Synthesis of C-X-C and C-C Chemokines by Human Peritoneal Fibroblasts

Induction by Macrophage-Derived Cytokines

Janusz Witowski,* Annette Thiel,* Ralf Dechend,[†]
Katharina Dunkel,* Nina Fouquet,*
Thorsten O. Bender,* Jan M. Langrehr,[‡]
Gerhard M. Gahl,* Ulrich Frei,* and Achim Jörres*

From the Department of Nephrology and Medical Intensive Care,*
the Department of Surgery,[‡] Campus Virchow-Klinikum, and the
Franz Volhard Klinik,[†] Campus Berlin-Buch, Medical Faculty
Charité, Humboldt-Universität zu Berlin, Berlin, Germany

Leukocyte accumulation during peritonitis is believed to be controlled by chemotactic factors released by resident peritoneal macrophages or mesothelial cells. Recent data indicate, however, that in many tissues fibroblasts play a key role in mediating leukocyte recruitment. We have therefore examined human peritoneal fibroblasts (HPFBs) for the expression and regulation of C-X-C and C-C chemokines. Quiescent HPFBs secreted monocyte chemoattractant protein (MCP)-1 and interleukin (IL)-8 constitutively. This release could be dose-dependently augmented with the pro-inflammatory cytokines IL-1 β and tumor necrosis factor- α . Stimulated IL-8 production reached a plateau within 48 hours while MCP-1 continued to accumulate throughout 96 hours. Induction of IL-8 and MCP-1 synthesis by HPFBs was also triggered by peritoneal macrophage-conditioned medium. This effect was partly related to the presence of IL-1 β as demonstrated by IL-1 receptor antagonist inhibition. Pretreatment of HPFBs with actinomycin D or puromycin dose-dependently reduced cytokine-stimulated IL-8 and MCP-1 secretion, which suggested *de novo* chemokine synthesis. Indeed, exposure of HPFBs to IL-1 β and tumor necrosis factor- α produced a significant up-regulation of IL-8 and MCP-1 mRNA. This effect was associated with the rapid induction of nuclear factor- κ B binding activity mediated through p65 and p50 subunits, and with a transient increase in the mRNA expression for RelB and inhibitory protein κ B- α proteins. These data indicate that peritoneal fibroblasts are capable of generating large quantities of chemokines under a tight control of nuclear factor- κ B/Rel transcription factors. Thus, peritoneal fibroblast-derived chemokines may contribute to the intraperitoneal recruitment of leukocytes during peritonitis. (*Am J Pathol* 2001, 158:1441–1450)

Peritonitis is the most common life-threatening pathology of the peritoneum. Its onset is accompanied by a massive leukocyte infiltration that is carefully orchestrated by a complex network of cytokines and chemotactic factors.¹ After the creation of chemotactic gradient, leukocytes migrate from blood vessels embedded in peritoneal interstitium across the monolayer of mesothelial cells into the peritoneal cavity. This chemotactic gradient is generated primarily by small polypeptide cytokines known as chemokines that display remarkable specificity toward various leukocyte subpopulations.² Chemokines are classified according to the position of cysteine residues in their amino terminal domain. Most C-X-C chemokines [eg, interleukin (IL)-8 or GRO α] act as potent chemoattractants for neutrophils whereas members of the C-C chemokine subfamily [eg, monocyte chemoattractant protein (MCP)-1 and RANTES] mediate the chemotaxis of mainly mononuclear cells.^{2–6} Accordingly, analyses of peritoneal effluent obtained during peritonitis from patients undergoing peritoneal dialysis have revealed that numbers and types of infiltrating leukocytes correlated with levels of specific chemokines.^{7–9} Increased intraperitoneal levels of chemokines have also been documented in animal models of septic peritonitis.¹⁰ Previous studies have provided ample evidence that the peritoneal mesothelium constitutes a major source of chemotactic activity in the inflamed peritoneum.^{11–14} It has been demonstrated that by secreting chemokines in a polarized manner mesothelial cells promote directed transmesothelial migration of both neutrophils and monocytes.^{15–17} On the other hand, our knowledge of how other peritoneal cell populations participate in the recruitment of inflammatory cells into the peritoneal cavity is limited. Fibroblasts, which are scattered in the submesothelial interstitium, have commonly been viewed as providing little more than a structural lattice for other cell types. The potentially important role of peritoneal fibroblasts in the intraperitoneal inflammatory response has

Supported by a grant from the Else-Kröner-Fresenius Foundation, Bad Homburg, Germany (to A. J.).

Accepted for publication December 27, 2000.

Address reprint requests to Dr. Achim Jörres, Dept. of Nephrology and Medical Intensive Care, Universitätsklinikum Charité, Campus Virchow-Klinikum, Augustenburger Platz 1, D-13353 Berlin, Germany. E-mail: achim.joerres@charite.de.

only recently begun to emerge.¹⁸ After the establishment of pure cultures of human peritoneal fibroblasts (HPFBs) in either two-dimensional¹⁹ or three-dimensional²⁰ systems, it has been demonstrated that HPFBs possess a significant biosynthetic capacity for producing cytokines and prostaglandins and are capable of responding to mitogen stimuli present in the milieu of peritonitis exudate.

Fibroblasts isolated from other tissues have been shown to generate a broad array of chemokines either constitutively or on stimulation.^{21–25} However, the degree to which these data can be extrapolated to the peritoneal fibroblast is rather limited; fibroblasts do not form a homogeneous population and marked differences in functional phenotypes have been observed between fibroblasts from different anatomical locations.^{26,27} In the present study we have therefore examined the chemokine production by cultured HPFBs and its regulation by peritoneal macrophage (PM Φ)-derived pro-inflammatory cytokines.

Materials and Methods

Materials

All chemicals, unless otherwise stated, were purchased from Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany. All tissue culture plastics were from Falcon; Becton-Dickinson GmbH, Heidelberg, Germany. Recombinant human IL-1 receptor antagonist (IL-1Ra) was kindly provided by Dr. P. Scholz (Schering AG, Berlin, Germany). All other recombinant materials were obtained from R&D Systems GmbH, Wiesbaden, Germany.

