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DISSERTATION

Functional characterization of human peritoneal fibroblasts: implications for peritoneal immunity in patients treated with peritoneal dialysis.

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SUMMARY

Zusammenfassung

Die Peritonealdialyse (PD) ist eine lebensrettende Nierenersatztherapie für Patienten mit Nierenversagen. Die langfristige Exposition der Peritonealmembran gegenüber bioinkompatiblen Peritonealdialyselösungen (PDF), aber auch wiederholte Peritonitisepisoden können strukturelle Veränderungen in Gang setzen, die letztendlich in einer peritonealen Fibrose und damit im Therapieversagen münden können. Wichtige zelluläre Elemente bei diesen Prozessen sind die peritonealen Fibroblasten. Das Ziel der vorliegenden Arbeiten war die Isolation, Identifikation und funktionelle Analyse von humanen peritonealen Fibroblasten (HPFB) speziell im Hinblick auf Eigenschaften, die für die erfolgreiche PD von Bedeutung sind.

Im Anschluß an die Identifikation von Fibroblasten in Zellkultur oder Peritonealbiopsien anhand ihrer Expression von Fibroblast-Specific Protein 1 (FSP-1) untersuchte ich die Produktion des Chemokins CCL5 in HPFB und fand, daß sie große Mengen an CCL5 nach Stimulation mit IL-1β und TNF-α und amplifiziert durch IFN-γ synthetisieren. Nach Exposition gegenüber hohen Glukosekonzentrationen (wie sie für PDF typisch sind) zeigten HPFB eine vermehrte, jedoch voneinander unabhängige, Expression des Transkriptionsfaktors NFAT5 und des Chemokins CCL2.

Eine besondere Eigenschaft von Fibroblasten ist ihre Heterogeneität zwischen verschiedenen anatomischen Lokalisationen innerhalb desselben Gewebes. Diesbezüglich konnte ich zeigen, daß die HPFBs im Peritoneum Unterschiede bezüglich ihrer Expression von Thy-1 aufweisen. Im Anschluß an ihre Isolation mittels magnetischer Zellsortierung zeigten Thy-1^+ HPFB eine vermehrte Expression von α-SMA, Kollagen 1 und TGF-β im Vergleich mit Thy-1^- HPFB. Weiterhin wiesen Thy-1^+ HPFB eine vermehrte Proliferationskapazität und Kontraktionsfähigkeit auf. Dies legt nahe, daß Thy-1^+ HPFB mehr zur Bildung eines myofibroblastoiden Phänotyps neigen und somit eher zur Entwicklung einer Peritonealfibrose beitragen können. Peritoneale Biopsien sowohl von urämischen Prädialysepatienten als auch von Patienten unter PD-Behandlung enthielten in Omentum und parietalem Peritoneum mehrheitlich FSP-1^+ Thy-1^+ HPFB. Im parietalen Peritoneum von nierengesunden Patienten fanden sich im Gegensatz hierzu keine FSP-1^+ Fibroblasten, doch fanden sich im Omentum eine vergleichbare Anzahl Thy-1^+ und Thy-1^- Zellen. Diese Beobachtungen deuten an, daß die Population Thy-1^+ Fibroblasten bei Patienten mit zunehmender Urämie sowie im Verlauf der Peritonealdialysetherapie zunehmen könnte. Insgesamt geben die Ergebnisse dieser Studie Hinweise auf eine Beteiligung von peritonealen Fibroblasten an inflammatorischen Prozessen im dialysierten Peritoneum und demonstrieren die Existenz einer Subpopulation von HPFB, die im Verlauf der Peritonealdialyse expandiert und zur Entwicklung einer peritonealen Fibrose beitragen kann.

Abstract

Peritoneal dialysis (PD) is a life-saving renal replacement therapy for patients suffering of kidney failure. Long-term exposure of the peritoneal membrane to bioincompatible peritoneal dialysis fluids (PDFs) and repeated peritonitis episodes may provoke structural alterations that eventually lead to the development of fibrosis and technique failure. Peritoneal fibroblasts are key cellular players involved in these processes. The aim of the present study was to purify, identify and functionally analyze human peritoneal fibroblasts (HPFB) with regard to features relevant to successful PD.

Having identified HPFB in cell culture and in peritoneal biopsies on the basis of fibroblast specific protein 1 (FSP-1) expression, I analyzed the capacity of HPFB for producing the chemokine CCL5 and found that HPFB synthesize large quantities of CCL5 upon stimulation with macrophage-derived IL-1β and TNF-α in a process that can be amplified by IFN-γ. Moreover, HPFB exposed to high glucose concentrations (corresponding to those in PDFs) showed increased expression of the transcription factor NFAT5 and the chemokine CCL2; the upregulation of these mediators appeared to be independent of each other.

An intriguing feature of fibroblasts is their heterogeneity between different anatomic locations and within the same tissue. I found that HPFB in the peritoneum differed in the expression of Thy-1. Following isolation by magnetic activated cell sorting, Thy-1^+ HPFB showed increased expression of α-SMA, collagen 1, and TGF-β in comparison to HPFB not expressing Thy-1. Furthermore, Thy-1^+ HPFB displayed greater proliferation and contraction ability, suggesting that they could be more prone to acquire myofibroblastic phenotype and thus contribute to peritoneal fibrosis. Peritoneal biopsies from uremic, non-dialyzed, and PD-treated patients showed that both in the omentum and the parietal peritoneum Thy-1^+ HPFB constituted the majority of fibroblasts as identified by the presence of FSP-1. In contrast, in healthy individuals FSP-1^+ fibroblasts could not be detected in the parietal peritoneum whilst those present in the omentum contained similar proportions of Thy-1^+ and Thy-1^- cells. These observations indicate that the number of Thy-1 expressing fibroblasts may increase as a result of both uremia and PD therapy. Taken together, the results presented in this thesis demonstrate the involvement of HPFB in inflammatory responses occurring in the dialyzed peritoneum and show the existence of a subset of HPFB that may expand during PD and contribute to the development of peritoneal fibrosis.

Introduction

Kidney failure with resulting uremia is a life-threatening condition requiring dialysis or transplantation. Haemodialysis is the most commonly used form of renal replacement therapy, however, peritoneal dialysis (PD) is gaining an increased popularity as an effective form of home dialysis. PD combines good clinical outcomes with simplicity, low costs, and ease, with which it can be performed at home by the patient himself. (1) In PD, the peritoneal membrane (PM) that covers visceral organs (visceral peritoneum) and lines the inner surface of the abdominal wall (parietal peritoneum), acts as a dialyzing organ. Seemingly the structure of PM is simple: under the monolayer of squamous, flattened mesothelial cells (HPMC) attached to basement membrane, there is a thin layer of submesothelial interstitium with embedded capillaries and scattered leukocytes and fibroblasts (HPFB) (Fig.1). The space between parietal and visceral peritoneum, termed the peritoneal cavity, contains a small amount of serous fluid that facilitates peristaltic movements of abdominal viscera. During PD the peritoneal dialysis fluids (PDFs) are infused into the peritoneal cavity enabling the removal of wastes and water from peritoneal capillaries. (40)

Unfortunately, long term PD may cause structural and functional alterations in the peritoneum that may eventually lead to technique failure. (12) The peritoneal membrane is continuously exposed to PDFs, which in spite of significant improvements, are still not fully biocompatible owing to the presence of high concentrations of glucose, glucose degradation products, and lactate, as well as low pH and high osmolality. Additionally, the peritoneum may be affected by episodes of peritoneal infection and the resulting peritonitis. (2)

Peritonitis is the commonest PD complication, caused typically by spreading of infectious agents through a peritoneal catheter. It is characterized by a massive infiltration of the peritoneum by various leukocyte subsets at a sequence controlled by a complex network of chemokines produced by peritoneal cells. (3) In the normal peritoneum there is a variable number of resident leukocytes, mostly macrophages, however, in response to pathogenic stimuli, peritoneal cells generate chemoattractants that recruit polymorphonuclear leukocytes (PMN). They phagocytose invading bacteria and then are cleared and replaced by a population of mononuclear cells. The principal source of chemokines are HPMC, however, also fibroblasts can significantly modulate the intraperitoneal inflammatory response. (23) HPFB release constitutively chemokines MCP-1/CCL2 and IL-8/CXCL8 and can further upregulate their synthesis upon stimulation with macrophage-derived proinflammatory cytokines, such as IL-1β and TNFα. IL-1β triggers expression of neutrophil-targeting cytokines by HPFB, whilst the production of mononuclear chemoattractant RANTES/CCL5 is controlled by IFN-γ, which

synergistically amplifies the effect of TNFα. (18; 19; 47) These observations confirm the role of fibroblasts as cells being able to control transperitoneal chemotactic gradients during peritonitis, crucial when mesothelial cells are damaged and exfoliated (Fig. 2). (45)

Chronic peritoneal inflammation during PD results from long term exposure to bioincompatible PDFs and repeated episodes of peritonitis. It promotes loss of mesothelial cells, fibrotic thickening of the submesothelial compact zone and increased peritoneal vascularization. (17) All these changes contribute ultimately to ultrafiltration failure (UFF), which is often a direct cause of withdrawal from PD therapy. (3)

Fibrosis is a pathological condition characterized by excessive accumulation of fibrous connective tissue. It can then lead to permanent scarring and severe organ malfunction. (44; 48) There appears to be two types of peritoneal fibrosis: (i) simple sclerosis and (ii) encapsulating peritoneal sclerosis (EPS). Simple sclerosis develops usually (although to a different degree) in all PD patients, progresses with time, but may decline after PD withdrawal. EPS is a very severe, but relatively uncommon, form of fibrosis that persists even after PD is stopped and progresses to create a cocoon surrounding and obstructing the bowel. (38)

