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Human Peritoneal Mesothelial Cells Synthesize Interleukin-8

Synergistic Induction by Interleukin-1β and Tumor Necrosis Factor-α

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The present study demonstrates the synthesis and secretion of the neutrophil-activating peptide/interleukin-8 (IL-8) by cultured human peritoneal mesothelial cells (HPMC) and examines the regulation of its production by other cytokines. Unstimulated HPMC under growth-arrested conditions released IL-8 in a constitutive and time-dependent manner. Stimulation of HPMC with IL-1β or TNF-α resulted in a time- and dose-dependent IL-8 generation; after 24 hours the levels induced by IL-1β and TNF-α (both at 1000 pg/ml) were (mean ± SEM, n = 5) 101 ± 26.6 (z = 2.023; P < 0.01) and 35 ± 8.09 (z = 2.023; P < 0.01) respectively. This release was inhibited following coincubation with the relevant anti-cytokine antibody or preincubation with either cycloheximide or actinomycin D. Treatment of HPMC with IL-1β or TNF-α resulted in increased levels of IL-8-specific mRNA. Stimulation of HPMC with combinations of IL-1β and TNF-α resulted in a synergistic increase in IL-8 release. This effect was significant at combined doses of IL-1β (50 pg/ml) and TNF-α (500 pg/ml) and above, when the release of IL-8 was 88 ± 27% above the additive IL-8 release values (z = 2.201; P < 0.01). Western blot analysis using specific anti-IL-8 antibody demonstrated the presence of two major immunoreactive bands between 9 and 10 kd, in HPMC culture supernatants. These data demonstrate that HPMC synthesize IL-8 and that its release can be regulated as a result of induction of mRNA expression and de novo protein synthesis by other cytokines. (Am J Pathol 1993, 142:1876–1886)

Neutrophil infiltration into the peritoneal cavity is a characteristic finding during the early stages of bacterial peritonitis in patients treated for end-stage renal failure with peritoneal dialysis. The precise cellular signaling mechanisms controlling this event are not yet fully characterized, however, although it is assumed that lipid mediators and chemotactic factors derived from the resident peritoneal macrophage (PMØ) population are important in this process.[1–5] An alternative source of chemotactic mediators could be the mesothelial cells lining the peritoneal cavity, although their role in the process of inflammatory cell recruitment is as yet undefined.

A neutrophil chemotactic factor derived from lipopolysaccharide-stimulated mononuclear phagocytes has been variously named NCF, MDNCF, NAF, TCF, and NAP or interleukin-8 (IL-8). IL-8 is a member of a group of related 8–10-kd peptides, many of which possess chemotactic activity toward polymorphonuclear leukocytes.[6] The primary biological activity of IL-8 appears to be the induction of neutrophil chemotaxis (which is evident at very low IL-8 concentrations). In addition, however, higher concentrations of IL-8 induce the neutrophil respiratory burst and degranulation and increase the killing of *Candida spp.*[6,7]

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Endothelial cell-derived IL-8 ($IL\text{-}8_{77}$), an NH_2-terminally extended 77-amino acid form of IL-8, in addition to its primary role as a neutrophil chemoattractant, may act to reduce neutrophil adherence endothelium and thus protect it from long-term inflammatory damage. In addition $IL\text{-}8_{77}$ also regulates adhesion molecule expression and promotes neutrophil diapedesis.[8] These data indicate that this cytokine may play both a pro- and an anti-inflammatory role in the control of inflammation.[9–11]

Intraperitoneal injection of IL-1β, tumor necrosis factor-α (TNF-α), or IL-8 in mice results in a rapid, dose- and time-dependent increase in neutrophil numbers in the peripheral circulation followed by a selective increase in the peritoneal cavity.[12,13] These experimental findings appear similar to those events occurring early in peritoneal dialysis patients who subsequently present with episodes of proven bacterial or fungal peritonitis. The similarity of these events, coupled with the fact that the mesothelium occupies a central position in the peritoneal cavity and can contribute to the control of peritoneal inflammation via the secretion of prostaglandins and interleukin-6 (IL-6),[14,15] led us to examine the possibility that the mesothelium contributes to the signal for neutrophil influx during peritonitis.