Isolation and Culture of HPFBs

HPFBs were isolated from the specimens of omentum obtained from consenting patients undergoing elective abdominal surgery. Cells were isolated and characterized as described in detail elsewhere.¹⁹ HPFBs were identified by uniform spindle-shape appearance, formation of parallel arrays and whorls at confluence, and by the uniform positive staining for vimentin. The presence of contaminating endothelial and/or mesothelial cells was excluded after negative staining for factor VIII-related antigen, cytokeratin 18, and desmin. Cells were propagated in Ham's F12 culture medium (ICN Biomedicals GmbH, Meckenheim, Germany) supplemented with L-glutamine (2 mmol/L), penicillin (100 U/ml), streptomycin (100 μ g/ml) (all from Seromed, Biochrom KG, Berlin, Germany), insulin (0.5 μ g/ml), transferrin (0.5 μ g/ml), hydrocortisone (0.4 μ g/ml), and 10% v/v fetal calf serum (Gibco BRL, Life Technologies GmbH, Eggenstein, Germany). HPFB cultures were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO₂. All experiments were performed using 1 to 3 passage cultures with cells derived from at least five separate donors.

Peritoneal Macrophage-Conditioned Medium (PM Φ -CM)

PM Φ -CM was kindly supplied by Dr. N. Topley (Institute of Nephrology, University of Wales College of Medicine, Cardiff, UK). PM Φ were harvested from peritoneal effluent drained from infection-free patients undergoing peritoneal dialysis, as previously described.²⁸ Conditioned medium was collected from adherent PM Φ cultures after a 3-hour incubation with Ham's F12 medium containing 0.1% fetal calf serum (which was necessary to maintain PM Φ baseline viability).

Induction of Chemokine Production

HPFBs were grown to confluence and then rendered quiescent by serum-deprivation for 48 hours before stimulation. Under these conditions the cells remained in a viable (as assessed by LDH release and intracellular ATP levels) and nonproliferative state (as measured by the incorporation of [³H]-thymidine).¹⁹ Quiescent HPFB cultures were then stimulated with recombinant IL-1 β and/or tumor necrosis factor (TNF)- α . In separate experiments cells were exposed to PM Φ -CM in the presence or absence IL-1Ra. In the inhibition studies HPFBs were pretreated with transcription (actinomycin D) or translation (puromycin) inhibitors for 1 or 2 hours before stimulation, respectively. Preliminary experiments revealed that concentrations of the inhibitors used did not affect the viability of HPFBs. At designated time intervals the supernates were removed, centrifuged at 12,000 $\times$ g to remove any cellular debris and stored at -70°C until assayed. The number of cells in representative HPFB monolayers was estimated using the improved Neubauer chamber.

Chemokine Measurements

Concentrations of IL-8 and MCP-1 in HPFB-derived supernates were measured with specific sandwich-type immunoassays using enzyme-linked immunosorbent assay-matched antibody pairs against IL-8 (R&D Systems) or MCP-1 (PharMingen GmbH, Hamburg, Germany). The assays were designed and performed according to the manufacturer's instructions. Sensitivity of the systems was 2.4 and 4.5 pg/ml for IL-8 and MCP-1, respectively. Intra- and interassay precision was <5% and <12%, respectively.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total cellular RNA from HPFB cultures was extracted with the RNA Isolator (Genosys Biotechnologies Ltd., Cambridge, UK) and purified according to the manufacturer's protocol. One microgram of the isolated RNA was then reverse-transcribed into cDNA with random hexamer primers, as previously described.¹⁹

PCR amplification was performed in a total volume of 50 μ l containing 36.25 μ l H₂O, 2.5 μ l sense/antisense

Table 1. Primer Sequences and PCR Conditions

Sequence	Product size	PCR cycles	Annealing temperature	Reference
β-actin F: 5'-ATCCCCCAAAGTTCACAA-3' R: 5'-CTGGGCCATTCTCCTTAG-3'	147bp	30	55°C	29
IL-8 F: 5'-TGACTTCCAAGCTGGCCGTG-3' R: 5'-TCTTCACAACCCTCTGCACC-3'	270bp	26	55°C	30
MCP-1 F: 5'-TCGCCTCCAGCATGAAAGTCT-3' R: 5'-TGGGGAAAGCTAGGGGAAAT-3'	369bp	23	55°C	31
RelB F: 5'-ACCGCCAGATTGCCATTGTGTTC-3' R: 5'-AGTGTGGGGGCCGTAGGGTCGTAG-3'	433bp	33	58°C	32
IκBα F: 5'-ACTCGTTCCTGCACTTGGCC-3' R: 5'-TGCTCACAGGCAAGGTGTAG-3'	237bp	26	60°C	33

primers (20 μ mol/L), 4 μ l dNTPs (2.5 mmol/L), 5 μ l 10 $\times$ PCR buffer with 1.5 mmol/L MgCl₂, 0.25 μ l Taq polymerase (1.25 U, AmpliTaq; Perkin Elmer), and 2 μ l of reverse transcription product. Specific oligonucleotide primer pairs were synthesized by TIB MolBiol SyntheseLabor, Berlin, Germany (Table 1). Polymerase chain reaction was performed on Perkin Elmer 480 Thermocycler (Perkin Elmer Cetus, Applied Biosystems, Weiterstadt, Germany). An initial 3-minute denaturation step was followed by 23 to 33 cycles of denaturation at 94°C for 40 seconds, annealing at 55 to 60°C for 1 minute, and extension at 72°C for 1 minute. The final cycle was 94°C for 40 seconds and 60°C for 10 minutes. Preliminary experiments determined the number of cycles so that PCR products were generated during the exponential phase of amplification. PCR products were then separated by electrophoresis on ethidium bromide-stained 3% agarose gels (FMC Bioproducts; Biozym Diagnostic GmbH, Hess Oldendorf Germany) and visualized under UV illumination. Expression of target mRNAs was assessed by comparison with the expression of the housekeeping gene of β -actin in the same sample. The bands corresponding to the intended products were analyzed using Scanpack 14.1A27 software (Biometra, Göttingen, Germany).