Peritoneal fibrosis is largely driven by myofibroblasts that are key effectors of fibrotic tissue remodeling. (15; 20) The origin of myofibroblasts in the peritoneum is still under debate. Partially, they can arise from mesothelial cells that acquire a mesenchymal phenotype through a process known as epithelial-to-mesenchymal transition (EMT) (or more accurately – mesothelial-to-mesenchymal transition (MMT). (26) Resident peritoneal fibroblasts are another possible source of myofibroblasts. Under certain circumstances they become activated and express α-smooth muscle actin (α-SMA). However, there is little data on the mechanism that controls the function of resident peritoneal fibroblasts in this respect. (48)

Fibroblasts are only scarcely dispersed in the normal peritoneum. Thus their isolation, characterization and analysis is challenging and technically demanding. Typically fibroblasts are identified by their spindle-shape appearance, the expression of mesenchymal markers and the lack of expression of markers of other cell lineages. Recently, fibroblast-specific protein-1 (FSP-1) emerged as a useful marker for fibroblasts not only in culture, but also in biopsy specimens. FSP-1 is a 10-kDa cytoskeletal protein belonging to the calmodulin-S100-troponin C superfamily of calcium binding proteins. These proteins are closely associated with cytoskeletal fibers, cell motility, and a mesenchymal phenotype. (39) Although, the specificity of FSP-1 has been questioned, the advantages of using it for identification of fibroblasts are persuasive. In combination with other biomarkers, FSP-1 can be used as a reliable marker of cells that has acquired a myofibroblastic phenotype. (8; 31)

A very intriguing feature of fibroblasts is their heterogeneity. It exists to be seen not only in different organs, but also within the same tissue. The analysis of gene expression patterns has even led to a suggestion that fibroblasts from different anatomic sites should be considered distinct differentiated cell types. (35) One of the distinguishing features of fibroblasts can be the presence of the surface receptor Thy-1 (CD90). (32) Thy-1 is a glycophosphatidylinositol-linked outer membrane protein expressed by many cell types, including hematopoietic and stromal stem cells, neurons, and fibroblasts. (5; 30; 34; 42) Fibroblast subsets that differ in Thy-1 expression have been detected in the lungs, orbital cavity, spleen and female reproductive tract. (5; 14; 21; 22) They have different capacity for producing collagens and fibronectin (11), expressing cyclooxygenases (21) and a receptor for platelet-derived growth factor-alpha. (13) It appears, however, that Thy-1 expression determines fibroblast properties in a tissue-specific manner so that Thy-1^{+} and Thy-1^{-} subsets may play different roles in different tissues. (24; 29; 41)

Difficulties in HPFB isolation and characterization as well as limited information on their role in the peritoneum exposed to PD treatment have encouraged me to study HPFB in more detail.

Objectives

1. To assess the usefulness of fibroblast specific protein-1 for identification of fibroblasts in cultures of omentum – derived peritoneal cells.
2. To examine the mechanisms by which proinflammatory cytokines present in the inflamed peritoneum regulate the production of the chemokine CCL5 by peritoneal fibroblasts.
3. To assess the effect of hyperosmolality on activation of NFAT5 and expression of CCL2 by peritoneal fibroblasts.
4. To isolate and functionally characterize fibroblast subsets differing in the expression of Thy-1.

Methodology

Isolation and culture of HPFB. Cells were isolated from specimens of the normal greater omentum obtained from consenting patients undergoing elective abdominal surgery. Cells were isolated by multi-step enzymatic digestion with trypsin/EDTA (0.05%/0.02%, Biochrom AG, Berlin, Germany), collagenase type 1 (from Clostridium histolyticum, 1 mg/mL, Sigma Aldrich, MO, USA) and hyaluronidase (0.1 mg/mL, Sigma Aldrich). Released cells were suspended and propagated in Fibroblast Medium supplemented with penicillin (100 U/mL), streptomycin (100 µg/mL), Fibroblast Growth Supplement and 2% (v/v) fetal bovine serum (FBS) (all from ScienCell, Carlsbad, CA, USA).

Isolation and purification of HPFB subsets. Fibroblasts obtained by enzymatic digestion were further purified by magnetic activated cell sorting (MACS®, Miltenyi Biotec, Bergisch Gladbach, Germany) and separated into Thy-1^+ and Thy-1^- fractions using CD90 MicroBeads (Miltenyi Biotec).

Immunostaining. Cells were grown on Lab-Tek™ Chamber Slides (Nunc, Langenselbold, Germany), fixed in 3.7% PFA, permeabilized, blocked, incubated with primary antibodies for 1 hour at 37°C, next with appropriate fluorescent Alexa dye-labeled secondary antibodies and counterstained with 4',6-diamidino-2-phenylindoleI (DAPI) nuclear stain (all from Invitrogen/ ThermoFisher Scientific**,** Waltham, USA).

Flow cytometry. Cells were washed, suspended in Cell Staining Buffer (Biolegend, San Diego, CA, USA), stained with FITC-conjugated anti-CD90 or isotype control IgG (Biolegend) for 30 min at 4°C and analyzed using BD FACS Aria III cell sorter and FACS Diva Software (Becton Dickinson, UK).

Western blotting. The cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then Western blotted using nitrocellulose membranes (Hybond-ECL; Amersham Biosciences, Freiburg, Germany) and incubated with antibodies of interest. The bands were visualized by chemiluminescence using ECL detection system (ThermoFisher Scientific) and analyzed using ImageJ 1.43 software (National Institutes of Health, USA).

Gene expression analysis. The expression of target genes was assessed with reverse transcription and quantitative PCR. The relative amount of gene transcript was calculated by the cycle threshold method using the Applied Biosystems 7500 System v.1.2.3 software and normalized for the endogenous reference (*β2-microglobulin*). Quantitative PCR data for CCL5 were normalized based on GAPDH transcript levels. Run data were analysed by "second derivative maximum" with the quantification program Quant versions 2.7 and 3.0.

Gel contraction assay. HPFB were suspended in 2x concentrated medium and mixed (1:1 v/v) with collagen solution (PureCol® Bovine Collagen Solution, Type I, 3 mg/ml, Advanced BioMatrix, Carlsbad, CA, USA). The gels were placed in culture wells pre-coated with 2% BSA in PBS and allowed to polymerize for 1 hour. Thereafter, the gels were released and left free-floating for contraction for 24 hours. Subsequently, they were photographed and analyzed for size using ImageJ software.

Proliferation assays. Proliferating HPFB were visualized by EdU (5-ethynyl-2´-deoxyuridine) incorporation using Click-iT® EdU Imaging Kit ThermoFisher Scientific, Waltham, USA), as per manufacturer's instructions. In addition, HPFB proliferation capacity was assessed by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolinum bromide) conversion assay.

Peritoneal biopsies. Biopsy samples were obtained from the International Pediatric Peritoneal Biopsy Registry (www.pedpd.org) that collects specimens of parietal peritoneum and omentum from children undergoing PD. The study has been registered at www.clinicaltrials.gov (NCT01893710). The biopsies were collected from aged matched 1) control patients, who underwent surgery for incidental conditions not affecting the peritoneum, 2) uremic patients, who donated the tissue at the time of PD catheter insertion, 3) PD patients who underwent surgery at various times on PD for kidney transplantation or for a PD-related problem (catheter repositioning or removal). Immunostaining was performed on formalin-fixed, paraffin-embedded samples according to standard methods.

HPFB exposure to glucose. HPFB were exposed for up to 96 h to media containing D-glucose at concentrations ranging from 10 mM (control) to 125 mM. At designated time points total RNA was extracted with RNA Bee (Tel-Test, Friendswood, TX). Cells were then stored at -80°C until assayed.

CCL5 Protein measurement. Concentrations of CCL5 protein secreted by HPFB were measured with the DuoSet Immunoassay Development Kit (R&D Systems). The assay was designed and performed according to the manufacturer's instructions. Sensitivity of the assay was 5 pg/mL.

Statistics. Statistical analysis was performed using GraphPad Prism 6.00 software (GraphPad Software, La Jolla, CA, USA). The data were analyzed with either the t-test or analysis of variance (ANOVA), as appropriate. A P-value <0.05 was considered significant. Results are presented as means ± SEM of the results obtained in independent experiments with cells from different donors.

Results

1. Identification of HPFB in cell culture

Methods used for the identification of various omentum-derived peritoneal cells in culture were described in the article "The proto-oncogene c-Fos transcriptionally regulates VEGF production during peritoneal inflammation" (Catar et al, Kidney Int. 2013). Stepwise enzymatic digestion of omentum resulted in a release of peritoneal mesothelial cells and fibroblasts, which were further identified using immunofluorescence staining against a panel of cell markers. Depending on the digestion phase, the cells isolated differed in morphology and biomarkers expression. Pure HPMC were obtained after short digestion with trypsin/EDTA. They displayed a uniform cobblestone appearance at confluence and expressed calretinin and cytokeratin, but not FSP-1. By contrast, confluent HPFB were obtained after prolonged digestion with collagenase/hyaluronidase and tended to acquire a spindle-like shape in culture. They expressed fibroblast specific protein 1 (FSP-1) but not calretinin and cytokeratin. Mesodermal origin of both mesothelial cells and fibroblasts was verified by the presence of vimentin. Any potential contamination with omentum-derived endothelial cells was excluded following negative staining for von Willebrand factor (vWF).