The aim of the present study was therefore to characterize the synthesis of IL-8 by human peritoneal mesothelial cells (HPMC) and examine its regulation at the mRNA and protein levels in response to the macrophage-derived inflammatory cytokines IL-1β and TNF-α. Our data demonstrate significant synthesis of IL-8 by HPMC. The synergistic increase in IL-8 induced by combinations of IL-1β and TNF-α further suggests that the mesothelium not only participates in a peritoneal cavity cytokine network regulating inflammation therein but also contributes directly to the amplification of those processes involved in the recruitment of polymorphonuclear leukocytes and the elimination of infection.

Materials and Methods

All chemicals unless otherwise stated were obtained from the Sigma Chemical Company (Poole, Dorset, England). Recombinant human IL-1β was a kind gift from Dr. D. Boraschi (Sclavo Research Center, Siena, Italy). Its specific activity was 2.5×10^7 U/mg as assessed in the EL-4 16/cytotoxic T-lymphocyte line bioassay. Human TNF-α was from BASF AG (Ludwigshafen, Germany). Its specific activity was 8×10^7 U/mg protein (as assessed in a 48-hour L929 bioassay in the absence of actinomycin D). All cytokine preparations were batched and stored at −70 C and freshly thawed for each experiment. Endotoxin contamination of recombinant material was <70 pg/mg as assessed by amebocyte lysate assay (Kabi Vitrum, Stockholm, Sweden). Human recombinant IL-8, goat anti-human IL-1β, goat anti-human IL-6, and goat anti-human IL-8 antibodies were a kind gifts from the National Institute of Biological Standards and Control (Potters Bar, Hertfordshire, England), and monoclonal anti-TNF-α antibody (mono Ab 195) was kindly provided by BASF AG.

Isolation and Culture of Human Peritoneal Mesothelial Cells

HPMC were obtained from the omental tissue of consenting patients undergoing elective abdominal surgery, isolated, and characterized essentially as described previously.[16] Cells were maintained in Ham's F12 medium (CM) (ICN/Flow, High Wycombe, England) supplemented with penicillin (100 U/ml), streptomycin (100 mg/ml), L-glutamine (2 mmol/L) (Gibco, Uxbridge, England), transferrin (5 µg/ml), insulin (5 µg/ml), hydrocortisone (0.4 µg/ml) (all from Sigma), and 10% v/v fetal calf serum (FCS) (ICN/Flow). Cells were passaged using trypsin: EDTA:glucose (0.125% w/v:0.01% w/v:0.1% w/v) as previously described.[16]

Identification and Characterization of HPMC

The cells cultured were pure mesothelial cells as assessed by their uniform cobblestone appearance at confluence, by the presence of surface microvilli, by the lack of staining for factor VIII-related antigen, and by the uniform positive staining for cytokeratins 8 and 18.[16] The presence of contaminating macrophages was excluded following examination of Fc receptor expression (using a red cell rosetting assay) and Ia (class II major histocompatibility complex) antigen immunostaining, both of which were negative in all mesothelial cell cultures examined. It has been suggested that cells derived from the digestion of omental tissue might be microvascular endothelial cells; recent evidence, however, suggests that these cells are in fact of mesothelial and not endothelial origin.[17–19]

All experiments were performed with cells from the second passage which had previously been growth arrested as described below. There was no difference in the reactivity of mesothelial cell cultures irrespective of passage number; cells were

used from the second passage and not later to maximize cell numbers but to avoid using cultures containing senescent cells.

Assay for Endothelin-1

Growth-arrested HPMC were exposed to control medium, IL-1β (0.1–100 pg/ml), or TNF-α (0.1–100 pg/ml) for 24 h at 37 C. Following stimulation the supernatants were removed and analyzed for their content of endothelin-1[20] using an endothelin-1-specific kit (RPA 555, Amersham International plc, Newbury, England). The detection limit of the assay was 5 pg/ml. No endothelin was detected in any HPMC supernatants under any of these conditions, providing further evidence that HPMC cultures were unlikely to be contaminated with endothelial cells.