Electrophoretic Mobility Shift Assay

For electrophoretic mobility shift assay cells were harvested at different time points after stimulation with IL-1 β (1,000 pg/ml) and then lysed in whole-cell lysate buffer, containing 20 mmol/L Hepes (pH 7.9), 350 mmol/L NaCl, 20% glycerol, 1 mmol/L MgCl₂, 0.5 mmol/L ethylenediaminetetraacetic acid, 0.1 mmol/L EGTA, 1% Nonidet P-40, and a mixture of protease inhibitors.^{34,35} Labeling and binding reactions were performed essentially as described previously.³⁶ The DNA probe containing the κ B site from the major histocompatibility complex-enhancer (H2K) was end-labeled with ³²P-dATP. Protein fractions and the probe were incubated for 30 minutes at 30°C in 20 μ l of reaction buffer containing 2 μ g poly (dl-dC), 1 μ g bovine serum albumin, 1 mmol/L dithiothreitol, 20 mmol/L

Hepes (pH 8.4), 60 mmol/L KCl, and 8% Ficoll. In antibody supershift experiments 1 μ l of the antisera against relevant nuclear factor (NF)- κ B proteins (Santa Cruz Biotechnology, Heidelberg, Germany) were added. The DNA-protein complexes were analyzed on 5% polyacrylamide/Tris borate-ethylenediaminetetraacetic acid gels. All electrophoretic mobility shift assay experiments were performed in triplicates.

Statistical Analysis

Statistical analysis was performed using nonparametric tests for paired data (GraphPad Prism 3.00; GraphPad Software Inc., San Diego, CA). Repeated measures analysis of variance with Friedman modification or Wilcoxon signed rank test were used when appropriate. A *P* value of less than 0.05 was considered as significant. All data are presented as mean ($\pm$ SEM).

Results

Constitutive Chemokine Secretion by HPFB

All HPFB cultures examined (*n* = 26) released MCP-1 and IL-8 spontaneously. After a 48-hour incubation under serum-free conditions and in the absence of any additional stimulation the levels of these chemokines ranged from 18 to 620 pg/10⁴ cells (median value, 118) for MCP-1 and from 10 to 170 pg/10⁴ cells (median value, 55) for IL-8. There was a significant correlation between the concentrations of MCP-1 and IL-8 released (Spearman's *r* = 0.846, *P* < 0.001).

Induction of MCP-1 and IL-8 Production in HPFB by Recombinant Cytokines

Stimulation of HPFBs with either IL-1 β or TNF- α resulted in a time-dependent generation of IL-8 and MCP-1 (Figure 1). The release of IL-8 in response to IL-1 β (1,000 pg/ml) was significantly greater than background levels

Figure 1. Time course of chemokine secretion by HPFB. Quiescent HPFB were stimulated with either IL-1 β or TNF- α (both at 1,000 pg/ml). Data represent a cumulative release of IL-8 (**left**) and MCP-1 (**right**). Data are expressed as the mean ($\pm$ SEM) from seven experiments with cells isolated from separate donors. Asterisks represent a statistically significant difference compared to the unstimulated controls at the same time point.

after 6 hours of incubation and reached plateau within 48 hours. The IL-1 β -induced MCP-1 release also became significantly elevated by 6 hours, however, the accumulation of MCP-1 continued to rise throughout the whole time course studied. Analysis of the chemokine secretion rate (calculated by dividing the net chemokine release during each experimental period by the number of hours of the respective time interval) revealed that the most pronounced IL-1 β -driven IL-8 release occurred within the first 12 to 24 hours and then declined toward basal values. In contrast, the MCP-1 secretion rate remained elevated throughout the whole period of 96 hours. The time course of IL-8 and MCP-1 generation in response to TNF- α (1,000 pg/ml) followed a similar pattern (Figure 1).

The magnitude of cytokine-stimulated IL-8 and MCP-1 release was also dose-dependent. Significant increase in chemokine secretion was achieved with IL-1 β at 10 pg/ml and above and with TNF- α at 100 pg/ml and above. Interestingly, although the absolute concentrations of MCP-1 released in response to all doses of IL-1 β or TNF- α tested were higher than those of IL-8, the fold increase above control levels was more prominent for IL-8 than for MCP-1 (eg, IL-1 β at 10 ng/ml triggered a 41 ± 6 -fold increase in IL-8 release compared to 13 ± 2 -fold increase in MCP-1 secretion).

Exposure of HPFB to a combination of IL-1 β together with TNF- α resulted in an additive release of IL-8 and MCP-1. This additive effect was evident for IL-1 β and TNF- α combined at the range of either low (1 to 25 pg/ml; data not shown) or higher doses (100 to 1,000 pg/ml; Figure 2).

Induction of MCP-1 and IL-8 by PM Φ -Conditioned Medium

After a 3-hour incubation the mean concentrations of IL-8 and MCP-1 in undiluted conditioned medium pooled from PM Φ preparations ($n = 6$) were 913 and 41 pg/ml, respectively. When HPFBs were incubated in the presence of PM Φ -CM these values were subtracted from those detected in HPFB supernates to estimate the specific release of chemokines by HPFB. The concentration of

IL-1 β in PM Φ -CM was 448 pg/ml, as measured by enzyme-linked immunosorbent assay (R&D Systems).

Exposure of HPFB to PM Φ -CM resulted in time- and dose-dependent stimulation of both IL-8 and MCP-1 secretion. At the optimal dilution (1:4), PM Φ -CM triggered the release of HPFB-derived IL-8 and MCP-1 that was, respectively, 50 ± 12 and 24 ± 4 -fold greater than control levels within 24 hours ($n = 6$, $P < 0.05$ for both). Incubation of HPFBs with PM Φ -CM in the presence of IL-1ra (100 ng/ml) significantly reduced the capacity of PM Φ -CM to stimulate both IL-8 and MCP-1 release (Figure 3). The HPFB secretion of IL-8 was diminished by $31 \pm 19\%$ and of MCP-1 by $39 \pm 12\%$ ($n = 6$, $P < 0.05$ for both).