2. HPFB produce CCL5 in the inflamed peritoneum

The article "Regulation of chemokine CCL5 synthesis in human peritoneal fibroblasts: a key role of IFN-gamma" (Kawka et al Mediators.Inflamm. 2014), describes the role of HPFB in the production of CCL5 in the inflamed peritoneum. Quiescent HPFB released only barely detectable amounts of CCL5. However, CCL5 production increased greatly in a time- and dose-dependent manner after stimulation with recombinant pro-inflammatory cytokines: IL-1β and TNF-α. CCL5 was released within 12-24 hours in response to IL-1β and plateaued after 72 hours. A dose of 1 pg/ml was sufficient to induce the effect, which reached saturation at 100 pg/ml. Similarly, the exposure to TNF-α resulted in CCL5 generation in a dose and time dependent manner with effective TNF-α concentrations ranging from 100 to 10000 pg/ml. Pre-treatment of HPFB with actinomycin D caused a dose-dependent inhibition of IL-1β and TNF-α induced CCL5 secretion, demonstrating that the stimulatory effects of these cytokines were mediated at the transcriptional level.

Interestingly, HPFB did not respond directly to stimulation with IFN-γ at concentrations ranging from 0.01 to 100 U/ml. However IFN-γ was found to amplify synergistically CCL5 release induced by TNF-α and – to lesser extent – by IL-1β. The effect was time-dependent and was related to the dose of both IFN-γ and TNF-α. Specificity of the combined stimulation by IFN-γ and TNF-α was confirmed by experiments with blocking antibodies.

Pre-treatment of cells with IFN-γ up-regulated the expression of the CD40 receptor, enabling HPFB to respond to a recombinant ligand of CD40 (CD40L). Although, CD40L had almost no effect in control cells, it stimulated dose-dependently CCL5 release in HPFB pre-treated with IFN-γ.

3. HPFB produce CCL2 and NFAT5 following osmotic stress

The impact of osmotic stress on chemokine production by HPFBs was demonstrated in the article "Activation of Nuclear Factor of Activated T Cells 5 in the peritoneal membrane of uremic patients" (Kitterer et al., Am.J.Physiol Renal Physiol, 2015). The exposure of HPFB in culture to increasing concentrations of glucose, led to a time- and dose-dependent induction of NFAT5 mRNA. It peaked at 6 hours, but declined later to baseline values. By contrast, CCL2 mRNA induction culminated as late as after 96 h. These results were analyzed in relation to findings in peritoneal biopsies from uremic and PD patients. The biopsies demonstrated that the expression of NFAT5 was greater in uremic patients compared with patients with normal renal function. However, it did not increase further in uremic patients undergoing PD. In contrast, the expression of CCL2 was not greater in pre-dialyzed uremic patients compared with healthy controls, but increased significantly when patients initiated PD.

4. Isolation and characterization of HPFB Thy-1 subsets in vitro

The paper "Thy-$1^{+/-}$ fibroblast subsets in the human peritoneum" (Kawka et al., Am. J.Physiol Renal Physiol, 2017) reports on the existence and role of different HPFB subsets. HPFB were identified by FSP-1 staining and separated by MACS technology into cells bearing or not bearing Thy-1. The purity of the subpopulations isolated was verified by immunocytochemical staining and flow cytometry. Thy-1^{+} and Thy-1^{-} subsets were found to exist in the omentum at almost equal proportions. However, they displayed different morphology with Thy-1^{+} cells having a typical elongated and spindle-shaped appearance, and Thy-1^{-} fibroblasts being more outspread and irregular. These morphological features were paralleled by different organization of cytoskeleton filaments, as visualized by vimentin immunostaining.

When analyzed for the expression of pro-fibrotic factors, Thy-1^{+} HPFB were found to express approximately twice as much of α-SMA (both at the level of mRNA and protein) as Thy-1^{-} HPFB. Moreover, Thy-1^{+} HPFB showed increased mRNA expression for collagen 1 and TGF-β1. By contrast, expression of fibronectin did not differ between the subsets.

Furthermore the proliferation capacity of Thy-1^{+} HPFB (as assessed by both EdU staining and MTT conversion) was found to be greater compared with Thy-1^{-} HPFB. Similarly, Thy-1^{+} HPFB displayed greater contractile properties than Thy-1^{-} cells, as visualized by the collagen gel contraction assay.

5. Characterization of HPFB Thy-1 subsets in peritoneal biopsies

The same paper ("Thy-$1^{+/-}$ fibroblast subsets in the human peritoneum") described also the localization of Thy-1 subsets of HPFB in peritoneal biopsies. Approximately 45±6% of FSP-1^{+} HPFB in normal omentum from healthy individuals stained also for Thy-1. In biopsies from uremic patients the overall number of detected FSP-1^{+} HPFB was marginally greater but contained the same proportion of Thy-1^{+} cells. In contrast, both the total number of FSP-1^{+} HPFB and the percentage of Thy-1^{+} cells was significantly greater (77±4%) in biopsies from patients undergoing PD.

In biopsies of the parietal peritoneum from healthy individuals, the presence of FSP-1^{+} HPFB could not be detected. In uremic and in PD patients FSP-1^{+} HPFB were found sporadically, but almost all of them (95±2% and 99±1%, respectively) were of the Thy-1^{+} fraction.

Discussion

As fibroblasts are such a heterogeneous cell population, the results of studies on fibroblasts from various tissues may not necessarily be equivalent. Fibroblasts are only sparsely distributed in the peritoneum, nevertheless they provide a crucial tissue support through the production of extracellular matrix components. Furthermore they participate in immune processes and pathological fibrosis by producing a broad array of growth factors and cytokines. (6; 20) Here, I present evidence for HPFB involvement in the cytokine network controlling the course of peritoneal inflammation and fibrosis in the context of PD. First, I have established a reliable method to identify fibroblasts obtained from omentum by using prolonged digestion with collagenase/hyaluronidase and cell selection with anti-fibroblast specific protein 1 (FSP-1) antibodies. HPFB isolated from omentum may be occasionally contaminated with mesothelial cells and less often with endothelial cells. Both fibroblasts and mesothelial cells arise from the mesoderm, which is confirmed by positive staining for vimentin. The contamination by endothelial cells can be excluded following negative staining for von Willebrand Factor (vWF). Normal mesothelial cells do not express FSP-1, but express cytokeratin and calretinin. In contrast, fibroblasts do express FSP-1 but not cytokeratin and calretinin. Thus, FSP-1 could be considered as a good marker to identify peritoneal fibroblasts in culture. Noteworthy, however, cells identified as HPFB must not express markers of other cell types. It is important, since FSP-1 can also appear in mesothelial cells undergoing fibroblastic conversion both in vitro and in vivo. (25; 27)

Previous studies have clearly demonstrated the potential of HPFB to contribute to peritoneal inflammation in PD by their ability to produce various chemokines. (46; 47) CCL5 is a chemokine involved in the recruitment of T lymphocytes and it is produced at the sites of injury typically after 3-5 days to maintain and amplify the immune response. Production of CCL5 was earlier demonstrated in peritoneal mesothelial cells, and in fibroblast from tissues other than the peritoneum. I found that peritoneal fibroblasts in culture produced significant quantities of CCL5, and demonstrated a key role of IFN-γ in the process. CCL5 is a chemoattractant mainly for T cells, which in turn produce large amounts of IFN-γ. Although IFN-γ on its own did not display any effect on CCL5 production, it amplified the effect of TNF-α through synergistic induction of CCL5 mRNA. Similar results were observed in mesothelial cells, synovial fibroblasts, endothelial cells, and alveolar epithelial cells. (7; 28; 33; 36) CD40L (CD40 ligand), a member of the TNF-α family, is a ligand for CD40 receptor and it is expressed by T-

lymphocytes recruited by CCL5 during peritonitis. I showed that the exposure to IFN-γ upregulated CD40 mRNA by HPFB and made them responsive to CD40L resulting in an increased CCL5 production. The same effect was previously observed in fibroblasts from inflamed colonic mucosa, and in peritoneal mesothelial cells. (4; 10)

Peritoneal cells are exposed during PD to high concentrations of glucose from PD fluids. Glucose is used at such high concentrations in order to generate an osmotic gradient that can drive ultrafiltration. (9) I found that the incubation of HPFB with glucose led to a dose-dependent and early induction of the nuclear factor of activated T cells (NFAT5), and more sustained rise in CCL2 mRNA expression. NFAT5 acts as a transcription factor that can activate many genes in response to osmotic stress. It is however unclear whether glucose-induced increase in CCL2 mRNA was mediated by NFAT5. The delay between NFAT5 mRNA induction and upregulation of CCL2 may suggest that either the effect of glucose on CCL2 expression occurs in a NFAT5–independent fashion or that the activity of NFAT5 requires some time to exert an effect on CCL2. (37) This problem could be clarified by future experiments assessing for example whether silencing of NFAT5 impacts on CCL2 production driven by increased osmolality. I looked into expression of NFAT5 and CCL2 in cultured fibroblasts because an increased expression of both NFAT5 and CCL2 was detected in peritoneal biopsies from PD patients. However, the increase in NFAT5 appeared to be related to uremia, while upregulation of CCL2 occurred only after exposure to PD. These observations may correspond to my in vitro results and support the hypothesis that CCL2 induction by high glucose is independent of NFAT5.