Establishment of Growth-Arrested HPMC

HPMC were grown to confluence in rat-tail type I collagen-treated[21] 250-ml flasks or in multiwell plates and transferred to CM containing 0.1% v/v FCS (rest medium) for 48 hours prior to stimulation. Under these conditions the cells remain in a non-proliferative, viable condition (as assessed by lack of lactate dehydrogenase release) for up to 96 hours.[14]

Western Blotting

Molecular weight characterization of secreted IL-8 of was examined by Western blot analysis using specific sheep anti-human IL-8 (National Institute of Biological Standards and Control, Potters Bar, England). Ten to 20 µl of neat or concentrated (10×) (Centricon 3 micro-concentrators, Amicon, Ltd., Stonehouse, England) culture supernatant from growth-arrested, control and cytokine-stimulated HPMC were subjected to electrophoresis on 1.5-mm 5–20% v/v sodium dodecyl sulfate gradient polyacrylamide gels (mini-Protean system, Bio-Rad Laboratories, Ltd., Hemel Hempstead, England). After electrophoresis the gels were equilibrated in blot buffer (7.81 mmol/L Tris, 60 mmol/L glycine, 20% v/v methanol, pH 8.3) and transblotted for 4 h at 0 C at 200 mA constant current (Bio-Rad blot chamber) onto prewetted nitrocellulose (Immobilon P, Millipore, Watford, England). The blotted membrane was placed in blocking buffer (phosphate-buffered saline containing 1% w/v bovine serum albumin) (ICN/Flow) and 0.1% v/v Tween 20 (Sigma) overnight at 4 C. The blots were washed six times with phosphate-buffered saline (PBS) and incubated overnight at 4 C with the primary antibody (1:1500) dissolved in PBS, Tween 20 (0.1% v/v), and bovine serum albumin (1% w/v) and incubated (60 minutes at room temperature) with alkaline phosphatase conjugated rabbit anti-goat IgG (1:5000, Sigma), in Tris-buffered saline containing 0.1% v/v bovine serum albumin.

The blot was washed again (four times with PBS-Tween 20) and developed following incubation with substrate buffer (0.1 mol/L Tris-HCl, pH 9.5; 0.1 mol/L NaCl; 5 mmol/L $MgCl_2$) containing nitroblue tetrazolium (0.33 mg/ml), 5-bromo-4-chloro-3-indolyl phosphate (170 µg/ml). The blots were dried overnight and photographed. The molecular weights of developed bands were compared to those of prestained sodium dodecyl sulfate-polyacrylamide gel electrophoresis standards (low range, Bio-Rad).

RNA Isolation, Reverse Transcription, and Polymerase Chain Reaction Amplification

HPMC were grown to confluence in multiwell plates (Falcon, Becton-Dickinson, Oxford, England), growth arrested as previously described, and stimulated with CM, IL-1β (1000 pg/ml), or TNF-α (1000 pg/ml) for 6 h at 37 C. Total cellular RNA was extracted from both control and cytokine-treated HPMC following lysis with 4 mol/L guanidine isothiocyanate[22] and centrifugation through 5.7 mol/L cesium chloride in 0.1 mol/L EDTA. Total RNA was reverse transcribed into complementary DNA (cDNA) with M-MLV reverse transcriptase (Gibco Life Technologies, Ltd., Paisley, Scotland), using the random hexamers method as previously described.[23] Briefly, the reaction mixture contained 1 µl random hexamers (100 µmol/L, Pharmacia Biosystems, Ltd., Milton Keynes, England), 5 µl nucleotide triphosphates (2.5 mmol/L, Gibco/BRL Life Technologies, Ltd.), 2 µl 10× polymerase chain reaction (PCR) buffer (100 mmol/L Tris/HCl, 500 mmol/L KCl, 15 mmol/L $MgCl_2$, and 0.01% w/v gelatin), 2 µl dithiothreitol (0.1 mol/L, Gibco/BRL Life Technologies, Ltd.), 1 µl RNAase inhibitor (Promega, Southampton, England), 1 µl M-MLV Superscript reverse transcriptase (200 U, Gibco/BRL Life Technologies, Ltd.), and 1 µg of total RNA.