Figure 2. Effect of combined IL-1 β and TNF- α stimulation on IL-8 and MCP-1 release by HPFB. Quiescent cells were exposed to either control medium (**open bar**) or a combination of IL-1 β and TNF- α (both at 1,000 pg/ml; **filled bar**) for 48 hours. Composite bars represent calculated additive values for IL-1 β (**hatched bars**) and TNF- α (**gray bars**) at the same dose. Data were obtained from six experiments performed with cells from separate donors.

Figure 3. Effect of PMΦ-CM on HPFB IL-8 and MCP-1 secretion. Chemokine generation was measured after a 48-hour exposure of HPFB to PMΦ-CM in the presence (open square) or absence (filled square) of IL-1ra (100 ng/ml). Data were derived from six separate experiments performed with cells from different donors. Asterisks represents a statistically significant difference compared to the control.

Effect of Transcription and Translation Inhibitors

Pre-exposure of HPFBs to actinomycin D for 60 minutes at 37°C resulted in a dose-dependent inhibition of cytokine-driven but not of constitutive IL-8 and MCP-1 release (Figure 4). Maximal inhibition was obtained with a dose of 10 μ g/ml when IL-1 β -stimulated generation of IL-8 was reduced from 725 $\pm$ 209 to 225 $\pm$ 88 pg/10⁴ and MCP-1 levels were reduced from 5629 $\pm$ 1450 to 671 $\pm$ 191 pg/10⁴ ($n = 6$, $P < 0.001$ for both). Inhibition of TNF- α -stimulated chemokine synthesis was of a comparable magnitude (data not shown).

Pretreatment of HPFBs with puromycin decreased the cytokine-stimulated production of both IL-8 and MCP-1 (Figure 4). In addition, puromycin was capable of reduc-

ing constitutive MCP-1 but not IL-8 release. At the highest dose of puromycin tested (25 μ g/ml) the basal secretion of MCP-1 was reduced by 95.0 $\pm$ 3.9% ($n = 5$, $P < 0.05$).

PCR Analysis of Chemokine Gene Expression in HPFB

HPFBs constitutively expressed low basal levels of mRNA transcripts for IL-8 and MCP-1. Exposure of HPFB to IL-1 β or TNF- α (both at 1,000 pg/ml) resulted in a transient increase in the expression of both chemokines as assessed by comparison with the expression of the housekeeping gene β -actin. IL-8 mRNA was rapidly up-regulated (within 1 hour), and maximal increase in MCP-1

Figure 4. Effect of actinomycin D and puromycin on cytokine-induced IL-8 and MCP-1 release from HPFB. Cells were pretreated with increasing doses of either actinomycin D (left) for 60 minutes or puromycin (right) for 120 minutes and then stimulated with IL-1 β (1,000 pg/ml) for 48 hours. The data were obtained from six experiments with cells isolated from different donors, and were expressed as a percentage of IL-8 (open square) or MCP-1 (filled square) release in cells not exposed to the inhibitors. Asterisks represent significant differences *versus* respective controls.

Figure 5. Time course of IL-8 and MCP-1 mRNA expression in HPFB. After timed exposure of HPFB to control medium or IL-1 β and TNF- α (both at 1,000 pg/ml) total RNA was extracted and analyzed by semiquantitative RT-PCR as described in Materials and Methods. The figure shows the results of a representative experiment of two performed with PCR products separated on an ethidium bromide-stained agarose gel.

mRNA expression was observed 3 hours after the stimulation (Figure 5). The effect triggered by IL-1 β and TNF- α also seemed to be dose-related; after 2 hours increased expression of chemokine mRNA transcripts was recorded in response to IL-1 β or TNF- α at concentrations as low as 1 pg/ml (data not shown).

Activation of NF- κ B in HPFB

Gel retardation assay revealed that stimulation of HPFBs with IL-1 β (1,000 pg/ml) resulted in a rapid increase in binding of cell extract proteins to the specific NF- κ B-binding site (Figure 6). Enhanced DNA-binding activity was clearly observed within 5 minutes, reached maximum after 45 minutes, and after 90 minutes was already reduced. Specificity of NF- κ B-binding was confirmed by competitive inhibition by unlabeled oligonucleotides (Figure 6). The nature of proteins responsible for the NF- κ B-activity was further characterized by supershift assays. These experiments demonstrated that the addition of antibodies to p65/RelA and p50, but not to RelB, had the effect of hampering the migration of DNA-protein complexes, resulting in a clear supershift (Figure 6).

Kinetics of RelB and I κ B α mRNA Expression in HPFB

Stimulation of HPFBs with IL-1 β led to substantial but transient increases in the mRNA expression for I κ B α (Figure 7A) and RelB (Figure 7B). Elevated mRNA levels were recorded within 1 hour, peaked at 3 hours, and then returned to basal levels within 12 hours.

Discussion

Research and clinical observations in recent years have clearly documented the key role of PM Φ and the peritoneal mesothelium in maintaining intraperitoneal microenvironment and controlling peritoneal host defense.^{37–39} In contrast, peritoneal fibroblasts received little attention as a cell population primarily involved in the inflammatory response and as such were strikingly absent from recent reviews on pathobiology of peritonitis and peritoneal immune system.^{40,41} However, increasing experimental evidence suggests that in many tissues the function of interstitial fibroblasts may be of paramount importance

Figure 6. IL-1 β -induced NF- κ B activity in HPFB. Quiescent cells were stimulated with IL-1 β (1000 pg/ml). Nuclear extracts were obtained at designated time intervals and analyzed with electrophoretic mobility shift assay for specific κ B-binding activity. The figure illustrates time course of NF- κ B activation (**left**) and characterization of NF- κ B complexes in extracts obtained 45 minutes after stimulation (**right**).