The origin and activation of peritoneal myofibroblasts is of practical importance given their role in PD-associated peritoneal fibrosis. I showed that the presence of Thy-1 identified different subsets of HPFB with different pro-fibrotic features. I have established that both Thy-1^+ and Thy-1^- fibroblasts could be isolated from normal human omentum. I have observed that although the numbers of Thy-1^- and Thy-1^+ cells in the omentum did not differ significantly, they displayed conspicuous differences in morphology and their ability to acquire features of profibrotic myofibroblasts. In this respect, Thy-1^+ cells displayed increased expression of α-SMA, as well as collagen 1 and TGF-β1, compared with Thy-1^- fibroblasts. Moreover, they exhibited increased contractile and proliferative capacities. These observations suggest that Thy-1^+ HPFBs may be predominant effector cells in peritoneal fibrosis.

There is a correlation between the duration of PD and an expansion of the extracellular matrix causing the thickening of the submesothelial compact zone. (43) I therefore looked into the presence of fibroblast subsets in the peritoneal biopsies of dialyzed patients. I used a PD

registry that collects samples from dialyzed children, as in this patient population the potential effect on the peritoneal structure of age- and life style-related comorbidities is minimal. As in my in vitro studies, I identified HPFBs by the presence of FSP-1, and the absence of biomarkers of other cell lineages, such as cytokeratin, CD45 (leukocytes), and CD68 (macrophages). Approximately 45% of FSP-1^+ fibroblasts detected in normal omentum expressed Thy-1, which corresponded to the percentage I observed earlier when isolating HPFB from adult patients. I did not detect FSP-1^+ cells in the parietal peritoneum. This could be expected, however, based on earlier reports from both human and animal studies. (16; 25) Interestingly, FSP-1^+ cells were present in the parietal peritoneum of uremic and PD patients and nearly all of them were also Thy-1^+. In biopsies of the omentum from PD patients, FSP-1^+ fibroblasts were also more abundant and contained more Thy-1^+ cells. This indicates that PD therapy may lead to the expansion of FSP-1^+ and Thy-1^+ HPFBs.

In conclusion, my studies demonstrate that:

1. Fibroblast specific protein 1 is a useful marker for identification of fibroblasts in cultures of peritoneal cells and in peritoneal biopsies.
2. Peritoneal fibroblasts are able to produce CCL5 upon stimulation with proinflammatory cytokines IL-1β and TNF-α, and this effect is critically regulated by IFN-γ.
3. Exposure of HPFB to high concentrations of glucose results in upregulation of both NFAT5 and CCL2; however, the time course of induction and the pattern of expression of NFAT5 and CCL2 in peritoneal biopsies does not support the hypothesis that glucose-induced expression of CCL2 is mediated by NFAT5.
4. There exist HPFB subpopulations in the peritoneum that differ in the expression of surface protein Thy-1; it is possible to isolate these subsets with high specificity by combining multistep enzymatic digestion with MACS. Cells of the Thy-1^+ HPFB subpopulation have greater ability to acquire a myofibroblastic phenotype, which predisposes them to contribute to the development of peritoneal fibrosis.
5. The majority of FSP-1^+ fibroblasts found in the parietal and visceral peritoneum of patients treated with PD is Thy-1 positive.

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Figures and legends

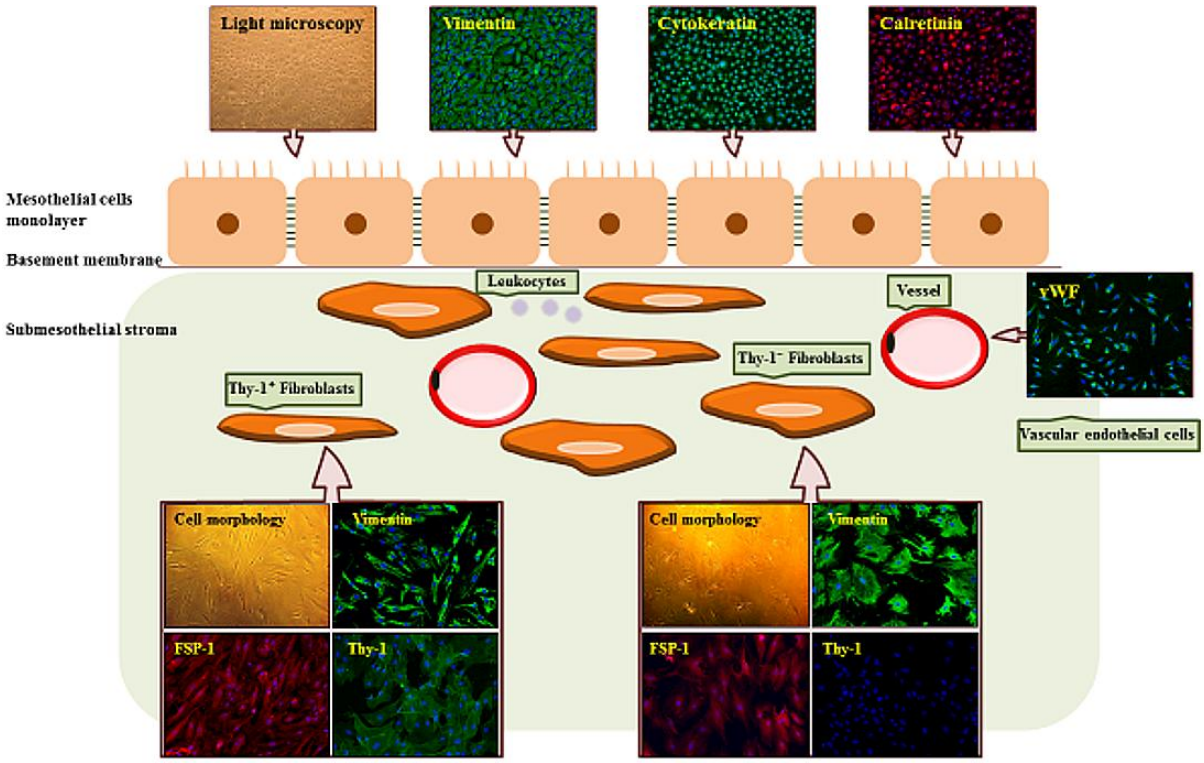

Fig.1 Structure of the peritoneal membrane and identification of peritoneal cells. Mesothelial cells rest on the basement membrane. They display an uniform cobblestone morphology (light microscope, magnification 200x) and stain positively for vimentin (green), cytokeratin (green), and calretinin (red). Resident fibroblasts and leukocytes, and blood vessels are localized in the interstitium under the basement membrane. Fibroblasts are identified by the presence of FSP-1 (red) and vimentin (green). Two subsets of fibroblasts differing in the expression of Thy-1 are identified by staining for Thy-1 (green) and by cell morphology (light microscope, magnification 200x, for immunofluorescence staining nuclei were counterstained with DAPI, blue; fluorescence microscope, magnification 200x)

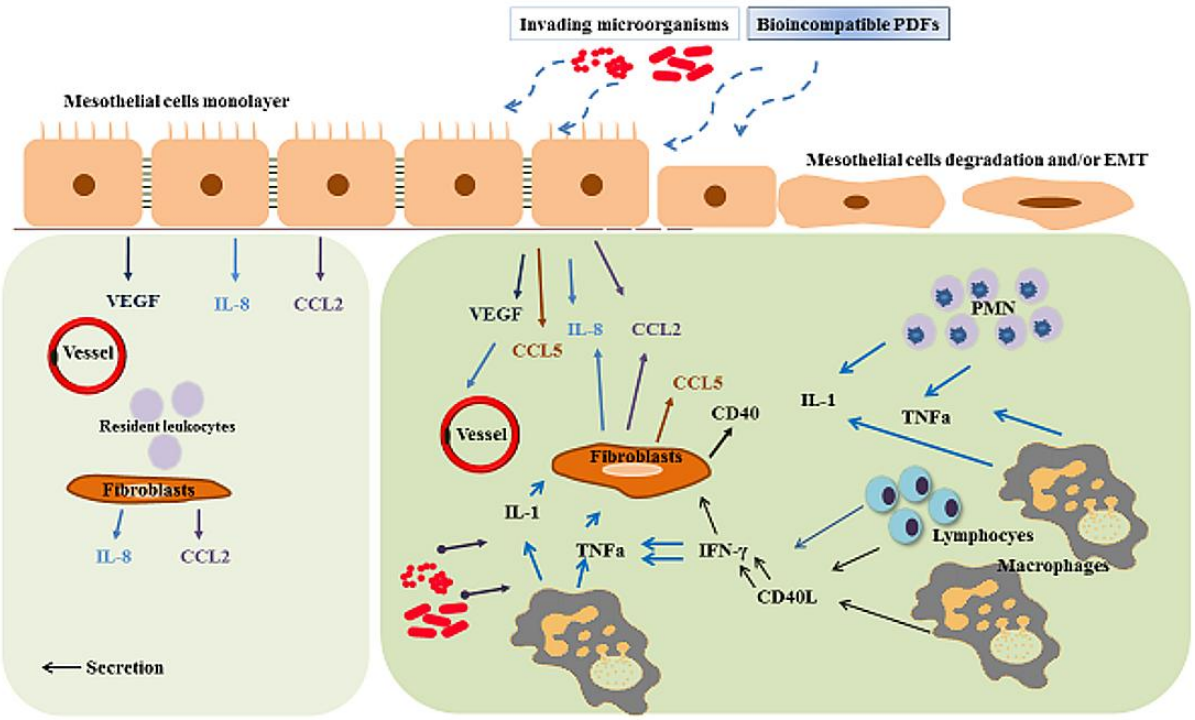

Fig.2 The cytokine network in the peritoneum. Mesothelial cells and fibroblasts are capable of producing IL-8, CCL2 and VEGF constitutively. However, infusions of bioincompatible PDFs and repeated episodes of bacterial infections lead to peritoneal inflammation, during which mesothelial cells are stimulated to produce large amounts of chemokines CCL2, IL-8, CCL5 and growth factors, such as VEGF. Unfortunately, in the course of peritonitis mesothelial cells may exfoliate and/or undergo epithelial-to-mesenchymal transition. The inflammatory response affects also the interstitium and leads to the activation of fibroblasts. Fibroblast-derived chemokines CCL5, CCL2, and IL-8 attract leukocytes that in turn produce cytokines (IL-1, TNF-α, IFN-γ, and CD50L) that can further modulate or amplify the course of inflammation.