PCR amplification was performed in a total volume of 50 µl (2 µl of reverse transcription product and 48 µl of master mix [36.25 µl H_2O, 1.25 µl 5′-primer (20 µmol/L), 1.25 µl 3′-primer (20 µmol/L), 4

μl nucleotide triphosphate, 5 μl 10× PCR buffer, and 0.25 μl Taq polymerase (2.5 U, Amplitaqγ, ILS, Ltd., London, England)] using a Perkin Elmer Thermocycler (Perkin Elmer Cetus, ILS, Ltd., London, England). The PCR protocol was as follows: first cycle, 94 C for 3 min, 55 C for 1 min, 72 C for 1 min; second through 24th cycles, 94 C for 1 min, 55 C for 1 min, 72 C for 1 min. The final cycle was 94 C for 1 min and 60 C for 10 min. PCR was performed for 25 cycles with α-actin and 35 cycles for IL-8. One-tenth of the PCR reaction from both test (IL-8) and control (α-actin) products was mixed and separated by flatbed electrophoresis in 1.5% (w/v) NuSieve GTG agarose gels (Flowgen Instruments, Ltd., Sittingbourne, England), stained with ethidium bromide (Sigma), and photographed. The negatives were scanned using a densitometer (model 620 video densitometer, Bio-Rad), and the density of the bands was compared to those of the housekeeping gene.

The oligoneucleotide primers for IL-8 were complementary to bases 104–124 in exon 1 (bases 2–21 in the IL-8 cDNA) of the nucleotide sequence in the case of the forward primer and bases 1442–1462 in exon 3 (bases 252–271 in the IL-8 cDNA) in the case of the reverse primer. These oligoneucleotides amplified a 270-bp fragment in the IL-8 sequence. The sequences of the amplification primers were as follows:

Gene	Primers	Product	Reference
α-Actin	Forward-GGAGCAATGATCTTGATCTT Reverse-TCCTGAGGTACGGGTCCTTCC	204 BP	23
IL-8	Forward-TGACTTCCAAGCTGGCCGTG Reverse-CCACGTCTCCCAACACCTCT	270 bp	24

Induction of IL-8 Production by HPMC

HPMC monolayers were grown to confluency in collagen-coated 24-well plates (Falcon, Becton-Dickinson) and growth arrested for 48 h in CM containing 0.1% v/v FCS, washed four times with rest medium, and then incubated at 37 C in the presence or absence of the appropriate cytokine. At specific time intervals, HPMC supernatants were removed, centrifuged at 12,000 × *g*, and then stored at −70 C until assayed. At the end of the incubation period the cells were washed with PBS (pH 7.3) (Dulbecco; Oxoid, Ltd., Basingstoke, England), and the cellular protein was solubilized with 0.1 N NaOH. Total cellular protein was estimated in these supernatants using the modified Bradford method.[25] Repeated cell counts revealed that 1 μg of cellular was equivalent to 3.76 ± 0.56 × 10^5 cells (n = 5). All data for IL-8 production are expressed as pg/μg of cellular protein. Incubation of HPMC with recombinant cytokines for up to 48 hours did not have any significant effect on cell viability or on the levels of cellular protein per well as compared to cells treated with medium alone (data not shown).

In separate experiments HPMC were pretreated with either the transcription inhibitor actinomycin D (Sigma) or the translation inhibitor cycloheximide (Sigma) for 30 minutes at 37 C prior to cytokine stimulation.