Figure 7. Time course of $\text{IkB}\alpha$ and RelB induction in HPFB. Quiescent cells were stimulated with IL-1 β (1,000 pg/ml) for the time periods indicated. Expression of mRNA for $\text{IkB}\alpha$ (A) and RelB (B) was analyzed by RT-PCR as described in Materials and Methods.

for initiating and regulating infiltration of immune cells during inflammatory reactions.⁴² Our data indicate that also in the peritoneum fibroblasts may provide signals for the intraperitoneal recruitment of inflammatory bone marrow-derived cells.

Initiation of peritonitis has been associated with increased levels of the potent proinflammatory mediators IL-1 β and TNF- α in the peritoneum.^{43–45} Stimulation of quiescent HPFBs with these cytokines led to a significant time- and dose-dependent increase in IL-8 secretion. Comparison of the amount of IL-8 (on the pg/ μ g cell protein basis) produced under similar *in vitro* conditions by peritoneal fibroblasts and peritoneal mesothelial cells¹³ indicated that HPFB was at least as potent in IL-8 generation as a mesothelial cell. This release of IL-8 from HPFB could be inhibited in a dose-dependent manner after the pretreatment of HPFBs with both transcription and translation inhibitors that suggested that at least part of IL-8 secreted was the product of *de novo* protein synthesis. PCR amplification of reverse-transcribed total HPFB RNA with specific IL-8 primers confirmed that exposure to IL-1 β and TNF- α resulted in a rapid and transient up-regulation of IL-8 mRNA. Secretion of IL-8 by HPFBs could be further augmented by simultaneous addition of IL-1 β together with TNF- α . The rise in IL-8 secretion recorded under these conditions approximated the sum of releases triggered by the two cytokines alone. Interestingly, Topley and colleagues¹³ have demon-

strated that in peritoneal mesothelial cells stimulated with a combination of IL-1 β and TNF- α the release of IL-8 was significantly greater than the predicted additive value. This observation may suggest that in HPFB the mechanisms modulating signal transduction from IL-1 β and TNF- α receptors are different from those operating in mesothelial cells.

Cytokine stimulation of HPFBs was also capable of inducing significant MCP-1 expression and release. The amount of MCP-1 secreted by HPFBs in response to IL-1 β and TNF- α was comparable to that recorded in mesothelial cells.¹¹ Similarly to the induction of IL-8 this effect was inhibited by actinomycin D and puromycin and seemed to be additive when IL-1 β and TNF- α were applied in combination. The rate of MCP-1 secretion was, however, different such that MCP-1 release continued to increase throughout the whole time course of a 96-hour period studied. In contrast, induction of IL-8 was more transient with IL-8 levels reaching plateau within 48 hours. Likewise, in HPFB treated with IL-1 β the MCP-1/IL-8 concentration ratio rose from 1.6:1 after 12 hours to 4.2:1 after 96 hours. Thus, at the onset of peritonitis HPFBs may provide relatively more signals triggering the intraperitoneal influx of neutrophils while subsequently the secretion of HPFB-derived chemokines gradually shifts toward mononuclear cell chemoattractants. This finding is in line with observations on the dynamics of leukocyte subpopulations infiltrating the peritoneum during peritoneal dialysis-associated peritonitis^{1,46,47} where IL-8 and MCP-1 were identified as being the major chemokines responsible for the influx of neutrophils and monocytes, respectively.^{7–9} Furthermore, Lu and colleagues⁴⁸ have demonstrated that on appropriate stimulation MCP-1-deficient animals were not able to recruit monocytes and macrophages to the peritoneum.

Increasing evidence suggests that the course and outcome of the inflammatory response may primarily depend on the interaction between infiltrating immune cells and structural cells such as fibroblasts.⁴⁹ We found that peritoneal fibroblasts responded to macrophage-derived stimuli by increasing chemokine production. It has previously been demonstrated that the potential of PM Φ -CM to trigger cytokine and prostaglandin synthesis in mesothelial cells was partly related to the presence of IL-1 β .^{14,50} Indeed, we found that PM Φ -driven HPFB chemokine production could be reduced by the simultaneous administration of IL-1Ra. Moreover, recent data indicate that in interactions between these two cell populations fibroblasts play a more active role than previously imagined. Steinhauser and colleagues⁵¹ have recently shown that the generation of MIP-1 α chemokine by macrophages depended critically on the direct contact with fibroblasts maintained in the co-culture system. These observations suggest the possibility that activation of PM Φ in the peritoneal interstitium and interaction with HPFB may serve to amplify the chemotactic gradient during acute phase of peritonitis.

The transcriptional control of MCP-1 and IL-8 genes is believed to be mediated via NF- κ B/Rel proteins.^{52–54} These proteins form homo- or heterodimer complexes that bind DNA in a sequence-specific manner. The iden-

