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Human peritoneal fibroblast proliferation in 3-dimensional culture: Modulation by cytokines, growth factors and peritoneal dialysis effluent

M. JANINE BEAVIS, JOHN D. WILLIAMS, JÜRGEN HOPPE, and NICHOLAS TOPLEY

Institute of Nephrology, University of Wales College of Medicine, Cardiff Royal Infirmary, Cardiff, Wales, United Kingdom, and Theodor Boveri Institut, Universität Wurzburg, Wurtzburg, Germany

Human peritoneal fibroblast proliferation in 3-dimensional culture: Modulation by cytokines, growth factors and peritoneal dialysis effluent. Structural and functional alterations of the peritoneal membrane are a significant problem in long-term peritoneal dialysis patients. The present study has established a 3-dimensional (3D) cell culture system to study the human peritoneal fibroblast (HPFB) and to examine its proliferative responses to cytokines and growth factors as well as dialysis effluent obtained from patients during peritoneal infection. PDGF-$_{AB}$, basic FGF and IL-1β induced a time and dose dependent increase in 3D-HPFB proliferation. At day 9 proliferation, as assessed by MTT uptake, was increased by 2.4-, 2.3- and 1.5-fold above control by PDGF-$_{AB}$ (50 ng/ml), bFGF (50 ng/ml) and IL-1β (10 ng/ml), respectively ($N = 5$, $P = 0.04$ for all). These effects could be inhibited by co-incubation with anti-PDGF-$_{AB}$ antibody, anti-bFGF or IL-1ra, respectively. Exposure of 3D-HPFB to TGF-β_1 did not result in an increase in cell proliferation. Incubation of 3D-HPFB with peritoneal macrophage (PMØ) or human peritoneal mesothelial cell (HPMC) conditioned medium also resulted in a time and dose dependent increase in proliferation. At day 9, proliferation was maximally increased 1.65- and 1.92-fold by peritoneal macrophage- and mesothelial cell-conditioned medium, respectively. Cell free PDE, obtained from CAPD patients during episodes of peritonitis, induced 3D-HPFB proliferation above control values (2- to 6.5-fold increases, $N = 5$, $P < 0.05$ for all). This mitogenic potential of PDE was reduced following dilution, and with time following peritonitis there was a gradual decrease in the mitogenic effect of PDE. The proliferative potential of PDE was significantly reduced following co-incubation with IL-1ra (45.7% inhibition), anti-bFGF (34.9% inhibition) and anti PDGF-$_{AB}$ (27.4% inhibition). These data indicate that infected PDE causes fibroblast hyperplasia which might potentially contribute to pro-fibrotic processes during CAPD.

Continuous ambulatory peritoneal dialysis (CAPD) is a major therapy for the treatment of end-stage renal disease. In these patients the peritoneal membrane functions as a dialyzing organ and forms the permeability barrier across which ultrafiltration and diffusion occur. Loss of ultrafiltration capacity is a major complication associated with long-term CAPD [1–9]. The ultrastructural changes observed in peritoneal biopsy studies include mesothelial desquamation and sub-mesothelial fibrosis resulting from fibroblast hyperplasia and increased extracellular matrix deposition [2, 6].

Although the initiation and progression of fibrosis is poorly understood, an ongoing acute or chronic inflammatory response, with the infiltration of leukocytes, appears to be a prerequisite for its initiation [10]. Data from studies of pulmonary, hepatic, renal and skin fibrosis indicate that the fibrotic process involves a series of overlapping phases, which eventually lead to irreparable damage to the interstitium of the tissue involved [4, 11–14]. The initial activation stage appears to be followed by interstitial cell hyperplasia and subsequent matrix remodeling, increased collagen deposition and eventually tissue fibrosis. Peritoneal fibrosis is also characterized by the presence of a prominent mononuclear cell infiltrate [4]. Biopsies obtained at autopsy from patients previously maintained on CAPD revealed the presence of chronic mononuclear cell infiltration suggesting that these cells may be the source of the mediators (cytokines and growth factors) responsible for initiating and/or perpetuating the fibrotic response. Support for this concept comes from earlier studies which demonstrated that mononuclear derived cytokines can alter fibroblast proliferation [15–18], proteoglycan synthesis [19] and chemotaxis [20, 21]. A recent study has confirmed that activated peritoneal macrophages reside within the peritoneal membrane of CAPD patients [22].

The potential of the mesothelial cells that line the pleural and peritoneal cavities to contribute to inflammation has recently been revealed [23–30]. In contrast, the contribution of the peritoneal fibroblasts, which reside within the sub-mesothelial interstitium surrounded by extracellular matrix, to intraperitoneal inflammatory and pro-fibrotic processes remains to be fully elucidated.

The majority of *in vitro* fibroblast studies to date have utilized monolayer cell culture systems. These, however, poorly mimic their *in vivo* state, where peritoneal fibroblasts are embedded within the extracellular matrix. Culturing HPFB within a 3-dimensional matrix might therefore more closely mimic their *in vivo* state, since it is clear that the extracellular matrix acts not only as the structural scaffold of tissues but also provides signals via integrin receptors that control cellular functions [31], including chemotaxis [21, 32], proliferation [33, 34], extracellular matrix production as well as morphology.

In the present investigation we describe the establishment of a

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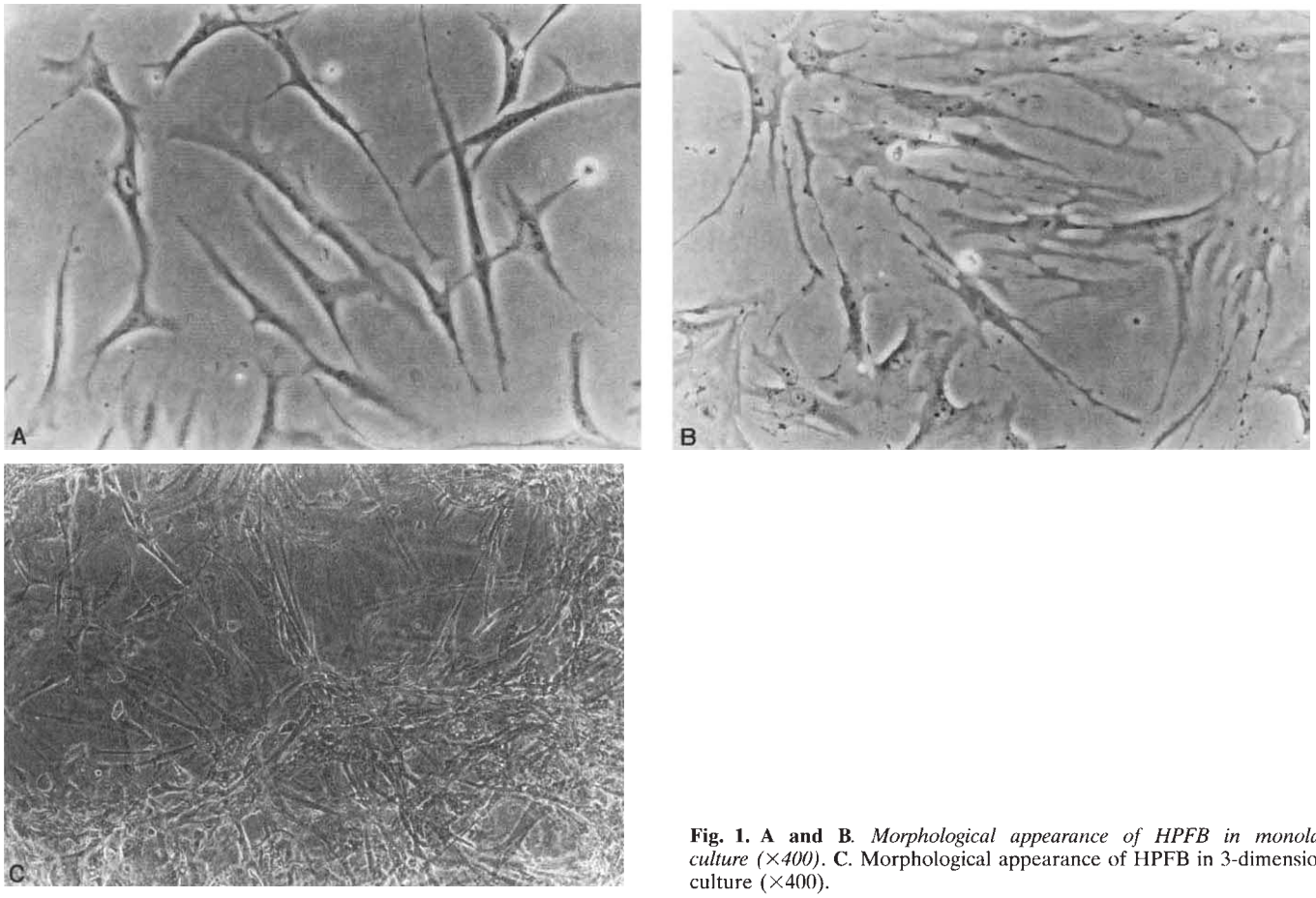