Affidavit

I, Edyta Kawka certify under penalty of perjury by my own signature that I have submitted the thesis on the topic "Functional characterization of human peritoneal fibroblasts: implications for peritoneal immunity in patients treated with peritoneal dialysis" I wrote this thesis independently and without assistance from third parties, I used no other aids than the listed sources and resources.

All points based literally or in spirit on publications or presentations of other authors are, as such, in proper citations (see "uniform requirements for manuscripts (URM)" the ICMJE www.icmje.org) indicated. The sections on methodology (in particular practical work, laboratory requirements, statistical processing) and results (in particular images, graphics and tables) correspond to the URM (s.o) and are answered by me. My contributions in the selected publications for this dissertation correspond to those that are specified in the following joint declaration with the responsible person and supervisor. All publications resulting from this thesis and which I am author of correspond to the URM (see above) and I am solely responsible.

The importance of this affidavit and the criminal consequences of a false affidavit (section 156,161 of the Criminal Code) are known to me and I understand the rights and responsibilities stated therein.

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Date Signature

Declaration of any eventual publications

Edyta Kawka had the following share in the following publications:

Publication 1: Catar R, Witowski J, Wagner P, Annett Schramm I, **Kawka E**, Philippe A, Dragun D, Jörres A.,The proto-oncogene c-Fos transcriptionally regulates VEGF production during peritoneal inflammation, Kidney Int, 2013

Contribution in detail: Designing and performing experiments to establish the immunofluorescence staining as a method for identification of fibroblast in cell culture. Isolation, culture and staining of mesothelial cells, fibroblasts and endothelial cells with antibodies against cellular makers, observation under microscope, analyzing obtained data and preparing figure for publication.

Publication 2: **Kawka E**, Witowski J, Fouqet N, Tayama H, Bender TO, Catar R, Dragun D, Jörres A. Regulation of chemokine CCL5 synthesis in human peritoneal fibroblasts: a key role of IFN-γ. Mediators Inflamm. 2014

Contribution in detail : Designing and performing experiments, analyzing data, preparing the manuscript.

Publication 3: Kitterer D, Latus J, Ulmer C, Fritz P, Biegger D, Ott G, Alscher MD, Witowski J, Kawka E, Jörres A, Seeger H, Segerer S, Braun N. Activation of Nuclear Factor of Activated T Cells 5 in the peritoneal membrane of uremic patients. Am J Physiol Renal Physiol. 2015

Contribution in detail : Designing and performing in vitro experiments with HPFB, such as isolation, culture and exposure to increasing dosage of glucose and collecting total RNA in set time points.

Publication 4: Kawka E, Witowski J, Bartosova M, Catar R, Rudolf A, Philippe A, Rutkowski R, Schaefer B, Schmitt C.P, Dragun D, Joerres A. Thy-1+/- fibroblast subsets in the human peritoneum Am.J.Physiol Renal Physiol 2017

Contribution in detail : Designing, performing and analyzing in vitro experiments with HPFB as described in publication. Immunofluorescence staining of parietal peritoneum biopsies, observation under microscope, analyzing obtained data. Preparing the manuscript, figures, and response to reviewers.

Signature, date and stamp of the supervising University teacher

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Signature of the doctoral candidate

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SELECTED PUBLICATIONS

Catar R, Witowski J, Wagner P, Annett Schramm I, **Kawka E**, Philippe A, Dragun D, Jörres A. *The proto-oncogene c-Fos transcriptionally regulates VEGF production during peritoneal inflammation*. Kidney Int. 2013 Dec;84(6):1119-28

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Research Article

Regulation of Chemokine CCL5 Synthesis in Human Peritoneal Fibroblasts: A Key Role of IFN-γ

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Peritonitis is characterized by a coordinated influx of various leukocyte subpopulations. The pattern of leukocyte recruitment is controlled by chemokines secreted primarily by peritoneal mesothelial cells and macrophages. We have previously demonstrated that some chemokines may be also produced by human peritoneal fibroblasts (HPFB). Aim of our study was to assess the potential of HPFB in culture to release CCL5, a potent chemoattractant for mononuclear leukocytes. Quiescent HPFB released constitutively no or trace amounts of CCL5. Stimulation of HPFB with IL-1β and TNF-α resulted in a time- (up to 96 h) and dose-dependent increase in CCL5 expression and release. IFN-γ alone did not induce CCL5 secretion over a wide range of concentrations (0.01–100 U/mL). However, it synergistically amplified the effects of TNF-α and IL-1β through upregulation of CCL5 mRNA. Moreover, pretreatment of cells with IFN-γ upregulated CD40 receptor, which enabled HPFB to respond to a recombinant ligand of CD40 (CD40L). Exposure of IFN-γ-treated HPFB, but not of control cells, to CD40L resulted in a dose-dependent induction of CCL5. These data demonstrate that HPFB synthesise CCL5 in response to inflammatory mediators present in the inflamed peritoneal cavity. HPFB-derived CCL5 may thus contribute to the intraperitoneal recruitment of mononuclear leukocytes during peritonitis.

1. Introduction

Peritoneal dialysis (PD) is an effective alternative to haemodialysis as a life-saving renal replacement therapy for patients with chronic kidney disease. However, the technique may fail as a result of repeated episodes of peritoneal infection that lead to peritoneal membrane damage and loss of its ultrafiltration capacity [1, 2]. The peritoneal cavity contains normally variable numbers of resident leukocytes, predominantly macrophages but also lymphocytes (mostly memory T cells), dendritic, and natural killer (NK) cells [3]. In contrast, acute peritonitis is characterized by a massive influx of polymorphonuclear leukocytes (PMN) [4]. PMN ingest invading microorganisms and then are gradually cleared and replaced by mononuclear cells (monocytes, macrophages, and lymphocytes) so that the intraperitoneal homeostasis is restored. The whole process is governed by a complex network of cytokines, growth factors, adhesion molecules, and molecules derived from pathogens and damaged cells [5]. In this respect, chemokines of various classes create chemotactic gradients that mediate migration of specific leukocyte subpopulations into the peritoneal cavity. In early stages of peritonitis proinflammatory cytokines (TNF-α and IL-1β) derived from resident macrophages induce the expression of CXC chemokines that attract PMN. Then, upon the influence of IFN-γ and IL-6, the pattern of chemokine expression changes so that CC chemokines predominate and mediate mononuclear cell recruitment [6].

During peritonitis chemokines are produced mainly by cytokine-stimulated mesothelial cells that cover the peritoneal membrane. However, in recent years it has become clear that fibroblasts embedded in peritoneal interstitium act not only as structural cells but may also serve as an important source of chemokines [7]. Thus, by producing various chemokines fibroblasts may modify both the intensity and the duration of the inflammatory response [8]. We have previously demonstrated that human peritoneal fibroblasts (HPFB) generate significant quantities of CXC

chemokines that attract and promote survival of PMN during PD-associated peritonitis [9]. Moreover, HPFB are able to produce CCL2, which belongs to CC chemokines and acts mainly as a monocyte chemoattractant [7].

CCL5 (CC-chemokine ligand 5) is another member of the CC chemokine family. First identified in T cells and designated RANTES (Regulated upon Activation, Normal T cell Expressed and Secreted), CCL5 is an 8 kDa protein consisting of 68 amino acids [10]. In addition to lymphocytes, it was found to be also produced by stromal cells. Acting through three types of chemokine receptors (CCR1, CCR3, and CCR5), CCL5 is broadly chemoattractive for T lymphocytes and NK cells, monocytes, basophils, and eosinophils [11]. Interestingly, once T lymphocytes reach the site of injury and become activated with specific antigens, they start producing large amounts of CCL5 after 3–5 days, which maintains and amplifies the immune response. Although there is a great deal of overlapping in biological activities of CC chemokines, the experimental studies in mice demonstrate that CCL5 deficiency is associated with impaired T-cell proliferation and function [12]. This observation indicates that CCL5 is uniquely essential for T-cell recruitment in vivo. Therefore, in the present study we have analysed how proinflammatory cytokines known to be present in the inflamed peritoneum regulate CCL5 production by peritoneal fibroblasts.

2. Materials and Methods

Unless stated otherwise, all chemicals were from Sigma-Aldrich (St Louis, MO, USA) and all culture plastics were Falcon from Becton Dickinson (Heidelberg, Germany). Cell culture media and foetal calf serum (FCS) were from Invitrogen/Life Technologies (Darmstadt, Germany), and other cell culture reagents were from Biochrom AG (Berlin, Germany). Human recombinant cytokines and anticytokine antibodies were from R&D Systems (Wiesbaden, Germany). IFN-γ specific activity was 2×10^4 WHO standard units per 1 μg protein (1 U/mL = 50 pg/mL).

