could account for the increase in CA125 seen in dialysis effluent from patients using this solution in clinical practice.

Considering the effect of dialysate buffer on ECM and TGF-β production no difference was detectable between dialysate buffers. For both TGF-β and fibronectin, an increase in glucose concentration produced an increase in protein amount independent of the dialysate buffer. The magnitude of the effect was similar between dialysate buffer conditions, and the lack of statistic significance on the effect on TGF-β production reflects wide variation in the responses of these cells. As this was also true in the lactate-buffered dialysate conditions, where TGF-β amount has previously been shown to increase with increasing glucose concentration (section 4.2.1), it is reasonable to conclude that the same effect is likely to be present with bicarbonate:lactate dialysate.

Clinical data using bicarbonate:lactate bicarbonate has demonstrated that it is safe and well tolerated [157], and can improve surrogate markers of HPMC mass (CA125) and decrease a key constituent of the ground substance, hyaluronic acid over six months use [161]. No study has shown any difference in peritoneal membrane characteristics or membrane longevity, although the current studies have been limited to six months in upto 60 patients. Any difference in peritoneal membrane longevity would be likely to be small, and the variability between patients large, making the current studies unlikely to detect any

differences. This *in-vitro* data suggest however, that with the exception of HPMC proliferation, hyperosmolar glucose is a more important risk factor for peritoneal fibrosis than changes in dialysate buffer.

7 Discussion

World wide over 100,000 people are dependent on the technique of CAPD for treatment of end-stage renal failure. However, long-term technique survival is poor and estimated to be less than 50% at five years in some published series. Although multi-factorial this is in part due to progressive peritoneal fibrosis resulting in loss of the semi-permeable peritoneal membrane. Repeated episodes of peritonitis are one aetiological factor, but as already discussed, membrane failure can occur in the absence of infection. In this instance the most likely factor in the progressive membrane failure is constituents of the dialysis solutions themselves.

Hyperosmolar glucose, lactate based dialysate is the most widely used dialysis solution for patients on CAPD, and hence it is important to understand the effects of this solution on the resident peritoneal cells. These experiments suggest that the high glucose concentration of the solution may have a crucial role in promoting extra-cellular matrix (ECM) production by the mesothelial cells lining the peritoneal cavity, and that this effect is mediated via autocrine production of TGF-β.

Increasing glucose concentration resulted in an increase in both fibronectin protein production and gene expression by HPMC. This effect was independent of the increase in osmolality of the dialysis solution. In contrast the increase in LDH release and hence decrease in cell viability of mesothelial cells appears to be purely the result of increasing osmolality of the solution, with no specific effect of high glucose concentration. Results of fibronectin protein production were expressed corrected for total cell protein, and although cell viability decreased there was no detectable change in total cell protein. The changes in fibronectin protein production cannot therefore be accounted for simply by changes in either cell viability or total cell protein.

In the twelve-hour protocol a 15% increase in fibronectin protein production, and 1.4 fold increase in gene expression was observed. Although this represents a relatively small change it must be remembered that patients on CAPD will be repeatedly replacing dialysis bags 4 to 5 times per day, and hence any small change over twelve hours potentially represents a considerable alteration in extra-cellular matrix synthesis over many years of CAPD. Although no *in-vitro* cell culture protocol can truly mimic the condition *in-vivo,* the twelve-hour time point was chosen to mimic the long overnight CAPD dwell, after which *in-vivo*, a patient would change the dialysate for a fresh solution. Longer dwells without replacement of dialysate do not occur in clinical practice. Preliminary experiments showed the same effects are observed with time-courses of 48 and 72 hours indicating that there is little adaptation to the effect of high glucose even over longer dwells.

These experiments provide convincing evidence for the role of autocrine TGF-β production by HPMC to regulate extra-cellular matrix production in response to glucose. Hyperosmolar glucose produced an increase in TGF-β protein and also fibronectin protein, and the latter could be inhibited by the addition of an anti TGF-β antibody. This suggests that TGF-β has a key regulatory role in promoting extra-cellular matrix production by HPMC. It is unclear whether the increase in fibronectin mRNA levels in response to TGF-β results from increased gene expression or increased RNA stability. A high glucose concentration has been shown to promote an increase in extra-cellular matrix in other cell types [95-97]. Furthermore exposing cultured mesangial cells to high glucose concentration results in an increase in collagen III deposition, which could be prevented by the addition of an anti TGF-β antibody [98].