Fig. 1. A and B. *Morphological appearance of HPFB in monolayer culture (×400).* **C.** Morphological appearance of HPFB in 3-dimensional culture (×400).

3-dimensional human peritoneal fibroblast (HPFB) culture system in which the cells are maintained within a matrix of type I collagen. These experiments have examined the regulation of HPFB proliferation by growth factors and cytokines present in the peritoneal cavity during peritoneal inflammation and by peritoneal dialysis effluent (PDE) obtained from patients during episodes of peritonitis.

Our observations demonstrate that bFGF, PDGF and IL-1 induce 3D-HPFB hyperplasia. Peritoneal dialysis effluent obtained during episodes of peritonitis is a potent inducer of 3D-HPFB proliferation, a fact which appears to be due to its IL-1, bFGF and PDGF content.

Methods

All chemicals, unless otherwise stated, were obtained from Sigma Chemical Company (Poole, UK). All tissue culture plastics were purchased from Falcon, Becton-Dickinson (Oxford, UK).

PDGF-$_{AB}$, PDGF-$_{BB}$ and PDGF-$_{AA}$ were expressed in *E. coli* harvested and purified as previously described [35, 36]. IL-1ra was kindly supplied by Dr. Peter Scholtz (Schering AG, Berlin, Germany). Basic FGF and TGF-β_1, polyclonal goat anti-human PDGF-$_{AB}$ and polyclonal rabbit anti-human bFGF were from R&D Systems (Oxford, UK). IL-1β was purchased from Advanced Protein Products (APP, Brierly Hill, UK). Endotoxin contamination of recombinant material was < 0.7 pg/μg protein as assessed by the limulus amoebocyte lysate assay (Kabi Vitrum, Stockholm, Sweden). Lipopolysaccharide (*E. coli* 055 B5) was purchased from Sigma.

Isolation and culture of human peritoneal fibroblasts

Human peritoneal fibroblasts were isolated from omental tissue of consenting patients undergoing elective abdominal surgery (during the entire study tissue samples were obtained from more than 400 donors), using a modification of a method previously described for peritoneal mesothelial cells [37]. Omental tissue, previously processed to remove the mesothelial cells, was incubated with a solution of trypsin/EDTA (0.25% wt/vol/0.02% wt/vol; Life Technologies Ltd, Paisley, UK) at 37°C under continuous rotation for two 40-minute periods. Fresh trypsin/EDTA was used during the second incubation and cell pellets were washed with Hams F12 (Life Technologies) containing 10% fetal calf serum (FCS; Biological Industries, Cumbernauld, UK) at the end of each digestion. The isolated cells were plated onto 25 cm^2 tissue culture flasks and maintained in Hams F12 medium supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml, L-glutamine (2 mM) insulin (5 μg/ml), transferrin (5 μg/ml) and hydrocortisone (0.4 μg/ml) (all purchased from Life Technologies). Cells were propagated in culture medium containing 10% FCS in a 37°C, humidified, 5% CO_2 atmosphere, with a complete change to fresh medium every three to four days.

Establishment of homogenous HPFB cultures

Mesothelial cells, which have a cobblestone appearance at confluence, occasionally contaminated HPFB cultures. In such instances a differential sub-culture was performed. This procedure utilizes the differential rate of cell attachment/detachment of the two cell types to the plastic, with the HPFB attaching more rapidly than the mesothelial cells; hence the latter can be removed by washing prior to their attachment.

Immunocytochemical characterization of HPFB

Using indirect immunocytochemical techniques the HPFB were characterized by the presence or absence of cytokeratin, (epithelial/mesothelial cell marker), Factor VIII (endothelial cell marker), vimentin, desmin and myosin (myogenic markers) and α-smooth muscle actin. Cells were grown to confluence on 8-well glass chamber slides (Life Technologies Ltd.), washed in phosphate buffered saline (PBS) and fixed in acetone:methanol (1:1 vol/vol at 4°C) for five minutes. The cells were subsequently washed in PBS containing 0.9% bovine serum albumin prior to incubation in the primary antibodies at room temperature for 45 minutes. The antibodies were as follows: anti-human Factor VIII-related antigen (MoAb), rabbit anti-human vimentin, rabbit anti-human myosin, rabbit anti-swine desmin (IgG1) (all from Dako Ltd, High Wycombe, UK), rabbit anti-human cytokeratin (peptide 18, IgG1) and MoAb anti-human smooth muscle actin (Sigma). After a further wash the appropriate secondary fluorescein-conjugated antibodies were added for a further 45 minutes (FITC conjugated anti-rabbit IgG or anti-mouse IgG, both from Sigma). Finally the cells were washed, mounted and viewed under a Leitz Orthoplan fluorescence microscope (Leica UK Ltd, Milton Keynes, UK).

Identification and characterization of HPFB

HPFB in monolayer cultures showed both a bipolar (Fig. 1A) and multipolar (Fig. 1B) morphology and stained positively for vimentin and myosin and occasionally α-smooth muscle actin by immunohistochemistry. The expression of the latter varied between 15 to 20% of cells depending on the individual culture. Cultures were always negative for cytokeratin, Factor VIII and desmin excluding contamination of HPFB cultures with epithelial, endothelial or mesothelial cells.