2.1. Isolation and Culture of Human Peritoneal Fibroblasts (HPFB). HPFB were isolated from the specimens of apparently normal omentum obtained from consenting patients undergoing elective abdominal surgery. The tissue was treated with four rounds of digestion with trypsin, as described in detail elsewhere [13]. HPFB were identified by spindle-shape appearance, formation of parallel arrays and whorls at confluence [13], and positive immunostaining for fibroblast specific protein 1 (FSP-1) [14]. Cells were propagated in Ham's F12 culture medium supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL), hydrocortisone (0.4 μg/mL), and 10% (v/v) FCS. HPFB cultures were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2. All experiments were performed using cells from the first 3 passages and with cells derived from separate donors. Before the experiments, cells were rendered quiescent by reducing FCS concentration to 0.1% for 48 hours. Cells were then treated as specified in the figure legends. After the exposure, the supernatants were collected and stored in aliquots at −80°C until assayed.

2.2. CCL5 Protein Measurement. Concentrations of CCL5 protein secreted by HPFB were measured with the DuoSet Immunoassay Development Kit (R&D Systems). The assay was designed and performed according to the manufacturer's instructions. Sensitivity of the assay was 5 pg/mL.

2.3. Gene Expression Analysis. Expression of CCL5 gene was assessed with reverse transcription (RT) and PCR. Total RNA was extracted with RNA Bee (Tel-Test, Friendswood, TX, USA), purified with the RNeasy kit (Qiagen, Hamburg, Germany), and reverse transcribed into cDNA with random hexamer primers, as described in [15]. Conventional semi-quantitive PCR was carried out essentially as described by Robson et al. for CCL5 and β-actin [16], and by Abdel-Haq et al. for CD40 [17]. Precise quantitation of CCL5 mRNA was performed by real-time PCR. The reactions were carried out in Roche LightCycler II using 20 ng of cDNA and FastStart DNA Master SYBR Green I reagents according to the manufacturer's instructions (Roche Applied Science, Indianapolis, IN, USA). Primer pairs (TIB Molbiol, Berlin, Germany) spanned an intron to eliminate potential amplification of contaminating genomic DNA. The following primers were used: CCL5 (GenBank NM_002985.2) forward, GAGTATTTCTACACCAGTGGCAAG; reverse, TCCCGAACCCATTTCTTCTCT; GAPDH (GenBank NM_002046.4) forward, TGATGACATCAAGAAGGTGGTGAAG; reverse, TCCTTGGAGGCCATGTGGGCCAT. Cycle parameters were as follows: denaturation at 95°C for 10 s, annealing at 63°C for 5 s, and elongation at 72°C for 20 s for 40 cycles. Melting curve analyses were performed from 60°C to 95°C in 0.5°C increments. Quantitative PCR data for CCL5 were normalized based on GAPDH transcript levels. Run data were analysed by "second derivative maximum" with the quantification program Quant versions 2.7 and 3.0.

2.4. Statistical Analysis. Data are presented as mean ± SEM of the results obtained in independent experiments with cells from different donors. Statistical analyses were carried out using GraphPad Prism 5.00 software (GraphPad Software Inc., La Jolla, CA, USA). The data were compared with repeated measures analysis of variance with Newman-Keuls modification or the paired t-test, as appropriate. A P value of <0.05 was considered significant. Significant differences compared with appropriate controls were denoted with asterisks: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

3. Results

3.1. Induction of CCL5 Production in HPFB by IL-1β and TNF-α. The amount of CCL5 released constitutively by quiescent HPFB was barely detectable (Figure 1). In contrast, stimulation of HPFB with recombinant proinflammatory cytokines IL-1β and TNF-α resulted in a time- and dose-dependent CCL5 secretion. The release of CCL5 in response to IL-1β was significantly above the background levels within 12–24 hours of incubation and reached plateau after 72 hours. The time course of CCL5 generation in response to the same concentration of TNF-α followed a similar pattern; however, even greater amounts of CCL5 were produced (Figure 1(a)).

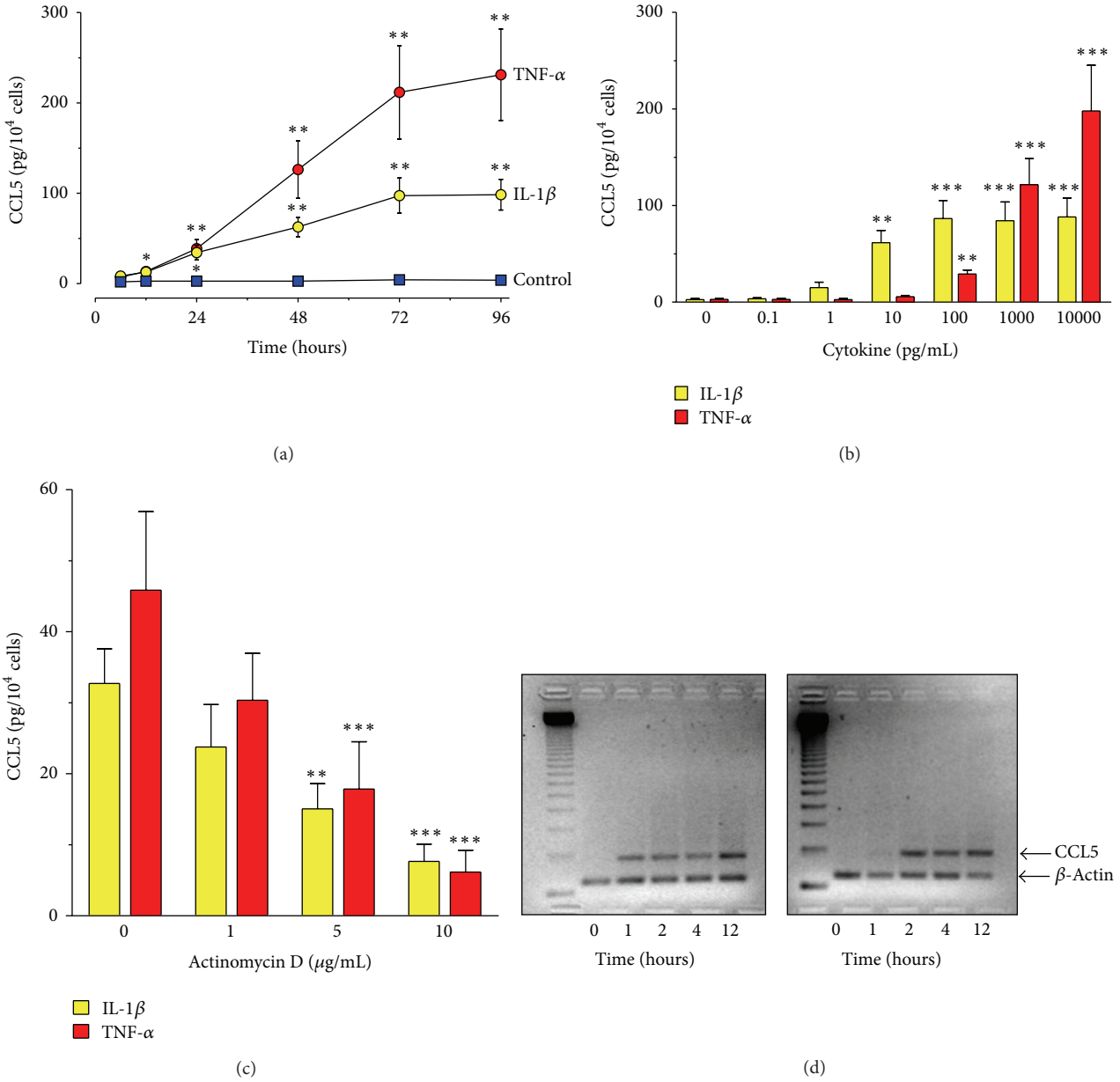

FIGURE 1: Effect of recombinant IL-1β and TNF-α on CCL5 expression and release by HPFB. Cells were exposed to either IL-1β or TNF-α. The data were derived from experiments with cells isolated from separate donors. (a) Kinetics of IL-1β-induced (1000 pg/mL) or TNF-α-induced (1000 pg/mL) CCL5 secretion ($n = 6$); (b) dose effect of IL-1β or TNF-α. Cells were stimulated for 48 hours ($n = 6$); (c) effect of actinomycin D on CCL5 release by HPFB. Cells were pretreated for 1 hour with actinomycin D and then exposed to either IL-1β or TNF-α (both at 1000 pg/mL) for 24 hours ($n = 6$); (d) time effect of IL-1β and TNF-α on CCL5 mRNA expression. HPFB were treated with cytokines at 1000 pg/mL for the times indicated. CCL5 mRNA expression was analysed by semiquantitative RT-PCR. Results of a representative experiment of three performed.

Experiments assessing the dose effect of cytokines revealed that IL-1β was effective already at concentrations as low as 1 pg/mL and the effect reached saturation at 100 pg/mL (Figure 1(b)). TNF-α was able to stimulate CCL5 release at concentrations ranging from 100 to 10000 pg/mL.

Pretreatment of HPFB with actinomycin D resulted in a dose-dependent inhibition of cytokine-induced CCL5 secretion, indicating that the stimulatory effects of IL-1β and TNF-α occurred at the transcriptional level (Figure 1(c)). Indeed, treatment of HPFB with either IL-1β or TNF-α resulted in a time-dependent upregulation of the CCL5 mRNA signal, as visualized by conventional semiquantitative PCR (Figure 1(d)).