Cell-Associated IL-8

HPMC were treated with control medium, IL-1β (500 pg/ml) or TNF-α (500 pg/ml) for 24 hours, washed extensively with PBS, and subsequently sonicated for 30 seconds on ice. These samples were subsequently centrifuged at 10,000 × *g*, and the supernatants were removed for IL-8 enzyme-linked immunosorbent assay (ELISA).

Cytokine Synthesis Measurements

Synthesized IL-8 was measured in the culture supernatants of control and simulated (growth-arrested) HPMC using a double-ligand ELISA as previously described.[26,27] Anti-human IL-8 mouse monoclonal antibody and goat anti-human IL-8 polyclonal antibody conjugated to alkaline phosphatase were kindly supplied by Sandoz Forschungsinstitut (Vienna, Austria).

Peritoneal Macrophage-Conditioned Medium

PMØ were harvested from infection-free peritoneal dialysis effluent of patients undergoing continuous ambulatory peritoneal dialysis (CAPD) at Cardiff Royal Infirmary and stimulated as previously de-

scribed.[1] More than 95% of the cells remaining adherent to the plates were PMØ as assessed morphologically following differential cell staining (neat stain; Guest Medical, Ltd., Sevenoaks, England).

Conditioned medium was collected from adherent PMØ following 3 hours of incubation with Ham's F12 (0.1% FCS) alone (PMØ-CM) or from PMØ stimulated with a strain of *Staphyloccocus epidermidis* isolated from the effluent of a CAPD patient with peritonitis, as previously described (PMØ-S.epi-CM),[1] and assessed for IL-8 content by ELISA.

Statistical Analysis

All statistical analysis was performed by using Wilcoxon's signed rank test for paired nonparametric data, a P value of less than 0.05 being considered as significant. All data are presented as mean ± SEM.

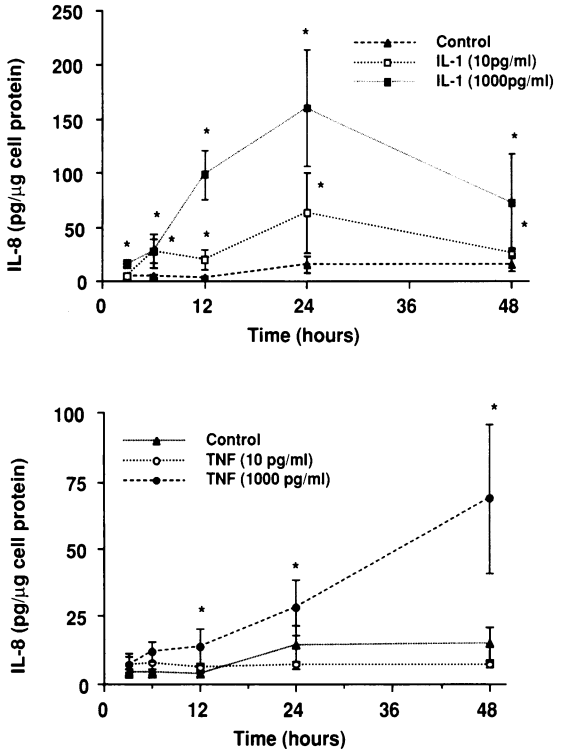

Figure 1. Top. *time-dependent generation of IL-8 from unstimulated HPMC (▲) or from HPMC stimulated with IL-1β (□, 10 pg/ml; ■, 1000 pg/ml). The data presented are the means ± SEM of IL-8 release expressed as pg/μg cell protein, from five separate experiments with HPMC prepared from separate omental specimens. *, statistically significant difference compared to unstimulated IL-6 release at the same time point.* Bottom. *time-dependent generation of IL-8 from unstimulated HPMC (○) or HPMC stimulated with TNF-α (▲, 10 pg/ml; ●, 1000 pg/ml). The data presented are the means ± SEM of IL-8 release expressed as pg/μg cell protein, from five separate experiments with HPMC prepared from separate omental specimens. *, statistically significant difference compared to unstimulated IL-8 release at the same time point.*

Results

Synthesis of IL-8 by HPMC

Unstimulated growth-arrested HPMC released low levels of IL-8. This release was significantly above the detection limit in the ELISA (1.25 ± 0.54 pg/μg cell protein (50 pg/ml) by 3 hours, when the amount released was 4.18 ± 1.48 pg/μg cell protein. Unstimulated IL-8 release increased up to 24 hours and leveled off thereafter. The release of IL-8 at 6, 24, and 48 hours was 3.67 ± 2.98, 14.6 ± 7.12, and 14.96 ± 6.02 pg/μg cell protein (mean ± SEM, n = 5), respectively.