Establishment of 3-dimensional HPFB cultures

Rat tail Type I collagen was prepared and lyophilized according to the method of Cawsten and Barrett [38]. The purity of the preparation was confirmed by SDS-polyacrylamide gel electrophoresis. HPFB were established in 3-dimensional culture using a modification of the method of Madri, Pratt and Tucker [39]. All initial procedures were carried out on ice to prevent collagen gel formation. Initial experiments established both the collagen gel concentration and the seeding cell density required to establish a reproducible 3D-HPFB culture system.

Briefly, lyophilized collagen was solubilized by the addition of 100 mM acetic acid followed by the addition of pyrogen free water (Baxter Healthcare Ltd., Egham, Surrey, UK), bringing the concentration of acetic acid to 16 mM. To 1 ml of collagen solution, 10× RPMI (110 μl) containing 27 μl sodium bicarbonate (7.5% vol/vol; Life Technologies) was added. The solution was then neutralized with sodium hydroxide solution (1 M, Life Technologies). Gel formation was facilitated by incubating this solution at 37°C for 10 minutes.

In experiments, using 24 well plates, 300 μl of collagen solution (4 mg/ml) was added to each well, while in 96 well plates, 40 μl is added per well. This dense collagen "plug" acts as a barrier to prevent fibroblast migration and attachment to tissue culture plastic. Once this dense gel was formed the HPFB suspension (3×10^4 in 125 μl in 24 well plates, 1×10^4 in 35 μl in 96 well plates) was mixed with an equal volume of the 4 mg/ml collagen solution on ice and plated on top of the collagen plug. In 24 well plates 250 μl was plated and 70 μl was plated in 96 well plates. These were allowed to gel and Hams F12 medium containing 10% FCS was added to the cultures. This medium was replaced every three days.

Once established in 3D culture, HPFB assumed an essentially bipolar morphology and were multiorientated throughout the gel (Fig. 1C). In 3D culture HPFB could be maintained in culture for extended time periods (> 30 days).

Establishment of growth arrested 3D-HPFB

Preliminary experiments revealed that HPFB in 3D culture could be rendered quiescent by incubating them for 72 hours in serum-free Hams F12 medium. Under these conditions the cells remained in a non-proliferative but viable state (as assessed by the lack of lactate dehydrogenase release and normal cellular ATP levels) for periods up to 30 days (Beavis and Topley, unpublished data).

Measurement of 3D-HPFB proliferation: Direct cell counting

After appropriate stimulation, medium was removed from above the 3D cultures by gentle aspiration, the gel was washed twice in Hams F12, removed from the well and placed into a sterile 1.5 ml micro-centrifuge tube (Eppendorf Safelock, Merck Ltd, Atherstone, UK) containing 1 ml of 0.4 mg/ml collagenase (Type VIII; Sigma Chemical). The gels were incubated at 37°C, with rotation, until the collagen was completely digested and the cells released. The cell pellet was then harvested by centrifugation (150 × g), washed in Hams F12 containing 10% FCS to inhibit the collagenase, and the cells counted using a modified Neubauer counting chamber.

^{3}H thymidine incorporation and DNA measurement

Following defined stimulation 3D-HPFB cultures were pulsed with 1 μCi/ml ^{3}H-thymidine (Amersham International PLC, Cardiff, UK) for 24 hours, washed twice with Hams F12 and the gels digested with collagenase as described above. Cell pellets were resuspended in 500 μl of PBS. One hundred microliter aliquots of released cells were solubilized with 0.1 N NaOH, neutralized with 1 M HCl and counted in a scintillation counter (Canberra Packard Instruments, Pangbourne, UK) following mixing with scintillant (Optiphase, Canberra Packard Instruments).

Total cellular DNA was also measured in these same samples using the method of Kissane and Robins [40]. Briefly, HPFB digested from the gels (100 μl) were extracted with 0.1 M potassium acetate in ethanol (on ice) and twice with 100% ethanol and subsequently air dried. Calf thymus DNA, (Sigma Chemical) dissolved in 1 M ammonium hydroxide, was used as standard. To the dried samples and standards 40 μl of a 30% (wt/vol) solution of 3,5-diaminobenzoic acid (dihydrochloride) (DabA) was added and incubated at 60°C for 30 minutes. Thereafter, 800 μl of 0.6 M

perchloric acid was added to each tube, vortexed and centrifuged at 13,000 × g for two minutes. Four hundred microliters of the supernatant was then transferred to fluorimeter tubes and the fluorescence measured on a Perkin-Elmer 150 fluorimeter (Applied Biosystems, Warrington, UK) using an excitation wavelength of 406 nm and an emission wavelength of 520 nm.

MTT assay

This assay determines viable cell numbers and is based on the mitochondrial conversion of the tetrazolium salt, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), into an insoluble formazan product. Briefly, after stimulation, culture medium was removed from 3D-HPFB and 100 μl of MTT (5 mg/ml) was added to 96 well plates for four hours at 37°C. The formazan product generated was solubilized by the addition of 100 μl of 20% SDS, 50% dimethylformamide, 2% acetic acid, 0.06% hydrochloric acid and incubated at 37°C overnight. The absorbance at 600 nm was recorded in an ELISA plate reader (Dynatech MR 5000/340; Dynatech Laboratories, Billingshurst, UK). There was a direct correlation between cell numbers assessed by direct cell counting of solubilized gels and the MTT assay irrespective of donor ($r = 0.995$, $P < 0.001$; Fig. 2).

Comparison of proliferation measurement techniques

MTT assay, ^{3}H-thymidine incorporation and DNA measurement. Initially HPFB proliferation in 3D culture was assessed by direct cell counting. This technique, however, required large numbers of cells and could only be performed successfully in gels established in 24 well plates. In order to maximize the potential of the system, alternative techniques were developed to assess HPFB proliferation in 3D culture.

In a series of five experiments ^{3}H-thymidine incorporation, cellular DNA content and measurement of proliferation using the MTT assay were compared to data obtained from parallel cell counting experiments. There was a positive correlation between cell numbers assessed by direct cell counting and the MTT assay ($N = 35$, $r = 0.636$, $P < 0.001$) and ^{3}H-thymidine incorporation/μg cellular DNA content ($N = 35$, $r = 0.436$, $P < 0.01$). Subsequent experiments utilised the MTT assay to assess 3D-HPFB proliferation.

Induction of 3D-HPFB IL-6 synthesis by cytokines and growth factors

3D-HPFB are grown in 24 well plates and growth arrested for 72 hours, washed twice, and subsequently stimulated for 24 hours with the cytokines or growth factors described. 3D-HPFB supernatants were removed, centrifuged at 13,000 × g and stored at −70°C until assayed. The cells were then washed in Hams F12, and the collagen gels digested with bacterial collagenase. The resulting cell pellet was washed with PBS and solubilized with 0.1 M sodium hydroxide. Total cellular protein was assessed using the modified Bradford method [41]. All data for IL-6 production are expressed as pg/μg cell protein.

IL-6 synthesis measurement

Synthesized IL-6 was measured in the culture supernatants of control and stimulated 3D-HPFB by a specific sandwich ELISA as previously described [42].