3.2. Effect of IFN-γ on CCL5 Production by HPFB. IFN-γ at concentrations ranging from 0.01 to 100 U/mL did not induce CCL5 production by HPFB. However, it amplified synergistically CCL5 release induced by TNF-α (Figure 2)

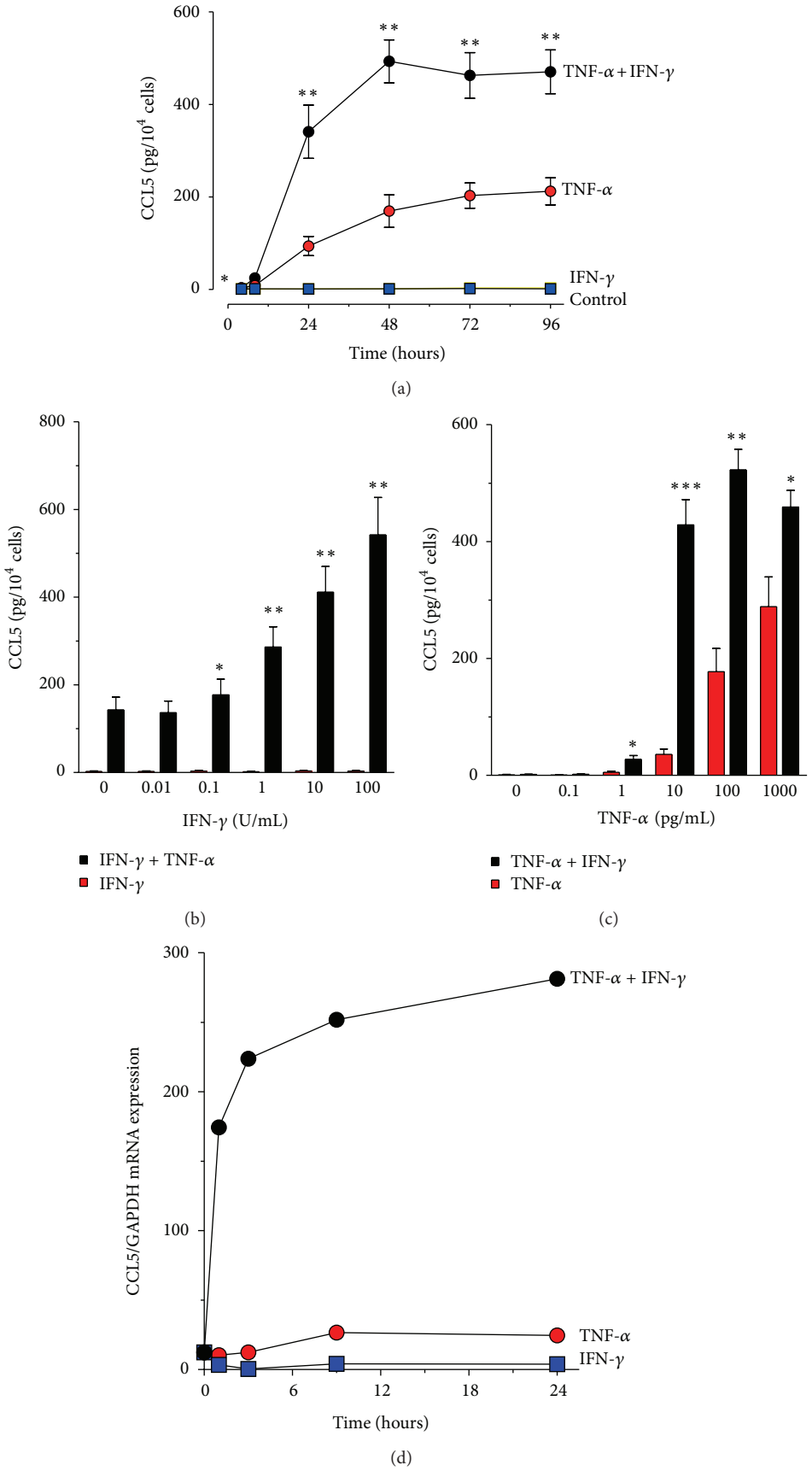

Figure 2: Continued.

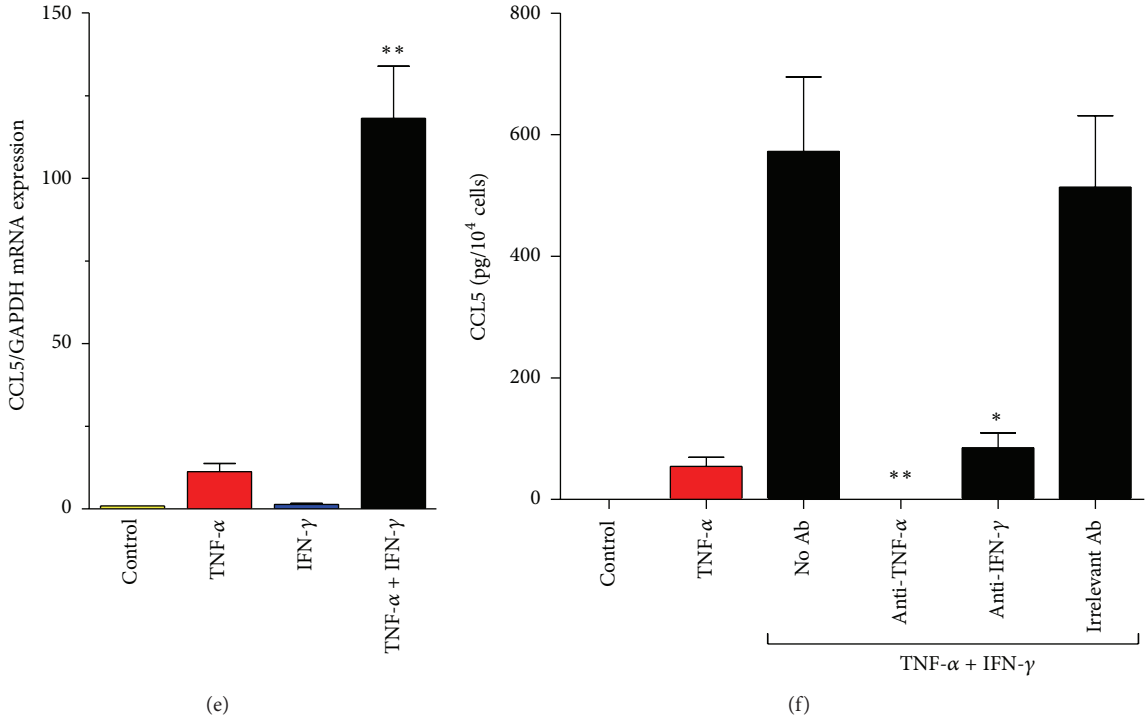

Figure 2: CCL5 induction in HPFB stimulated with TNF-α and IFN-γ. (a) Kinetics of CCL5 secretion by HPFB treated with TNF-α (1000 pg/mL) and IFN-γ (25 U/mL) alone or in combination. Asterisks represent a significant difference compared with the predictive additive values at each time point ($n = 6$); (b) dose effect of IFN-γ alone or with TNF-α (1000 pg/mL; $n = 7$); (c) dose effect of TNF-α alone or with IFN-γ (25 U/mL; $n = 5$). B and C cells were stimulated for 48 hours. Asterisks represent statistically significant differences compared to the predictive additive values; (d) kinetics of TNF-α and IFN-γ-induced CCL5 mRNA. Cells were treated with TNF-α and/or IFN-γ for the times indicated. Results of an exemplary experiment of two performed; (e) magnitude of CCL5 mRNA expression in HPFB treated for 24 hours with TNF-α and/or IFN-γ. Results of 4 experiments with cells from separate donors. Asterisks represent a significant difference compared to the predictive additive value. D and E cells were treated with TNF-α at 1000 pg/mL and IFN-γ at 25 U/mL. CCL5 mRNA expression relative to that of GAPDH was quantified with real-time PCR; (f) effect of neutralizing anti-TNF-α or anti-IFN-γ antibodies on synergistic CCL5 release by HPFB. Cells were incubated with antibodies (all at 1 μg/mL) for 48 h. Asterisks represent a significant difference compared with cells treated with a combination of TNF-α (1000 pg/mL) and IFN-γ (25 U/mL) in the absence of antibodies ($n = 4$).

and—to lesser extent—by IL-1β (not shown). The effect was time-dependent (Figure 2(a)) and related to the dose of both IFN-γ and TNF-α (Figures 2(b) and 2(c)). Concentration of IFN-γ as low as 0.1 U/mL was capable of amplifying the effect of 1000 pg/mL TNF-α. On the other hand, 25 U/mL IFN-γ magnified the effect exerted by 10 pg/mL TNF-α more than 10-fold.

Although IFN-γ alone did not induce CCL5 mRNA, it produced a rapid (within 1 hour) synergistic increase in TNF-α-driven CCL5 expression, which persisted over 24 hours (Figure 2(d)). Quantitative assessment showed that CCL5 mRNA expression in response to a combination of TNF-α + IFN-γ was approximately 10-fold greater than that induced by TNF-α alone (Figure 2(e)).