tified NF- κ B subunits include p65/RelA, p50, p52, RelB, and c-Rel.^{55,56} We found that stimulation of HPFB with IL-1 β rapidly activated a NF- κ B complex, which in supershift experiments could be further defined as being composed of p65 and p50 subunits. This is a classical association of NF- κ B proteins that seems to be particularly important in regulating the expression of genes during the inflammatory response.⁵⁵ In the absence of stimulation the NF- κ B (p50/p65) is sequestered in the cytoplasm through binding to the inhibitory protein κ B- α (I κ B α) that masks a nuclear localization signal in the NF- κ B sequence. Stimulation with pro-inflammatory mediators leads to a multistep I κ B α degradation that allows nuclear translocation of NF- κ B and its binding to target DNA motifs. These include the promoter of I κ B α and as a result I κ B α is rapidly resynthesized creating a feedback loop limiting prolonged activation of NF- κ B.^{55–57} Indeed, we observed the inducible expression of I κ B α mRNA in HPFB in response to IL-1 β that activated NF- κ B. Furthermore, the up-regulation of I κ B α mRNA correlated with a decrease in NF- κ B binding activity. However, the I κ B α pathway is probably not the only mechanism controlling NF- κ B activity and it does not seem to operate in all cell types.^{55,58} Recent data from Xia and colleagues²³ indicate that generation of chemokines in fibroblasts, but not in macrophages, may be specifically regulated by the RelB transcription factor that seems to suppress NF- κ B activity. Activation of normal fibroblast with LPS led to a transient increase in chemokine production and parallel induction of RelB expression. RelB^{-/-} fibroblasts displayed spectacular overproduction of chemokines (including murine equivalents of MCP-1 and IL-8) and *in vivo* RelB-deficient animals suffered from multifocal inflammation with massive interstitial infiltration by neutrophils and mononuclear cells.²³ We found that in peritoneal fibroblasts the expression of RelB rapidly increased after the stimulation with IL-1 β , however, the RelB-containing complexes did not seem to contribute to NF- κ B activity as demonstrated by supershift assays. A similar effect was also observed in murine kidney fibroblasts. Xia and colleagues²³ demonstrated that, although RelB was translocated to the nucleus after the stimulation, it did not directly participate in the NF- κ B binding. These observations indicate that in fibroblasts RelB could execute its inhibitory potential through a new, not yet identified, mechanism.

Under normal conditions the peritoneal cavity contains a small volume of serous fluid and a low number of resident leukocytes with macrophages forming ~90% of the population.⁵⁹ A significant proportion of macrophages has also been located in the submesothelial interstitium.⁶⁰ As peritoneal fibroblasts constitutively generated considerable amounts of MCP-1, one may hypothesize that the establishment of these resident macrophages in the peritoneum is mediated, at least partially, by peritoneal fibroblast-derived MCP-1. In this respect Koyama and colleagues²² have demonstrated that human pulmonary fibroblasts constitutively secrete MCP-1 at concentrations high enough to facilitate monocyte influx into the lung. On the other hand, although MCP-1 seems to be critical for intraperitoneal monocyte trafficking during the inflammatory response, it has been

suggested that the presence of resident macrophages in the peritoneum is independent of MCP-1.⁴⁸ The yield of PM Φ from unchallenged MCP-1-deficient mice did not differ from that in wild-type animals. However, even if MCP-1 is not responsible for basal monocyte recruitment in healthy humans, its function may still be important in patients treated with peritoneal dialysis for kidney insufficiency. The procedure produces constant loss of macrophages with the drained dialysate and is associated with a small but continuous migration of new mononuclear cell populations into the peritoneum.¹ As fibroblasts are uniquely positioned in the peritoneal interstitium between the vascular compartment and the peritoneal cavity, HPFB-derived MCP-1 may well be involved in monocyte recruitment under these conditions. In this respect, the presence of MCP-1 has been documented in peritoneal effluents from infection-free peritoneal dialysis patients.^{7,52} In addition, Tekstra and colleagues⁷ have shown that monocyte migration toward these dialysates during *in vitro* chemotaxis assay could be blocked with anti-MCP-1 antibodies.

Taken together our data indicate that although peritoneal fibroblasts constitute a small fraction of the peritoneal tissue mass, they may play a very special role in the recruitment of immune cells into the peritoneum. These observations also support the concept of fibroblasts as sentinel cells that combine structural and immunomodulatory function.⁴²

References

1. Topley N, Liberek T, Davenport A, Li FK, Fear H, Williams JD: Activation of inflammation and leukocyte recruitment into the peritoneal cavity. *Kidney Int* 1996, 56(Suppl):S17–S21
2. Rollins BJ: Chemokines. *Blood* 1997, 90:909–928
3. Schlondorff D, Nelson PJ, Luckow B, Banas B: Chemokines and renal disease. *Kidney Int* 1997, 51:610–621
4. Baggiolini M, Dewald B, Moser B: Human chemokines: an update. *Annu Rev Immunol* 1997, 15:675–705
5. Ward SG, Westwick J: Chemokines: understanding their role in T-lymphocyte biology. *Biochem J* 1998, 333:457–470
6. Luster AD: Chemokines—chemotactic cytokines that mediate inflammation. *N Engl J Med* 1998, 338:436–445
7. Tekstra J, Visser CE, Tuk CW, Brouwer-Steenbergen JJ, Burger CW, Krediet RT, Beelen RH: Identification of the major chemokines that regulate cell influxes in peritoneal dialysis patients. *J Am Soc Nephrol* 1996, 7:2379–2384
8. Betjes MG, Visser CE, Zemel D, Tuk CW, Struijk DG, Krediet RT, Arisz L, Beelen RH: Intraperitoneal interleukin-8 and neutrophil influx in the initial phase of a CAPD peritonitis. *Perit Dial Int* 1996, 16:385–392
9. Zemel D, Krediet RT, Koomen GCM, Kortekaas WMR, Geertzen HGM, ten Berge RJM: Interleukin-8 during peritonitis in patients treated with CAPD: an in-vivo model of acute inflammation. *Nephrol Dial Transplant* 1994, 9:169–174
10. Walley KR, Lukacs NW, Standiford TJ, Strieter RM, Kunkel SL: Elevated levels of macrophage inflammatory protein 2 in severe murine peritonitis increase neutrophil recruitment and mortality. *Infect Immun* 1997, 65:3847–3851
11. Visser CE, Tekstra J, Brouwer Steenbergen JJ, Tuk CW, Boorsma DM, Sampa Sardjoepersad SC, Meijer S, Krediet RT, Beelen RH: Chemokines produced by mesothelial cells: huGRO- α , IP-10, MCP-1 and RANTES. *Clin Exp Immunol* 1998, 112:270–275
12. Visser CE, Steenbergen JJ, Betjes MG, Meijer S, Arisz L, Hoefsmit EC, Krediet RT, Beelen RH: Interleukin-8 production by human mesothelial cells after direct stimulation with staphylococci. *Infect Immun* 1995, 63:4206–4209