HPMC conditioned medium

Human peritoneal mesothelial cells (HPMC) were grown to confluence in 24 well plates and growth arrested in M199 containing 0.1% FCS for 48 hours as previously described [25–27, 43]. Cells were then washed three times in rest medium and stimulated with either IL-1β (100 pg/ml) or TNFα (1000 pg/ml), or control medium. Following a 24 hour incubation the HPMC supernatants were removed, centrifuged at 13,000 × g and stored at −70°C until required.

Peritoneal macrophage conditioned medium

Peritoneal macrophages (PMØ) were harvested on ice, from infection-free peritoneal dialysis effluent of patients undergoing CAPD as previously described [44–46]. Isolated cell populations were plated onto 35 mm Petri dishes and incubated at 37°C in a 5% CO_2 incubator for 90 minutes. After this period the non-adherent cells were removed by washing twice with warm Hams F12. Conditioned medium was collected from adherent PMØ following 18 hours incubation with Hams F12 alone. The PMØ-CM was centrifuged (13000 × g), aliquoted and stored at −70°C until required.

Infected spent dialysate

Spent dialysate (PDE) was collected as previously described [47] from CAPD patients. In the first group (patients A, B, D, E and F), PDE was collected on the day of presentation with peritonitis (day 1) and subsequently from the overnight dwells over the next five days. In these same patients, infection free PDE was collected three months after the resolution of infection (A1-F1) only if they had remained infection free for that three month period. PDE was also collected from a second group of patients with no history of peritonitis. PDE was collected on ice, centrifuged at 2000 × g to remove cellular material and subsequently filtered (0.2 μM Filter, Sartorius Ltd, Epsom, UK). The sterile cell free PDE was aliquoted and stored at −70°C until required.

Blocking and depletion experiments

In a separate series of experiments the proliferative responses of 3D-HPFB to cytokines, growth factors or PDE were examined in the presence of increasing concentrations of IL-1ra, anti-PDGF-$_{AB}$, or anti-bFGF (either alone or in combinations). Antagonists or antibodies were added to the agonist (IL-1ra, anti-PDGF or anti-bFGF) and incubated for 60 minutes at 37°C prior to the commencement of the experiment. Cell proliferation was assessed by MTT assay as described previously.

Statistical analysis

All data are presented from experiments performed in triplicate with HPFB prepared from at least five separate omental donors. Statistical analyses were performed using the Wilcoxon signed rank test for paired non-parametric data. All data are presented as means ± SEM.

Results

Establishment of 3D-HPFB proliferative capacity

Initial experiments demonstrated that following exposure to FCS, HPFB numbers, as determined by cell counting, increased over a period of 12 days. Cell numbers increased from 2.69 ± 0.49

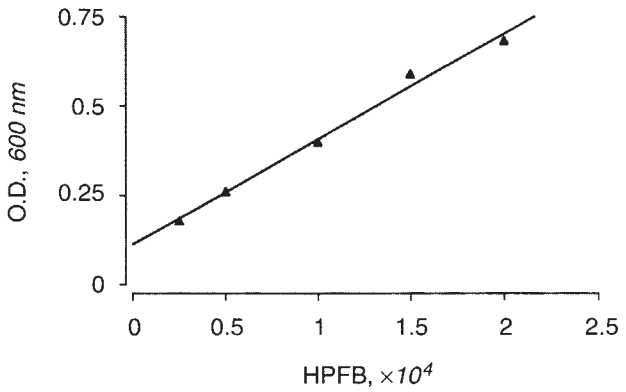

Fig. 2. *Correlation between the number of 3D-HPFB (seeded in a 96-well plate) and MTT absorbance at 600 nm.* Data are presented from a single representative experiment of 6 performed with cells from different donors. y = 0.296x + 0.110; $r = 0.995$; $P < 0.001$.

($\times 10^4$) at the start of the experiment to 3.98 ± 1.32 ($\times 10^4$; $N = 5$, z = 1.96, $P = 0.04$) after three days. By day 9 HPFB numbers had further increased to 6.3 ± 1.39 ($\times 10^4$; $N = 5$, z = 1.96, $P = 0.04$), a 2.3-fold increase above control.

Growth arrest of 3D-HPFB cultures

To examine the proliferative responses of HPFB to growth factors and cytokines in the absence of the confounding influence of serum-derived growth factors, HPFB were established in 3D cultures in a subconfluent state and subsequently growth arrested under serum free conditions. After 72 hours, of serum free culture, cell numbers were not significantly different from controls, (3.36 ± 0.34 vs. 3.18 ± 0.49 ($\times 10^4$; $N = 5$ experiments) and remained constant for extended periods (at day 9, 3.07 ± 0.31; $\times 10^4$). There was no evidence of cell toxicity as assessed by LDH release (data not shown). As a result of these experiments, a growth arrest period of 72 hours was judged to be sufficient to render cells quiescent.

It was then necessary to establish whether 3D-HPFB were capable of resuming their proliferative capacity following this 72 hours growth arrest period. After growth arrest the addition of 10% FCS resulted in a time dependent increase in 3D-HPFB numbers. After nine days 3D-HPFB numbers had increased threefold (6.639 ± 2.12; $\times 10^4$) at day 9 versus 2.095 ± 0.64 ($\times 10^4$) in control ($N = 5$, z = 1.96, $P = 0.04$). These levels were comparable to 3D-HPFB maintained in the continuous presence of 10% FCS after seeding.

Mitogenic effect of cytokines and growth factors on 3D-HPFB

The effects of PDGF-$_{AB}$, IL-1β, bFGF and TGF-β_1 on 3D-HPFB proliferation were studied using the MTT assay. All three isoforms of PDGF induced a time and dose dependent increase in 3D-HPFB proliferation, after nine days PDGF-$_{AB}$, the most potent isoform, increased 3D-HPFB numbers by 2.4-fold above control ($N = 5$, z = 1.96, $P = 0.04$; Fig. 3A). IL-1β induced a smaller but significant increase in proliferation (Fig. 3B). The maximum response was achieved at the highest dose tested (10 ng/ml) where a 1.5-fold increase above control was observed ($N = 5$, z = 2.02, $P = 0.04$).

Stimulation of 3D-HPFB with bFGF also induced a dose dependent increase in 3D-HPFB proliferation (Fig. 3C). This was significant for all doses of bFGF above 1 ng/ml and was maximal at 50 ng/ml (2.3-fold above control; $N = 5$, z = 2.02, $P = 0.04$). In contrast, stimulation of 3D-HPFB with TGF-β_1 did not induce a mitogenic response irrespective of the dose tested (Fig. 3D).

The addition of lipopolysaccharide (LPS) to 3D-HPFB (at doses up to 10 μg/ml) did not have any effect on 3D-HPFB proliferation (Control O.D. 0.242 ± 0.05 vs. 0.231 ± 0.05 at 10 μg/ml).

Mesothelial cell conditioned medium

Conditioned medium obtained from control or cytokine stimulated, growth arrested, mesothelial cells (HPMC-CM) all induced a dose dependent increase in 3D-HPFB proliferation. These increases were significant with all HPMC-CM tested, with increases of 1.9-, 1.8- and 1.6-fold being induced by undiluted HPMC-CM pre-stimulated with IL-1β, TNFα and control, respectively ($N = 5$, z = 1.96, $P = 0.04$ for all; Fig. 4A). These effects were reduced following dilution of HPMC-CM.