Hyperosmolar glucose is implicated in the pathogenesis of other important conditions, in particular diabetes mellitus. Consequently, the mechanism by which hyperosmolar glucose

influences cell functions has been widely studied. Two glycolytic pathways, the polyol and the hexosamine pathways have been investigated. Sorbitol (an intermediate of the polyol pathway) has been shown to accumulate in mesothelial cells exposed to high concentrations of glucose and cause cellular dysfunction [102]. In contrast, the hexosamine pathway has been explored in mesangial cells. There is evidence that both high glucose concentration, or the intermediate metabolite of the hexosamine pathway glucosamine can stimulate mesangial cell TGF-β production [101]. These data suggest that it is metabolism of glucose via the sorbitol pathway that stimulates mesothelial cell extra-cellular matrix protein production. In HPMC sorbitol was able to reproduce the effect of hyperosmolar glucose on fibronectin protein production whilst glucosamine had no effect. Further work has shown that the aldose-reductase inhibitor zopolrestat inhibited HPMC fibronectin protein production when exposed to hyperosmolar glucose [111]. These data are consistent with work in the diabetic eye has shown the clinical benefit of preventing metabolism of glucose through sorbitol by using an aldose-reductase inhibitor [163].

The two newer dialysis solutions; amino acid dialysate, lactate-bicarbonate dialysate are in increasingly widespread clinical use. These experiments show that they have differential effects on cell viability, and extra-cellular matrix turnover. All would appear to have theoretical advantages and limitations, although none have been demonstrated in clinical practice. Amino acid dialysate offers improvement in cell viability in this cell culture model. However, as with glucose dialysate it does increase fibronectin mRNA levels and also fibronectin protein. Amino acid dialysate also causes an increase in HPMC TGF-β protein, and the addition of a pan specific anti TGF-β antibody prevented the amino acid stimulated fibronectin production. It appears that although offering improvements in cell

viability amino acid dialysate inhibits proliferation, and stimulated TGF-β and ECM production.

The mechanism behind the TGF-β mediated increase in ECM with amino acid dialysate is considered. The nitric oxide precursor L-arginine, is present in high concentration in amino acid dialysate. *In-vivo* L-arginine has been shown to increase NO synthesis in patients with diabetes mellitus [138], and *in-vitro* an increase in NO has been shown to regulate TGF-β and ECM in mesangial cells [139] and chondrocytes [140]. However, in these other cell types increases in NO have been associated with an inhibition of TGF-β, and would be unable to explain the increase in TGF-β and fibronectin seen in HPMC exposed to amino acid dialysate. The explanation for amino acid induced ECM production remains unclear, but worthy of further investigation.

Lactate-buffered dialysate has been shown to be inhibitory to *in-vitro* cell function independent of glucose concentration because of the high lactate concentration of 40mmol/L [47]. Bicarbonate:lactate (25:15mmol/L) buffered dialysate is now available for clinical use in selected patients. In clinical trials it is well tolerated and safe [164]. *In vivo* studies in rabbits [154] and rats [155] showed conflicting results with the study in rats showing improvements in the population of cells shed in the PD effluent, whilst the rabbit study showed no differences in peritoneal histology after one month of either solution. *In-vitro* studies have suggested that bicarbonate or bicarbonate:lactate dialysate is less inhibitory to ATP production at 1.36% glucose, but no difference was seen at 3.86% glucose [66].

In this *in-vitro* model of CAPD with 50:50 dialysate with M199 dialysate buffer appeared to have little influence on HPMC cell viability or production of TGF-β and ECM. Increases in glucose concentration resulted in decrease cell viability, and increases in TGF-β and

fibronectin of a comparable amount despite changes in dialysate buffer between bicarbonate:lactate 40:0 to 0:40 mmol/L. HPMC proliferation was greater in bicarbonate:lactate 25:15mmol/L dialysate compared with conventional lactate dialysate (40mmol/L) with approximately twice the proliferation rate measured by ^{3}H-thymidine incorporation. In addition, increase in dialysate glucose did not cause the same decrease in proliferation seen with lactate-buffered dialysate. This effect is likely to be the result of differences in test medium pH which was much closer to physiological pH with the bicarbonate:lactate buffer (7.44) versus 7.07 with the lactate buffer. With the exception of the improved proliferation rate it would appear that hyperosmolar glucose is a more important risk factor for peritoneal fibrosis than dialysate buffer.