Cytokine Induction of HPMC IL-8

Stimulation of HPMC with IL-1β or TNF-α induced a time-dependent generation of IL-8. This release was significantly above background generation by 3 hours for IL-1β and 12 hours for TNF-α (both at 1000 pg/ml) (Figure 1); thereafter IL-8 release continued to rise up to 24 hours following IL-1β stimulation and over the whole time course studied (up to 48 hours) following TNF-α treatment.

The release of IL-8 in response to IL-1β and TNF-α was also dose dependent; significant 24-hour release was achieved with doses of IL-1β at 10 pg/ml (z = 2.023, $P < 0.01$) and above and with TNF-α doses of 100 pg/ml (z = 1.96, $P < 0.05$) and above (Figure 2). Maximal generation of HPMC IL-8 was stimulated with an IL-1β dose of 5000 pg/ml (Figure 3) when the release was 153.8 ± 34.2 (mean ± SEM, n = 5) pg/μg cellular protein, 11-fold above background levels (z = 2.201, $P < 0.01$). The 24-hour IL-8 release in response to TNF-α was also maximal at the highest dose tested (5000 pg/ml) when IL-8 levels had reached 44.77 ± 11.02

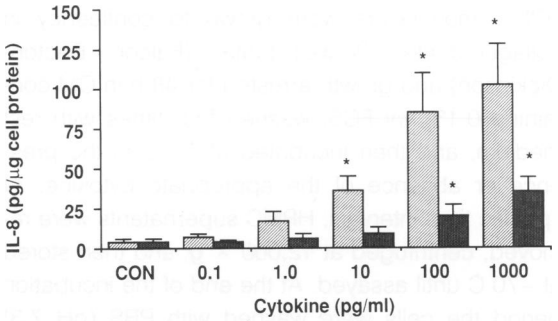

Figure 2. *Dose effect of IL-1β (0.1–1000 pg/ml) and TNF-α (0.1–1000 pg/ml) on the 24-hour generation of IL-8 from HPMC. The data presented are the means ± SEM of IL-8 release expressed as pg/μg cell protein, from five separate experiments with HPMC prepared from separate omental specimens. *, statistically significant difference compared to the control unstimulated IL-8 release value.*

(mean ± SEM, $n = 5$) pg/μg cellular protein, 3-fold above background levels ($z = 2.201$, $P < 0.01$) (Figure 3).

Combined Cytokine Stimulation

Stimulation of HPMC with combinations of IL-1β and TNF-α resulted in the release of immunoreactive IL-8 above the levels generated by either cytokine alone. The release of IL-8 in response to combined cytokine stimulation always exceeded that inducible by the addition of individual cytokine stimulatory capacities (Figure 3). At the highest combined doses tested (IL-1β, 50 pg/ml, and TNF-α, 500 or 5000 pg/ml) the amounts of released IL-8 were 153 ± 33 pg/μg (88.8 ± 27% above additive value) and 214 ± 44 pg/μg (114.2 ± 44% above additive value), respectively. This stimulation was synergistic since the levels of IL-8 generated by combined stimulation were significantly higher than the additive values of the individual cytokine IL-8 stimulatory capacity ($z = 2.201$, $P < 0.01$ for both).