13. Topley N, Brown Z, Jörres A, Westwick J, Davies M, Coles GA, Williams JD: Human peritoneal mesothelial cells synthesize interleukin-8. Synergistic induction by interleukin-1 beta and tumor necrosis factor-alpha. *Am J Pathol* 1993, 142:1876-1886
14. Betjes MG, Tuk CW, Struijk DG, Krediet RT, Arisz L, Hart M, Beelen RH: Interleukin-8 production by human peritoneal mesothelial cells in response to tumor necrosis factor-alpha, interleukin-1, and medium conditioned by macrophages cocultured with *Staphylococcus epidermidis*. *J Infect Dis* 1993, 168:1202-1210
15. Zeilemaker AM, Mul FP, Hoyneck van Papendrecht AA, Kuijpers TW, Roos D, Leguit P, Verbrugh HA: Polarized secretion of interleukin-8 by human mesothelial cells: a role in neutrophil migration. *Immunology* 1995, 84:227-232
16. Zeilemaker AM, Mul FP, Hoyneck van Papendrecht AA, Leguit P, Verbrugh HA, Roos D: Neutrophil adherence to and migration across monolayers of human peritoneal mesothelial cells. The role of mesothelium in the influx of neutrophils during peritonitis. *J Lab Clin Med* 1996, 127:279-286
17. Li FK, Davenport A, Robson RL, Loetscher P, Rothlein R, Williams JD, Topley N: Leukocyte migration across human peritoneal mesothelial cells is dependent on directed chemokine secretion and ICAM-1 expression. *Kidney Int* 1998, 54:2170-2183
18. Jörres A, Ludat K, Sander K, Dunkel K, Lorenz F, Keck H, Frei U, Gahl GM: The peritoneal fibroblast and the control of peritoneal inflammation. *Kidney Int* 1996, 56(Suppl):S22-S27
19. Jörres A, Ludat K, Lang J, Sander K, Gahl GM, Frei U, DeJonge K, Williams JD, Topley N: Establishment and functional characterization of human peritoneal fibroblasts in culture: regulation of interleukin-6 production by proinflammatory cytokines. *J Am Soc Nephrol* 1996, 7:2192-2201
20. Beavis MJ, Williams JD, Hoppe J, Topley N: Human peritoneal fibroblast proliferation in 3-dimensional culture: modulation by cytokines, growth factors and peritoneal dialysis effluent. *Kidney Int* 1997, 51:205-215
21. Sempowski GD, Rozenblit J, Smith TJ, Phipps RP: Human orbital fibroblasts are activated through CD40 to induce proinflammatory cytokine production. *Am J Physiol* 1998, 274:C707-C714
22. Koyama S, Sato E, Masubuchi T, Takamizawa A, Nomura H, Kubo K, Nagai S, Izumi T: Human lung fibroblasts release chemokine activity for monocytes constitutively. *Am J Physiol* 1998, 275:L223-L228
23. Xia Y, Pauza ME, Feng L, Lo D: RelB regulation of chemokine expression modulates local inflammation. *Am J Pathol* 1997, 151:375-387
24. Yoshimura T, Leonard EJ: Secretion by human fibroblasts of monocyte chemoattractant protein-1, the product of gene JE. *J Immunol* 1990, 144:2377-2383
25. Elner VM, Burnstine MA, Kunkel SL, Strieter RM, Elner SG: Interleukin-8 and monocyte chemoattractant protein-1 gene expression and protein production by human orbital fibroblasts. *Ophthalmol Plast Reconstr Surg* 1998, 14:119-125
26. Smith TJ, Higgins PJ: Interferon gamma regulation of de novo protein synthesis in human dermal fibroblasts in culture is anatomic site dependent. *J Invest Dermatol* 1993, 100:288-292
27. Alvarez RJ, Sun MJ, Haverty TP, Iozzo RV, Myers JC, Neilson EG: Biosynthetic and proliferative characteristics of tubulointerstitial fibroblasts probed with paracrine cytokines. *Kidney Int* 1992, 41:14-23
28. Mackenzie RK, Coles GA, Williams JD: Eicosanoid synthesis in human peritoneal macrophages stimulated with *S. epidermidis*. *Kidney Int* 1990, 37:1316-1324
29. Ponte P, Ng SY, Engel J, Gunning P, Kedes L: Evolutionary conservation in the untranslated regions of actin mRNAs: DNA sequence of a human beta-actin cDNA. *Nucleic Acids Res* 1984, 12:1687-1696
30. Matsushima K, Morishita K, Yoshimura T, Lavu S, Kobayashi Y, Lew W, Appella E, Kung HF, Leonard EJ, Oppenheim JJ: Molecular cloning of a human monocyte-derived neutrophil chemotactic factor (MDNCF) and the induction of MDNCF mRNA by interleukin 1 and tumor necrosis factor. *J Exp Med* 1988, 167:1883-1893
31. Yoshimura T, Yuhki N, Moore SK, Appella E, Lerman MI, Leonard EJ: Human monocyte chemoattractant protein-1 (MCP-1). Full-length cDNA cloning, expression in mitogen-stimulated blood mononuclear leukocytes, and sequence similarity to mouse competence gene JE. *FEBS Lett* 1989, 244:487-493
32. Ruben SM, Klement JF, Coleman TA, Maher M, Chen CH, Rosen CA: I-Rel: a novel rel-related protein that inhibits NF-kappa B transcriptional activity. *Genes Dev* 1992, 6:745-760
33. Emmerich F, Meiser M, Hummel M, Demel G, Foss HD, Jundt F, Mathas S, Krappmann D, Scheidereit C, Stein H, Dörken B: Overexpression of I kappa B alpha without inhibition of NF-kappaB activity and mutations in the I kappa B alpha gene in Reed-Sternberg cells. *Blood* 1999, 94:3129-3134
34. Beraud C, Henzel WJ, Baeuerle PA: Involvement of regulatory and catalytic subunits of phosphoinositide 3-kinase in NF-kappaB activation. *Proc Natl Acad Sci USA* 1999, 96:429-434
35. Dechend R, Hirano F, Lehmann K, Heissmeyer V, Ansieau S, Wulczyn FG, Scheidereit C, Leutz A: The Bcl-3 oncoprotein acts as a bridging factor between NF-kappaB/Rel and nuclear co-regulators. *Oncogene* 1999, 18:3316-3323
36. Dechend R, Maass M, Gieffers J, Dietz R, Scheidereit C, Leutz A, Gulba DC: Chlamydia pneumoniae infection of vascular smooth muscle and endothelial cells activates NF-kappaB and induces tissue factor and PAI-1 expression: a potential link to accelerated arteriosclerosis. *Circulation* 1999, 100:1369-1373
37. Topley N, Mackenzie RK, Williams JD: Macrophages and mesothelial cells in bacterial peritonitis. *Immunobiology* 1996, 195:563-573
38. Topley N, Williams JD: Role of the peritoneal membrane in the control of inflammation in the peritoneal cavity. *Kidney Int* 1994, 48(Suppl):S71-S78
39. Holmes CJ: Peritoneal host defense mechanisms in peritoneal dialysis. *Kidney Int* 1994, 48(Suppl):S58-S70
40. Hall JC, Heel KA, Papadimitriou JM, Platell C: The pathobiology of peritonitis. *Gastroenterology* 1998, 114:185-196
41. Rapoport J, Hausmann MJ, Chaimovitz C: The peritoneal immune system and continuous ambulatory peritoneal dialysis. *Nephron* 1999, 81:373-380
42. Smith RS, Smith TJ, Bliden TM, Phipps RP: Fibroblasts as sentinel cells. Synthesis of chemokines and regulation of inflammation. *Am J Pathol* 1997, 151:317-322
43. Brauner A, Hylander B, Wretling B: Tumor necrosis factor-alpha, interleukin-1 beta, and interleukin-1 receptor antagonist in dialysate and serum from patients on continuous ambulatory peritoneal dialysis. *Am J Kidney Dis* 1996, 27:402-408
44. Moutabarrik A, Nakanishi I, Namiki M, Tsubakihara Y: Interleukin-1 and its naturally occurring antagonist in peritoneal dialysis patients. *Clin Nephrol* 1995, 43:243-248
45. Zemel D, Imholz AL, de Waart DR, Dinkla C, Struijk DG, Krediet RT: Appearance of tumor necrosis factor-alpha and soluble TNF-receptors I and II in peritoneal effluent of CAPD. *Kidney Int* 1994, 46:1422-1430
46. Brauner A, Hylander B, Wretling B: Interleukin-6 and interleukin-8 in dialysate and serum from patients on continuous ambulatory peritoneal dialysis. *Am J Kidney Dis* 1993, 22:430-435
47. Ho-Dac-Pannekeet MM, Krediet RT: Inflammatory changes in vivo during CAPD: what can the effluent tell us? *Kidney Int Suppl* 1996, 56:S12-S16
48. Lu B, Rutledge BJ, Gu L, Fiorillo J, Lukacs NW, Kunkel SL, North R, Gerard C, Rollins BJ: Abnormalities in monocyte recruitment and cytokine expression in monocyte chemoattractant protein 1-deficient mice. *J Exp Med* 1998, 187:601-608
49. Smith RE, Hogaboam CM, Strieter RM, Lukacs NW, Kunkel SL: Cell-to-cell and cell-to-matrix interactions mediate chemokine expression: an important component of the inflammatory lesion. *J Leukoc Biol* 1997, 62:612-619
50. Topley N, Petersen MM, Mackenzie RK, Neubauer A, Stylianou E, Kaever V, Davies M, Coles GA, Jörres A, Williams JD: Human peritoneal mesothelial cell prostaglandin synthesis: induction of cyclooxygenase mRNA by peritoneal macrophage-derived cytokines. *Kidney Int* 1994, 46:900-909
51. Steinhauser ML, Kunkel SL, Hogaboam CM, Evanoff H, Strieter RM, Lukacs NW: Macrophage/fibroblast coculture induces macrophage inflammatory protein-1alpha production mediated by intercellular adhesion molecule-1 and oxygen radicals. *J Leukoc Biol* 1998, 64:636-641
52. Roebuck KA: Regulation of interleukin-8 gene expression. *J Interferon Cytokine Res* 1999, 19:429-438
53. Ueda A, Ishigatsubo Y, Okubo T, Yoshimura T: Transcriptional regulation of the human monocyte chemoattractant protein-1 gene. Cooperation of two NF-kappaB sites and NF-kappaB/Rel subunit specificity. *J Biol Chem* 1997, 272:31092-31099