Other investigators have implicated plasticisers from the PD bags and advanced glycation end-products (AGE) as contributors to the abnormal function of the HPMC during PD. The bags containing the PD solution are made from polylvinyl chloride (PVC) and as a consequence release plasticisers such as acid esters of phthalic acid, and particularly bis-(2-ethylhexyl) phthalate (BEHP). The presence of these compounds have been shown to inhibit both HPMC and also peritoneal fibroblast function *in-vitro* [165]. It is unknown if their presence influences peritoneal fibrosis, but they do stimulate neutrophils and macrophages to release IL-1 and IF-gamma, which can be pro-fibrotic in other contexts [165].

Advanced glycation end-products are implicated in the pathogenesis of other conditions associated with hyperosmolar glucose such as diabetes [74]. AGE have been shown to be deposited in the peritoneum of patients on long-term CAPD using hyperosmolar glucose solutions [27,166], and *in-vitro* their amount to be increased by the normal heat-sterilisation of commercial PD solutions [78]. The presence of AGE in the peritoneum has been

correlated with time on dialysis and also episodes of peritonitis [166]. In addition the presence of AGE was felt to be associated with adverse changes in peritoneal permeability (decreased 4hour glucose on PET and decrease UF). The finding of AGE and permeability changes are not necessarily related, but this finding is supported by histological data which suggests that the AGE are deposited in proximity to peritoneal capillaries [27]. In mesangial cells *in-vitro* AGE have been associated with an increase in profibrotic growth factors and also ECM [98]. Therefore *in-vivo* during long-term CAPD, AGE may well be associated or even causal in the changes in peritoneal function observed clinically [167]. These effects are beyond the scope of this thesis. However, they are unlikely to be responsible for the findings in this *in-vitro* model in which test medium was manufactured in non-PVC containers, filter sterilised, and HPMC exposed for short time courses during which significant formation of AGE is unlikely.

In summary therefore, this study provides evidence that hyperosmolar glucose stimulates mesothelial cell ECM production, and that this effect is mediated through autocrine TGF-β production. It also demonstrates that this effect can be prevented by using an anti TGF-β blocking strategy. Sorbitol, an intermediate in the aldose reductase pathway caused a similar increase in fibronectin protein production by HPMC, an effect seen in other cells of interest in the investigation of the pathogenesis of diabetes. Amino acid dialysate causes less cytotoxicity, but also inhibits HPMC proliferation, and also stimulates TGF-β mediated an increase in fibronectin production. The mechanism for this increase remains unclear. These data raise the possibility of local anti TGF-β strategies may be useful in prolonging CAPD technique survival by decreasing the tendency for peritoneal fibrosis with these dialysis solution. Modifications to the dialysate buffer had little effect on HPMC viability, or TGF-β and fibronectin production. Bicarbonate:lactate dialysate did

cause less inhibition of proliferation which is likely to be an effect of more physiological pH.

7.1 Critique of methodology used

It is important in the discussion of this data to include a critical review of the methodology used, and consider alternative methods. The following methods will be reviewed.

7.1.1 Loss of mesothelial cell monolayer with time on dialysis

It is now appreciated that a cohort of patients on CAPD are able to remain in peritoneal dialysis for a long period of time. Although histological studies of patients failing on PD have shown total loss of the mesothelial cell monolayer [19,168], this does not appear to occur in all patients assessed in the ongoing Cardiff peritoneal membrane biopsy study (Professor J.Williams, personal communication). In-vitro studies to consider methods of further preserving the HPMC monolayer therefore remain clinically relevant.

7.1.2 Cell characterisation

The HPMC in this thesis were all obtained from uraemic patients undergoing PD catheter insertion. Within this group basal levels of fibronectin and TGF-β varied by 2-3 fold (and hence results are expressed as percentage changes). It is unclear what patient factors account for these differences, or if differences exist between cells from a uraemic or non-uraemic environment. However, as in clinical practice PD only occurs in patients who are uraemic, cell obtained in this way appear most appropriate for these experiments and were used consistently throughout the thesis.

Cells in this thesis were considered to be mesothelial in origin by co-expression of cytokeratin and vimentin, and absence of vWF and CD45. In addition they were

considered non-fibroblast like in morphological appearance. An additional check might have been to include the absence of staining for the fibroblast marker α-smooth muscle actin. In addition differences in cytokeratin expression in response to noxious stimuli (such as dialysate) could have been considered, or the change in morphology and phenotype with passage.