Peritoneal macrophage conditioned medium

Conditioned medium from PMØ induced 3D-HPFB proliferation, this effect was maximal with undiluted PMØ-CM, and was reduced to control levels following dilution. At day 9 3D-HPFB proliferation (expressed as OD_{600}) was increased from 0.17 ± 0.05 in control 3D-HPFB to 0.334 ± 0.054 following exposure to PMØ-CM (a mean 1.96-fold induction; $N = 5$, z = 2.02, $P = 0.04$; Fig. 4B).

Peritoneal dialysis effluent

Peritoneal dialysis effluent (PDE) samples obtained during acute infection induced an increase in 3D-HPFB proliferation as assessed by an increase in MTT absorbance. Initial experiments compared the mitogenic potential of pooled infected PDE (obtained from 5 patients on day 1 of infection), pooled non-infected PDE, (obtained from 5 patients who had no history of peritonitis) and 10% FCS. All induced a significant cell proliferation (as assessed by MTT assay) above control with mean increases of 3.5-, 1.6- and 1.7-fold being induced by infected PDE, infection free PDE and 10% FCS, respectively ($N = 5$ experiments, z = 2.023, $P = 0.04$ vs. control for all; Fig. 5A). Cell counting experiments confirmed that 3D-HPFB numbers increased during these experiments. Infected PDE, pooled from all patients, induced an increase in 3D-HPFB number from 3.45 ± 1.6 ($\times 10^4$) in control to 7.79 ± 1.8 ($\times 10^4$) after nine days of incubation (a mean 1.9-fold induction; $N = 5$, z = 2.02, $P = 0.04$).

The level of stimulation induced by individual infected PDE samples was greater than that seen with any individual growth factor or cytokine. 3D-HPFB proliferation increased by between 2-fold and 6.5-fold above control, and was significant with all infected PDE tested ($N = 5$ experiments, $P < 0.05$ for all). The mitogenic potential of infected PDE was maximal on the day of presentation; PDE obtained on subsequent days (from all patients) had significantly less mitogenic activity compared to day 1 and diminished with time after onset of infection.

In order to simulate the temporal changes occurring in the inflammatory milieu during peritonitis, growth arrested 3D-HPFB were successively exposed (for 24 hr in each case) to PDE obtained on days 1, 2, 3, 4 and 5 of infection. At the end of the experiment (day 6), cell proliferation was assessed by MTT assay. Peritonitis effluents (days 1 to 5 sequentially added to HPFB)

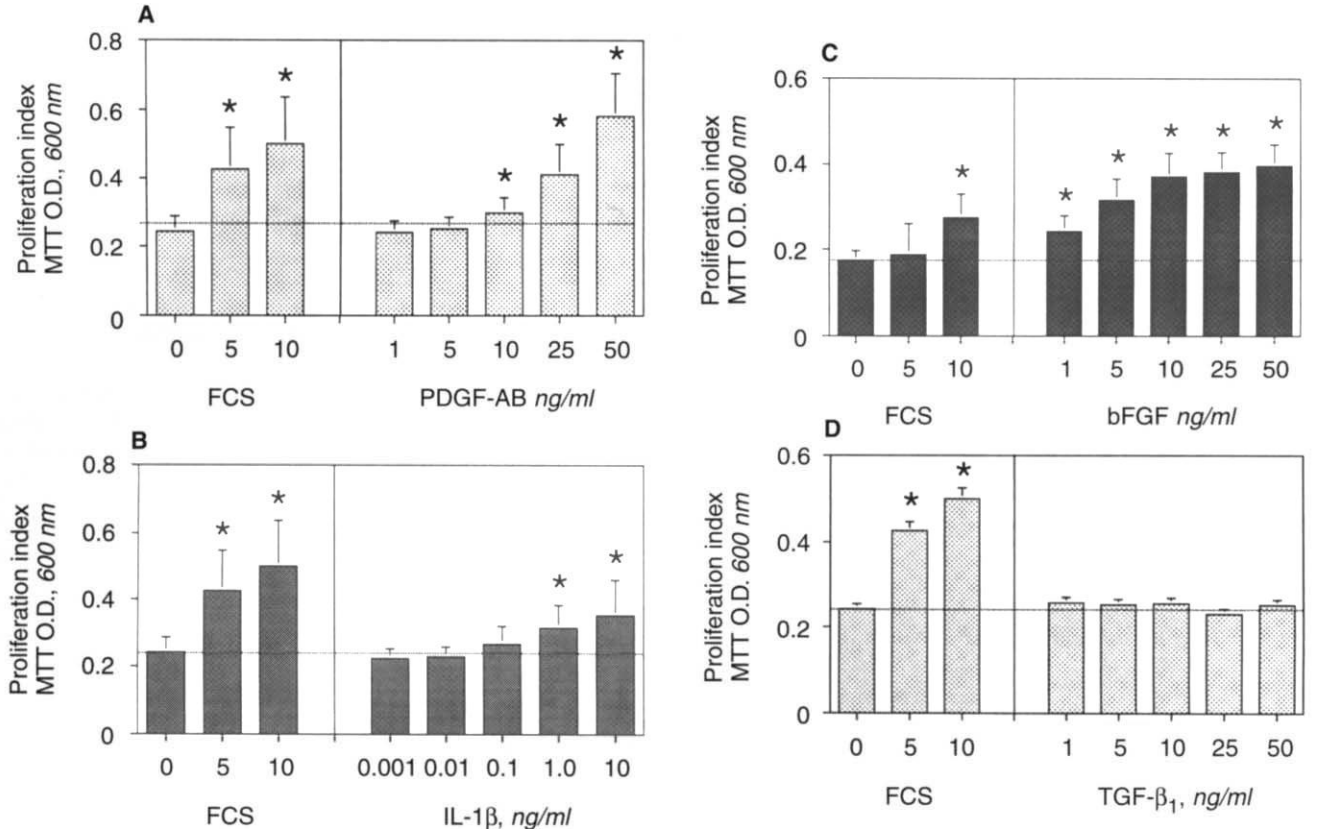

Fig. 3. *Effect of:* (**A**) *PDGF-$_{AB}$ (1 to 50 ng/ml),* (**B**) *IL-1β (0.001 to 10 ng/ml),* (**C**) *bFGF (1 to 50 ng/ml), and* (**D**) *TGF-β$_1$ (1 to 50 ng/ml) on 3D-HPFB proliferation at day 9, as assessed by MTT assay.* Data are presented as means ± SEM from 5 experiments performed with 3D-HPFB from different donors. *A statistically significant difference ($P < 0.05$) compared to growth arrested 3D-HPFB (Day 9).

from all patients (patients A, B, D, E and F) induced significant proliferative responses (between 2.2- and 4-fold increases) compared to control ($N = 5$, $z = 2.02$, $P = 0.04$ for all; Fig. 5B). Parallel 3D-HPFB cultures were exposed for the same time periods to PDE obtained from the same patients three months after resolution of infection (A1, B1, D1, E1 and F1). These effluents still contained significant mitogenic potential for 3D-HPFB. These increases (1.77- to 2.38-fold above control) were significantly less than those increases seen in response to the PDE obtained during peritonitis ($N = 5$, $z = 2.023$, $P = 0.04$ for all).