54. Blackwell TS, Christman JW: The role of nuclear factor-kappa B in cytokine gene regulation. *Am J Respir Cell Mol Biol* 1997, 17:3–9
55. Auwardt RB, Mudge SJ, Power DA: Transcription factor NF-kappaB in glomerulonephritis. *Nephrology* 2000, 5:71–82
56. Ghosh S, May MJ, Kopp EB: NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. *Annu Rev Immunol* 1998, 16:225–260
57. Baeuerle PA: IkappaB-NF-kappaB structures: at the interface of inflammation control. *Cell* 1998, 95:729–731
58. Auwardt RB, Mudge SJ, Chen C, Power DA: Inhibition with antisense oligonucleotide suggests that IkappaB-alpha does not form a negative autoregulatory loop for NF-kappaB in mesangial cells. *Exp Nephrol* 2000, 8:144–151
59. Brulez HF, Verbrugh HA: First-line defense mechanisms in the peritoneal cavity during peritoneal dialysis. *Perit Dial Int* 1995, 15:S24–S33
60. Suassuna JH, Das Neves FC, Hartley RB, Ogg CS, Cameron JS: Immunohistochemical studies of the peritoneal membrane and infiltrating cells in normal subjects and in patients on CAPD. *Kidney Int* 1994, 46:443–454