7.1.3 Assessment of Cell viability and proliferation

In this thesis, lactate dehydrogenase (LDH) is used as a marker of cell viability. LDH is an intracellular enzyme which is released into culture medium with disruption of the cell membrane as occurs on cell death. Other methods exist for measuring cell viability including trypan blue exclusion. Monolayers of cells are stained with trypan blue which is only taken up by cells without an intact cell membrane, and the remaining cells counter stained with giemsa. Cell viability is then assessed by counting trypan stained cells per high power field. Other alternatives would include assay of ATP amount which others have previously advocated in measuring cell viability in HPMC as it provides additional information about cell function [72,169].

^{3}H-Thymidine incorporation is a widely used means of measuring proliferation although this technique has potential flaws. ^{3}H-Thymidine is incorporated into the DNA of proliferating cells in the "S-phase" of the cell cycle. Unfortunately it is also incorporated into other non-DNA protein to a variable degree. This cell culture system investigates the hypothesis that TGF-β is an intermediary in the stimulation of HPMC ECM production and inhibition of proliferation. In this setting ^{3}H-Thymidine incorporation may additionally over estimate the inhibition of proliferation as TGF-β prevents cells moving out of the "S-phase" of the cell cycle. Finally proliferation is difficult to assess in confluent cells which form monolayers, as cell-cell contact inhibition is likely to inhibit cell

proliferation. A better technique would perhaps have been to growth arrest semi-confluent cells with 48 hour exposure to rest-medium before going on to measure proliferation. This may have resulted in greater differences between test conditions, and have allowed subtle differences in proliferation to have been detected. Validity of the ^{3}H-Thymidine incorporation assay could also have been improved by expressing results corrected for cellular DNA to account for differences in other cell proteins.

Any discussion of cell viability and proliferation should also include a discussion of apoptosis. This is the process by which cells are removed in a programmed manner rather than by necrosis. Others have measured differences in apoptosis in polymorphonuclear cells [170] and also HPMC [68,171,172] exposed to peritoneal dialysis solutions. In general these show that peritoneal dialysate incites apoptosis in HPMC, but there was no detectable difference in apoptosis with different glucose concentrations. Apoptotic cells can be identified by a variety techniques, including staining of apoptotic cells with Anexin V. Apoptosis can then be quantified either by counting staining cells per high power field, or FACS analysis. However such work is not novel, and was considered beyond the scope of this thesis.

7.1.4 Justification of the chosen in-vitro model

The model chosen in this thesis was predominantly a 50:50 mixture of dialysis solution and M199 with 0.1% FBS. Exposure of the confluent monolayer to test solutions was for twelve hours. A maximum glucose concentration of 40mmol/L was chosen to represent the glucose concentration 4 hours into a clinical dialysis exchange [87]. The following changes could have been considered to this methodology:

7.1.4.1 Using collagen IV (the predominate component of the basement membrane) rather than collagen I in the coating of cell culture plastics.

7.1.4.2 Using delipidated human albumin rather than FBS in the culture model (although it is acknowledged that cell viability necessitates the addition of at least some protective protein).

7.1.4.3 Considering using a buffered well-insert model. Although neat dialysate is cytotoxic to HPMC it is appreciated that this scenario does not occur in clinical practice due to the immediate buffering of the dialysate with a pool of several hundred millilitres of spend dialysate remaining in the patients abdomen. An alternative method using well-inserts might therefore be to use partially buffered dialysate in the central well. This would have avoided the limitation in the buffering capacity of the culture medium in the baso-lateral compartment with the repeated changes in the dialysis solution.

7.1.5 Use of fibronectin as a marker of ECM deposition

It is acknowledged that the major component of the ECM deposited in the fibrosing peritoneum is type I, III and IV collagen. It is likely, although not proven, that this ECM also contains some fibronectin. Fibronectin is thought to be one of the ECM proteins least influenced by the un-physiological environment of *in-vitro* experiments. In addition it proved the most reliable ECM for measurement of the protein by ELISA and mRNA by Northern analysis. Attempts were made to measure mRNA levels of other ECM proteins (collagens I,III and IV), but without consistent success. This is likely to be because only small amounts of mRNA could be extracted from HPMC making hybridisation patchy. With the availability now of real-time PCR these technical limits may be overcome. Clearly

other techniques exist to measure total de-novo synthesis of proteins, such as ^{35}S-methionine incorporation. However these techniques were considered beyond the scope of this thesis.