Blocking experiments

In order to define those factors present in infected PDE which might be responsible for inducing 3D-HPFB proliferation, blocking experiments were performed using increasing concentrations of IL-1ra, anti-PDGF-$_{AB}$ antibody or anti-bFGF antibody. Control experiments demonstrated that the proliferative responses induced by IL-1β, PDGF-$_{AB}$ and bFGF alone could be dose dependently inhibited by IL-1ra, anti-PDGF-$_{AB}$ or anti-bFGF, respectively (data not shown). Incubation of 3D-HPFB with the blocking agents alone did not modulate cell proliferation.

Mitogenic potential in PDE

Pre-incubation of pooled infected PDE with increasing concentrations of IL-1ra (0.1 to 10 μg/ml), anti-PDGF-$_{AB}$ (2.5 to 10 μg/ml) or anti-bFGF (12.5 to 50 μg/ml) all significantly reduced the proliferation in response to PDE (Fig. 6). Maximal inhibition was achieved with the highest dose of IL-1ra, which reduced infected PDE induced 3D-HPFB proliferation by a mean of 45.7% ($N = 5$, $z = 2.203$, $P = 0.027$). Pretreatment with anti-bFGF or anti-PDGF also significantly reduced the PDE induced proliferation by 34.9% and 27.4%, respectively ($N = 5$, $z = 2.023$, $P = 0.04$ for both). Combinations of all three blocking agents (at the highest doses tested) resulted in the largest degree of inhibition, with a mean (± SD) 75.84 ± 9.8% inhibition of pooled infected PDE induced 3D-HPFB proliferation ($N = 5$, $z = 2.203$, $P = 0.04$ vs. pooled infected PDE). Heat treatment (95°C for 1 hr) also reduced the mitogenic capacity of pooled infected PDE, and in two separate experiments (with different HPFB donors) the mitgenic capacity of infected PDE was reduced by 46.7 and 65.03%.

In additional experiments 3D-HPFB were stimulated with either IL-1β in the presence of anti-PDGF-AB or bFGF in the presence of IL-1ra. In neither case was 3D-HPFB proliferation reduced by a significant amount.

Induction of 3D-HPFB IL-6 synthesis

Stimulation of growth arrested 3D-HPFB with IL-1β or TNFα resulted in a dose dependent generation of IL-6 (Fig. 7). After 24 hours a significant release (expressed as pg/μg cell protein) above control levels (0.521 ± 0.08) was achieved with doses of IL-1β above 1 pg/ml and TNFα above 100 pg/ml ($N = 6$, $z = 2.023$, $P <$

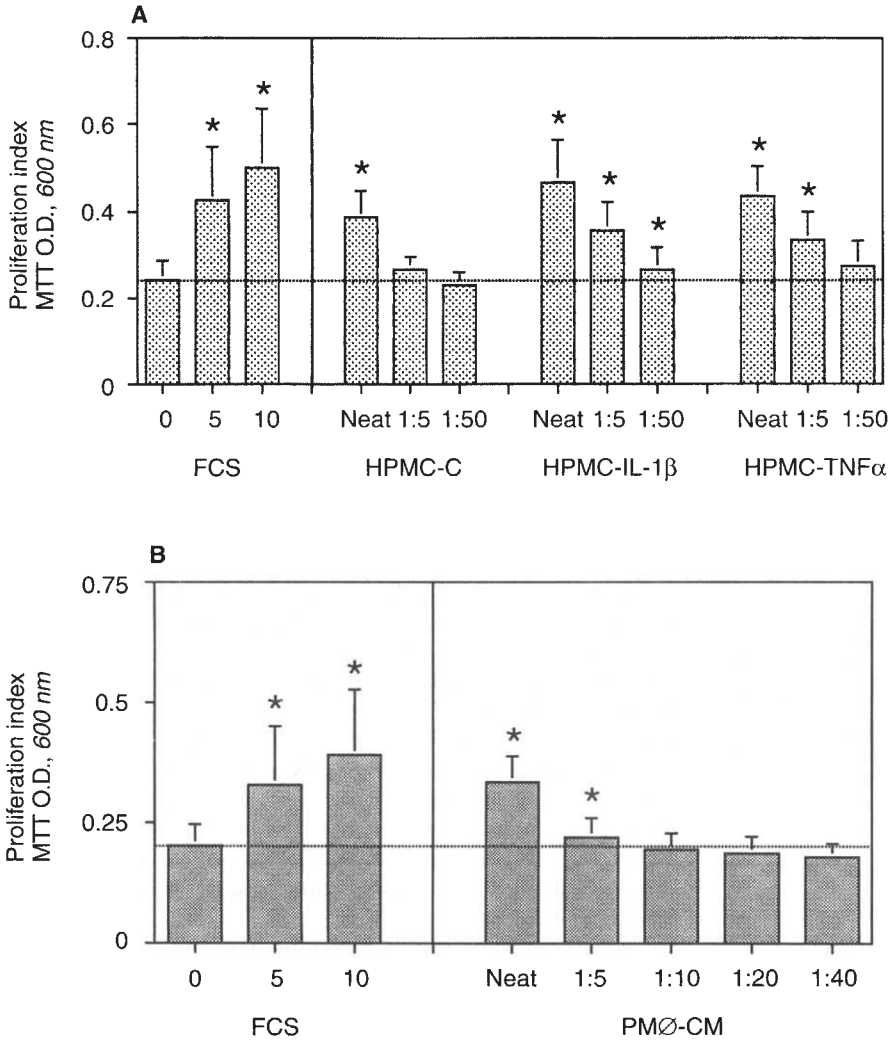

Fig. 4. A. *Effect of human peritoneal mesothelial cell (HPMC) conditioned medium (HPMC-Control, HPMC-IL-1β, HPMC-TNFα) on the proliferation at day 9 of 3D-HPFB as assessed by MTT assay.* Data are presented as (mean ± SEM) from 5 experiments with cells from different donors. *A statistically significant difference ($P < 0.05$) compared to growth arrested 3D-HPFB (Day 9). **B.** Effect of PMØ-CM conditioned medium on the proliferation at day 9, of 3D-HPFB as assessed by MTT assay. Data are presented as (mean ± SEM) from 5 experiments with cells from different donors. *A statistically significant difference ($P < 0.05$) compared to growth arrested 3D-HPFB (Day 9).

0.028 for both). Maximal IL-6 levels were stimulated by IL-1β (1000 pg/ml) and TNFα (1000 pg/ml) (6.47 ± 1.41 and 1.24 ± 0.315, respectively; $N = 6$, $z = 2.203$, $P < 0.028$ for both). The levels of IL-6 secreted by 3D-HPFB in response to stimulation with combinations of IL-1β and TNFα were significantly different from the IL-6 levels generated by the two cytokines alone, at the highest combined doses (IL-1β, 100 pg/ml and TNFα, 1000 pg/ml). Under these conditions the amount of IL-6 released (6.86 ± 0.911) was 1.5-fold above the additive values ($N = 6$, $z = 2.203$, $P < 0.027$).