3.3. Specificity and Timing of the Effects of IFN-γ and TNF-α on CCL5 Release by HPFB. Specificity of the combined stimulation by IFN-γ and TNF-α was verified in experiments using blocking antibodies. Neutralization of IFN-γ decreased CCL5 production to a level achieved by treatment with TNF-α alone (Figure 2(f)). In turn, anti-TNF-α antibodies totally abolished CCL5 secretion in response to TNF-α + IFN-γ. Control antibody of the same class did not affect CCL5 release. To determine whether the synergistic effect of TNF-α and IFN-γ was related to the sequence of stimuli, HPFB were incubated in the presence or absence of TNF-α or IFN-γ for 24 hours, then washed, and stimulated again for further 24 hours (Table 1). These experiments showed some degree of priming with either TNF-α or IFN-γ. However, the greatest synergy was observed when both cytokines were applied together. Interestingly, the effect of combined stimulation with TNF-α and IFN-γ for the first 24 hours still persisted during the next 24 hours, even in the absence of cytokines.

3.4. Effect of CD40 Ligand (CD40L) on CCL5 Induction in HPFB. CD40L, a member of the TNF-α family, is expressed by mononuclear cells infiltrating the peritoneum during peritonitis [18]. We have therefore examined if CD40L is able to induce CCL5 in HPFB. It turned out that CD40L had almost no effect in control cells but stimulated dose-dependent CCL5 release in HPFB pretreated with IFN-γ (Figure 3). We have then used PCR to assess the expression in HPFB of CD40, a receptor for CD40L. Unstimulated cells did not express CD40 mRNA; however its presence could be

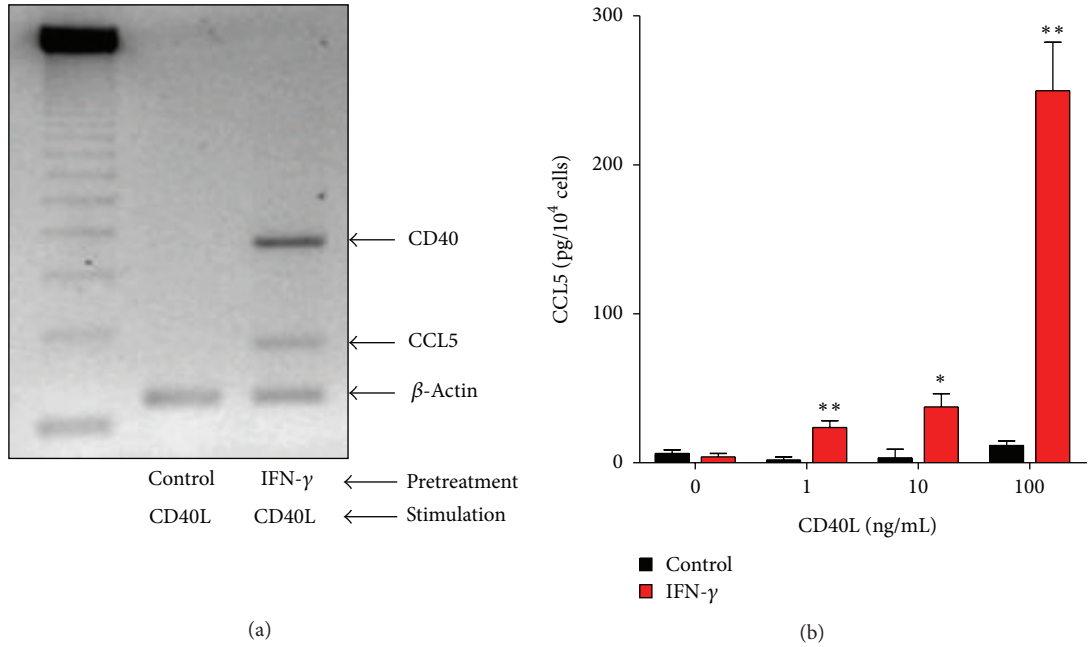

Figure 3: Effect of preexposure to IFN-γ on CD40L-induced CCL5 expression and release by HPFB. Cells were pretreated for 48 hours either with control medium or IFN-γ (100 U/mL). After that cells were stimulated with CD40L for the next 24 hours. (a) Expression of mRNA for CD40 and CCL5 was assessed by conventional RT-PCR. Results of a representative experiment of two performed. (b) CCL5 release was measured in HPFB cultures established from 5 separate donors. Asterisks represent a significant difference compared to cells not exposed to IFN-γ.

Table 1: Effect of sequential addition of TNF-α and IFN-γ on CCL5 release by HPFB.

Stimulus 1	Stimulus 2	CCL5 (pg/10^4 cells)
Medium	Medium	Undetectable
Medium	IFN-γ	Undetectable
Medium	TNF-α	4 ± 2
Medium	TNF-α + IFN-γ	24 ± 13
IFN-γ	Medium	1 ± 1
IFN-γ	TNF-α	9 ± 4
TNF-α	Medium	6 ± 4
TNF-α	IFN-γ	9 ± 7
TNF-α + IFN-γ	Medium	146 ± 20

Cells were incubated with TNF-α (1000 pg/mL) and/or IFN-γ (25 U/mL) for 24 hours (stimulus 1), washed, and incubated again for the next 24 hours in the presence or absence of these cytokines (stimulus 2). Data were derived from two independent experiments.

detected following the treatment with IFN-γ. Accordingly, subsequent stimulation with CD40L induced CCL5 mRNA expression in cells pretreated with IFN-γ.

4. Discussion

The ability of chemokines to recruit specific leukocyte subpopulations is crucial for controlling the course of inflammatory response. Thus, the regulation of chemokine production is equally important. We have shown that peritoneal fibroblasts produce significant quantities of chemokine CCL5. This observation is in keeping with the view of fibroblasts as sentinel cells providing address codes for migrating leukocytes [19]. It has previously been shown that peritoneal mesothelial cells synthesize CCL5 in response to inflammatory cytokines [16, 20, 21]. However, peritonitis may result in serious mesothelial cell damage and exfoliation [22, 23]. The function of peritoneal fibroblasts may then become essential, providing an alternative and/or additional source of chemokines. Although CCL5 production has been demonstrated in fibroblast from other locations, such as pancreas [24], skin [25, 26], gingiva [27], nasal mucosa [28, 29], and synovium [30], it is important to study the function of fibroblasts derived precisely from the tissue of interest. It is because fibroblasts display tissue-specific phenotypes that include different patterns of chemokine expression [31, 32], which may contribute to characteristic composition of leukocyte infiltrates.

Here, we show that HPFB in culture do not release CCL5 constitutively but are capable of producing this chemokine de novo in response to stimulation with proinflammatory cytokines IL-1β and TNF-α. Of those, TNF-α appears to be a more potent stimulus, which is in contrast to its effect on CXC chemokines, whose production in HPFB was found to be induced primarily by IL-1β [9]. This differential responsiveness to IL-1β and TNF-α may provide yet another level of regulation to chemokine release by HPFB.

CCL5 mediates the influx of mononuclear cells, including T cells, which are the main source of IFN–γ in the dialysed peritoneum [33]. IFN-γ can further amplify CCL5 production through synergistic induction of CCL5 mRNA. Interestingly, IFN-γ exerted this effect despite the fact that

when acting on its own, it did not stimulate CCL5. Similar results were observed in mesothelial cells [16], synovial fibroblasts [34], endothelial cells [35], and alveolar epithelial cells [36]. In contrast, in mouse macrophages IFN-γ was found to directly induce CCL5 [37]. Early induction of CCL5 gene in response to TNF-α and IFN-γ suggests that the effect is mediated by rapidly activated transcription factors that bind to CCL5 promoter. In this respect, nuclear factor κB (NF-κB) was found to be a chief mediator involved [36, 38]. It may further cooperate with interferon regulatory factors (IRF) [39, 40] and signal transducers and activators of transcription (STATs) [38].

In addition to T-cells, CCL5 attracts also eosinophils. This feature is interesting, as peritoneal eosinophilia may occur in the course of peritoneal dialysis [41] and may be related to exposure of the peritoneal membrane to foreign environment [42]. Interestingly, it has been demonstrated in an animal model of peritoneal dialysis that peritoneal eosinophilia and CCL5 elevation was particularly pronounced after exposure to dialysis fluids regarded as less biocompatible [43].

CCL5-induced leukocyte infiltrate contains T-lymphocytes that express a membrane-bound CD40L [18]. It has been demonstrated that fibroblasts from various sources express no or very little CD40 mRNA; however, it can be upregulated through IFN-γ [44]. This effect corresponds to an increase in CD40 cell surface expression [44–46]. We have found that exposure to IFN-γ increased CD40 expression in HPFB and made them responsive to CD40L. Ligation of thus induced CD40 by CD40L resulted in increased CCL5 production. Such an effect was observed previously in fibroblasts from inflamed colonic mucosa [47], but also in peritoneal mesothelial cells [45]. The underlying mechanism most likely involves NF-κB, which was shown to be activated by CD40 ligation [46]. CD40L-induced CCL5 may create positive feedback loop that further supports lymphocyte influx. In this respect, it has been shown that increased CD40L expression on peritoneal lymphocytes and macrophages supports the transition to mononuclear cell predominance in the late phase of peritonitis and timely resolution of inflammation [18].

In conclusion, our study demonstrates the great potential of peritoneal fibroblasts to generate CCL5 in response to activation by proinflammatory mediators encountered during peritonitis. By establishing a CCL5 gradient, HPFB may facilitate mononuclear leukocyte recruitment and successful resolution of inflammation. On the other hand, repeated and/or severe episodes of infection may injure the protective mesothelium and expose underlying HPFB to excessive stimulation. In those circumstances, HPFB-derived CCL5 may promote leukocyte infiltration into the peritoneal interstitium, which may lead to prolonged inflammation. In both scenarios HPFB would be actively involved in the cytokine network controlling the course of inflammation.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Edyta Kawka and Janusz Witowski contributed equally to this study.

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