7.1.6 Effects of FK506 on HPMC

The data presented in this section are a brief summary of the effects of FK506 at concentrations that could be usable in clinical practice without overt toxicity. Clearly much more extensive studies could be undertaken to include other known TGF-β receptor antagonists, and a more thorough dosing strategy to determine an effective drug concentration. However, these experiments would have been beyond the scope of this thesis, and the FK506 section is included as a potential (unsuccessful) clinical application of an anti TGF-β blocking strategy.

7.2 Ongoing Work (beyond the scope of this thesis)

Future work proposed includes investigation of the effect of glucose on ECM degradation, by studying the role of metalloproteinases and their tissue inhibitors (TIMPs). Preliminary work suggests that hyperosmolar glucose causes an increase in HPMC TIMP-1 measured by ELISA, but metalloproteinases-3 and 9 are undetectable by ELISA in HPMC under either control or hyperosmolar glucose conditions. The non-specific activator of protein kinase C, PMA in early work appears to be the only condition capable of increasing MMP3 or 9 measured by ELISA. Further work to investigate the functional changes in metalloproteinases (by zymography for example) would be necessary to validate these early findings. Additional experiments are also planned to further investigate the mechanism responsible for amino acid dialysate stimulated ECM, including NO donors (e.g. SNAP).

8 Appendices

8.1 List of solutions

Glucose PD4 dialysate (Dianeal®)	Anhydrous glucose Sodium chloride Calcium chloride dihydrate Magnesium chloride hexahydrate Sodium Lactate	13.6, 22.7, 38.6g/L 5.38g/L 0.184g/L 0.051g/L 4.48g/L
Amino-acid dialysate (Nutrineal®)	Mixed Amino acids Sodium chloride Calcium chloride dihydrate Magnesium chloride hexahydrate Sodium Lactate	11.0g/L 5.38g/L 0.184g/L 0.051g/L 4.48g/L
Bicarbonate:lactate dialysate (Physioneal®)	Anhydrous glucose Sodium chloride Calcium chloride dihydrate Magnesium chloride hexahydrate Sodium Bicarbonate Sodium Lactate	13.6, 22.7, 38.6g/L 5.38g/L 0.184g/L 0.051g/L 2.1g/L 1.68g/L
Denhardt's solution	2% BSA 2% Ficoll 2% Polyvinylpyrrolidone	
Diluent buffer 10X(for diluting cDNA)	NaCl TRIS EDTA (0.5M) made-up in 100ml DEPC water	0.29g 1.21g 200µl
ELISA plate coating buffer	In 100ml deionised water Sodium bicarbonate Sodium carbonate pH 9.6	 0.378g (45mM) 0.053g (5mM)
ELISA substrate solution	In 12ml deionised water 1,2-Phenylenediamine dihydrochloride (OPD) immediately before use 5µl 30% hydrogen peroxide added	8mg (4 tablets)
DNA Gel loading buffer	50% Glycerol 10mM Tris 1mM EDTA Bromophenol blue (tiny bit)	
Methylene blue	0.04% solution of methylene blue	

	in 0.5M sodium acetate Adjust pH to 5.2 with glacial acetic acid	
MOPS buffer 10X	Per litre DEPC water MOPS (Sigma A9539) Sodium Acetate (3M) EDTA (0.5M, pH8.0) Adjust to pH 7.0 with NaOH	 42g 16.7ml 10ml
Phosphate buffered saline	In 1L deionised water NaCl Na_2HPO_4 KCl KH_2PO_4	 8.00g (137mM) 1.15g (8mM) 0.20g (2.7mM) 0.20g (1.5mM)
SSC 20X	Per litre DEPC water NaCl (Sigma S3014) tri-sodium citrate (Sigma C8532) pH to 7.0	 175.3g 88.2g
SSPE 20X (Now bought as 20X solution from Sigma S2015)	Per litre DEPC water NaCl NaH_2PO_4 EDTA Adjust pH to 7.4 with NaOH	 175.3g 27.6g 7.4g
TBST wash buffer	Tris-HCl pH 7.6 NaCl Tween-20	20mM 150mM 0.05% (v/v)
TAE 50X (Now bought as 10X solution from Sigma T9650)	Per litre DEPC water Tris Glacial acetic acid EDTA (0.5M, pH8.0) Adjust pH to 7.6 with HCl	 242g 57.1ml 100ml
Tris HCl **0.1M pH 8.2**	In 200ml deionised water Tris base 1M HCl	 2.42g 10ml
Stripping solution	In 1L DEPC water 20% SDS 20X SSPE heated in waterbath to 95°C	 50ml 5ml
AP substrate	Naphthol AA-Mx phosphate In N,N dimethyl formamide Add to	0.02g 2ml