Discussion

Ultrafiltration failure is a major factor limiting the long-term use of peritoneal dialysis [1, 48]. It is assumed that progressive fibrotic events within the interstitium contribute to modulation of peritoneal membrane structure and function [4, 8]. Little is known, however, about how these events are initiated and progress, and to what extent the resident cells of the peritoneal membrane (resident and infiltrating macrophages, mesothelial cells and interstitial fibroblasts) contribute to this process. It is clear, however, that infection/inflammation (both its frequency and severity) plays a significant role [48].

The process of inflammation within the peritoneal cavity of CAPD patients has been characterized by both *ex vivo* measurements of effluent cytokine levels and *in vitro* cell culture of peritoneal membrane cell populations [23–27, 37, 49–54]. These observations suggest that control of peritoneal inflammation involves the products of the macrophage and the mesothelium [28].

Within the peritoneal membrane there exists a third population of cells, the peritoneal fibroblasts. This cell type has only recently been isolated and its contribution to the normal function of the peritoneal membrane as well as its responses to inflammatory processes remains to be determined. During peritoneal inflammation the levels of cytokines, especially IL-6, are elevated [53]. It has been previously suggested that the mesothelium is the major source of IL-6 present during peritoneal inflammation [25, 53, 55]. The synergistic induction of IL-6 synthesis in conditions that mimic peritoneal inflammation suggest that HPFB potentially contribute to this secretion. The role of IL-6 in the peritoneal cavity remains to be fully defined, however, it has been suggested that it plays both an anti-inflammatory role as well as being potentially involved in peritoneal transport [56].

Most *in vitro* fibroblast studies have utilized monolayer cell

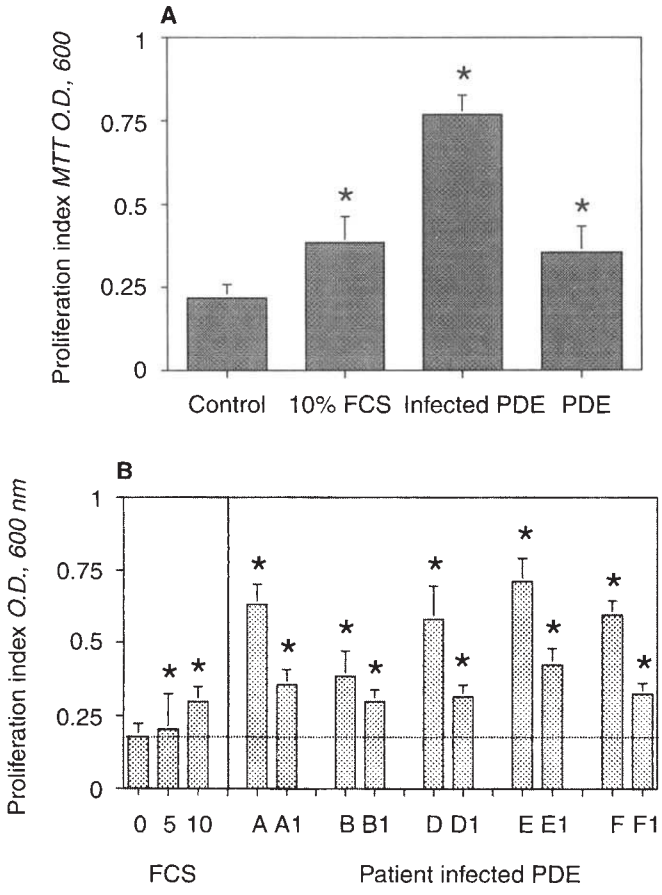

Fig. 5. A *Effect of pooled infected PDE (obtained from 5 patients at presentation of infection), pooled non infected PDE (obtained from 5 patients without a history of peritonitis) and 10% FCS on 3D-HPFB proliferation at day 6, as assessed by MTT assay.* Data are presented as (mean ± SEM) from 5 experiments with cells from different donors. *A statistically significant difference ($P < 0.05$) compared to growth arrested 3D-HPFB (Day 6). **B.** Effect of overnight infected PDE obtained from 5 different patients (A, B, D, E and F) (PDE was obtained on days 1, 2, 3, 4 and 5 of infection) and infection free PDE (A1, B1, D1, E1 and F1) obtained three months later from the same patients on 3D-HPFB proliferation. PDE was added to 3D-HPFB on 5 successive days (day 1 PDE on day 1, day 2 PDE on day 2, etc.) and 3D-HPFB proliferation assessed at day 6 by MTT assay. Data are presented as (mean ± SEM) from 5 experiments with cells from different donors. *A statistically significant difference ($P < 0.05$) compared to growth arrested 3D-HPFB (Day 6).

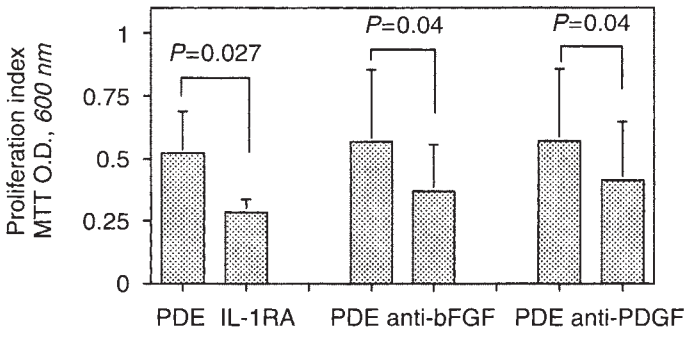

Fig. 6. *Effect of IL-1ra (10 μg/ml), anti-PDGF-$_{AB}$ (10 μg/ml) or anti-bFGF (50 μg/ml) on 3D-HPFB proliferation at day 6, induced by pooled infected PDE.* Data are presented as (mean ± SEM) from 5 experiments with cells from different donors. *A statistically significant difference ($P < 0.05$) compared to growth arrested 3D-HPFB (Day 6).

culture systems to investigate fibroblast function. As the extracellular matrix provides important signals that modulate cellular behavior [31] and peritoneal fibroblasts reside within the submesothelial interstitium embedded within the extracellular matrix, we reasoned that culturing HPFB within a 3-dimensional matrix might more closely mimic their *in vivo* state. This study describes, for the first time, the routine isolation and culture of 3D-HPFB and their establishment and propagation in a 3-dimensional collagen matrix.

In monolayer culture, 3D-HPFB exhibited either the classical bipolar spindle morphology, or more frequently, a flattened, multipolar/stellate appearance. Within the 3D collagen matrix, cell morphology was altered with cells displaying an accentuated spindle morphology. Immunocytochemical characterization of these cells, in monolayer culture, showed them to be negative for cytokeratin, desmin and Factor VIII, excluding contamination with endothelial and mesothelial cells, and positive for vimentin, myosin and occasionally, in proliferating cells, α-smooth muscle actin. This expression of muscle differentiation features in fibroblasts has previously been described in both physiological and pathological settings [57]. Fibroblasts expressing such features have been termed myofibroblasts, and are associated with pulmonary and renal fibrotic diseases [57]. It has previously been observed that the cytoskeletal profile of fibroblasts maintained in a 3D collagen matrix differs from that seen in monolayer culture [12], confirming that the interaction of cells with the collagen matrix modulates their behavior.