	Tris HCl 0.1M, pH 8.2 Levamasole 1M Add Fast Red TR salt and filter just before use	98ml 0.1ml 0.002g
Wash Buffer	In 1L deionised water NaCl Na_2HPO_4 KCl KH_2PO_4 Tween20	 28.00g 1.15g 0.20g 0.20g 1ml

8.2 List of suppliers

Sigma	Sigma-Aldrich Company Ltd. Fancy Road Poole Dorset, BH12 4QH
Life Technologies	Life Technologies Ltd. 3 Fountain Drive Inchinnan Business Park Paisley, PA4 9RF
BDH AnalaR	BDH Scientific supplies Ltd. Poole Dorset, BH15 1PD
Promega	Promega (U.K.) Ltd. Delta House Chilworth Research Centre Southampton, SO16 7NS
Amersham	Amersham Life Science Ltd. Amersham Place Little Chalfont Buckinghamshire, HP7 9NA
R&D systems	R&D Systems (Europe) Ltd. 4-10 The Quadrant Barton Lane Abingdon Oxfordshire, OX14 3YS
Phamacia Biotech	Pharmacia Biotech 23 Grosvenor Road St Albans Hertfordshire, AL1 3AW

8.3 Patient information sheet, ethical committee approval and consent form

Patient Information

The role of mesothelial cell extracellular matrix protein production in peritoneal fibrosis

The Department of Nephrology are currently running a study on the production of scar tissue. This information sheet outlines how you can help if you would like to. Just like all other research however you are under no obligation to take part.

What is the trial for

The study will be based in a laboratory, and will investigate the production of scar tissue by the cells which line the abdomen (mesothelial cells). It will try to find out what happens to these cells in people who have kidney failure and are on CAPD.

As you know patients on CAPD fill their abdomens with fluid to do the job of their kidneys. The fluid that is used at present works well, but in some people it can slowly damage the delicate cell lining of their abdomen. New fluids are being tested to see if they work better, and cause less problems.

Where do I fit in?

You are about to have an operation on your abdomen to insert a PD tube. Because you are not yet on CAPD the cells lining your abdomen are therefore perfectly normal.

If you agree to take part then when the surgeon is doing the operation he will also remove a small piece of the lining of your abdomen. This piece of tissue

will be taken to the laboratory and the mesothelial cells will be removed and allowed to grown on their own.

What will happen to my abdomen

The small piece of tissue (about 2.5cm by 2.5cm) will be taken from an area in your abdomen called the omentum. This is a large sheet which can move around freely in your abdomen. You have no sensation or feeling in this tissue and the small cut will heal with no long-lasting effects to you. The risks of this additional procedure are small.

What will happen to the cells you are growing

Over about two weeks the cells will be studied to see if different dialysis fluids cause them to produce scar tissue. After that the experiments on those cells will stop. This is because mesothelial cells are very fragile, and will only grow for this short time out of the body. After two weeks they gradually stop growing and die.

Do I have to take part in this trial?

No, you do not have to take part. Your participation is voluntary, and your relationship with the doctors and nurses will not be affected if you decide not to take part. If you wish to withdraw from the trial at any time you may do so without giving a reason.

James Medcalf (Dr)

Research Fellow

Consent form

I ..... hereby fully and freely consent to participation in the following study

"The role of mesothelial cell extracellular matrix protein production in peritoneal fibrosis."

The nature and purpose of this study has been explained to me by doctor ....., and all details of advantages and risks have been given.

I note that I may withdraw my consent at any stage in the investigation without it affecting the treatment that I am given.

Patient signature .....

Date.....

Physician signature.....

Date.....

8.4 Project funding and grants held

Mason Medical Research Foundation (1996)
Trent Research Scheme (1996)
Peel Medical Research Trust (1997)
Leicester General Hospital Research Award (1998)

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