The extracellular matrix is known to exert influence over many cellular functions, including proliferation and secretion. Several investigators have observed reduced proliferative potential of fibroblasts in 3D collagen gels as compared to monolayer cultures [58–60]. The turnover of extracellular matrix and matrix mRNA expression are also modified with various investigators observing modulation of collagen synthesis, mRNA expression, collagenase activity and proteoglycan composition [61–64].

Interstitial cell hyperplasia is thought to be an important initiating step in the fibrotic process [65, 66]. In the present study, we chose to focus on the proliferative responses of 3D-HPFB to cytokines and growth factors previously identified as being important in other fibrotic conditions. Initial experiments demonstrated that, in our extended culture system, IL-1β, all isoforms of PDGF, and bFGF were mitogenic for 3D-HPFB. All induced both a time and dose dependent increase in 3D-HPFB proliferation as assessed by a variety of standard techniques. Under the same conditions TGF-β1 was not mitogenic for 3D-HPFB.

Interleukin-1 has been shown to increase fibroblast proliferation in many systems [10, 67–69]. This mitogenic action of IL-1 on fibroblasts is postulated to be indirect and mediated by induction of PDGF-A chain synthesis [69]. In our studies, however, IL-1 induced 3D-HPFB proliferation could not be inhibited by anti-PDGF antibody. There is considerable evidence that PDGF and bFGF participate in the initiation of fibrotic diseases particularly in the kidney [70], and both are potent mitogens for many cell types [71, 72]. In our system both PDGF and bFGF were potent mitogens for 3D-HPFB over a wide dose range.

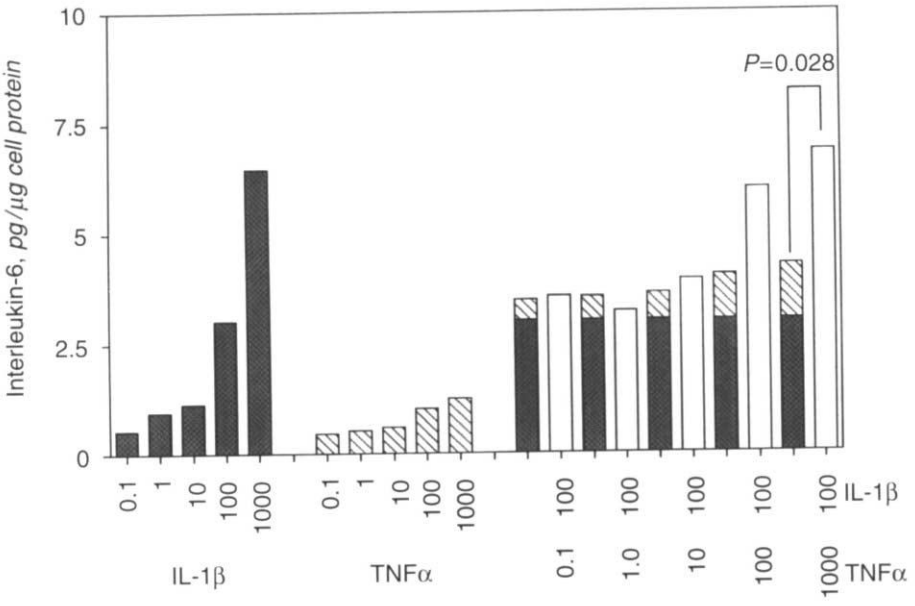

Fig. 7. *Induction of IL-6 synthesis by 3-D HPFB in response to IL-1β or TNFα combinations of the two.* Data are presented as the mean (± SEM) of 6 experiments performed with different cell preparations. Symbols are: (▨) IL-1β, (▧) TNFα, and (□) IL-1β + TNFα.

The most pronounced mitogenic effect upon 3D-HPFB, however, occurred following incubation of 3D-HPFB with infected spent dialysate. Dialysate collected on the first day of the infection induced the greatest mitogenic response, with fluids taken from subsequent days showing a significantly diminished effect. Interestingly, PDE obtained from patients three months after the resolution of infection or in patients without a history of peritonitis still contained significant mitogenic potential. These data concur with those of Selgas et al, who observed a highly variable effect of uninfected PDE on mouse fibroblast proliferation [73]. These authors, however, did not determine the nature of this mitogenic activity.

Inhibition experiments revealed that the mitogenic potential present in infected PDE was heat labile and related to its IL-1 and also to its bFGF and PDGF content. In this respect, it has recently been demonstrated that infected PDE contains significant quantities of IL-1 [50, 74] that are reduced as infection resolves. There are no reports on PDGF and bFGF levels in PDE; however, our preliminary Western blot data suggest that both proteins are detectable in both infected and noninfected PDE (Beavis and Topley, unpublished data).

As mentioned previously, PMØ and the mesothelium are believed to contribute to the control of peritoneal inflammation. We investigated whether these cells might be the source of the mitogenic molecules present in PDE. Conditioned medium obtained from both PMØ and HPMC induced 3D-HPFB proliferation, confirming that both cell types were capable of contributing to the mitogenic activity present in PDE.

The implications of these findings remain to be determined. Recent data, however, suggest that ultrafiltration failure in long-term CAPD is associated with the frequency and severity of peritonitis episodes [48]. These data suggest that during the process of infection, factors are released that contribute to the initiation of membrane dysfunction. Clearly, however, the peritoneal membrane is capable of recovering, at least in the short term, from a single infectious episode, but frequent infection is obviously detrimental to long-term membrane function. Loss of ultrafiltration, however, also occurs in patients without a history of peritonitis [1, 4, 48], which suggests that other mechanisms, such as continuous exposure to unphysiological dialysis fluids or ongoing sub-clinical inflammation, might contribute to this process.

The data from the present study suggest that significant mitogenic potential exists within PDE and that this is significantly increased during peritoneal infection. Within the peritoneum, therefore, there exist mechanisms by which inflammatory activation is controlled and the outcome in terms of peritoneal fibrosis depends on the balance of normal healing process versus those processes which result in the initiation and propagation of the fibrotic process. Our data suggest that the peritoneal fibroblast may be centrally involved in both the normal and pathological processes occurring within the CAPD peritoneum.

Acknowledgments

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Reprint requests to Dr. Nicholas Topley, Institute of Nephrology, Cardiff Royal Infirmary, Newport Road, Cardiff, CF2 1SZ, Wales, United Kingdom.

Appendix

Abbreviations are: HPFB, human peritoneal fibroblasts; 3D-HPFB, HPFB in 3-dimensional cell culture; bFGF, basic fibroblast growth factor; CAPD, continuous ambulatory peritoneal dialysis; FCS, fetal calf serum; HPMC, human peritoneal mesothelial cell; HPMC-CM, human peritoneal mesothelial cell conditioned medium; IL-1β, interleukin-1β; IL-1ra, interleukin-1 receptor antagonist; LPS, lipopolysaccharide; MTT, 3[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide; PDE, peritoneal dialysis effluent; PDGF, platelet-derived growth factor; PMØ, peritoneal

macrophage; PMØ-CM, peritoneal macrophage conditioned medium; TGF-β_1, transforming growth factor-β_1.

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