Antibody Inhibition Studies

The specificity of the IL-1β and TNF-α stimulation of HPMC IL-8 was confirmed in antibody inhibition experiments with specific anti-cytokine antibodies. Coincubation of HPMC with IL-1β (0.1–1000 pg/ml) or TNF-α (0.1–1000 pg/ml) in the presence of either polyclonal anti-IL-1β antibody in the case of IL-1β or monoclonal anti-TNF-α antibody in the case of TNF-α resulted in inhibition of IL-8 generation at all cytokine doses tested. At an IL-1β concentration of 100 pg/ml IL-8 synthesis was reduced by a mean of 94.4% (Figure 4). At a TNF-α concentration of 100 pg/ml IL-8 release was reduced by 93.9%. The degree of stimulation by either IL-1β or TNF-α was unaffected in the presence of excess polyclonal goat anti-human IL-6 antibody, irrespective of the dose of either cytokine.

Effect of Transcription and Translation Inhibitors

HPMC were preincubated with either cycloheximide (1.0–10 μg/ml) or actiomycin D (0.1–10 μg/ml) for 30 minutes at 37 C, washed, and subsequently stimulated with IL-1β or TNF-α (both at 500 pg/ml). Both inhibitors induced a dose-dependent inhibition of IL-8 generation by HPMC (Figure 5). At an actinomycin D dose of 5 μg/ml both IL-1β- and TNF-α-induced IL-8 release was inhibited to unstimulated

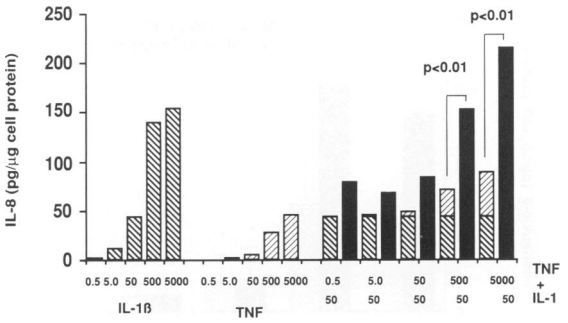

Figure 3. *Dose effect of either IL-1β (0.5–5000 pg/ml) or TNF-α (0.5–5000 pg/ml) either alone or in combination (black bars) on the capacity of HPMC to release IL-8. Data presented are the means of IL-8 release expressed as pg/μg cell protein, from five separate experiments performed with cells from separate donors. Statistical comparisons were made between the calculated additive values of IL-8 release (composite bars) and those levels detected by ELISA (black bars).*

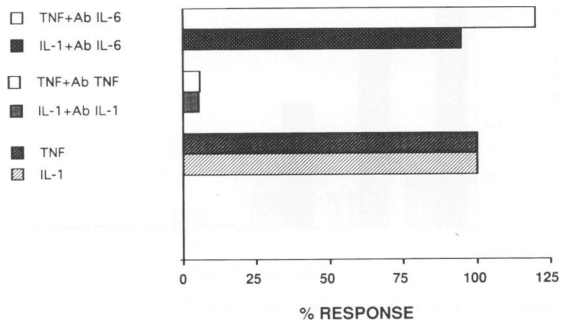

Figure 4. *Effect of anti-cytokine antibodies on the generation of IL-8 by HPMC. Cells were stimulated with IL-1β or TNF-α (both at 100 pg/ml) in the presence or absence of the antibodies indicated. Data presented as a percentage of control response (ie, 100%) are the mean of duplicate determinations from a single representative experiment of three performed.*

levels (2.7 ± 0.21 pg/μg cell protein). In the presence of cycloheximide at doses above 1 μg/ml IL-8 release was significantly reduced (Figure 5, top). At a cycloheximide concentration of 10 μg/ml, IL-1β- and TNF-α-induced IL-8 release was reduced by means of 91.5% and 83.6% respectively ($n = 5$ experiments). These inhibitory effects were not related to cell cytotoxicity since at these doses of inhibitors there was no increase in the release of the cytoplasmic enzyme lactate dehydrogenase above that in untreated cells (data not shown).

Western Blot Analysis

Western blot analysis of 10× concentrated HPMC supernatants using polyclonal anti-IL-8 antibody identified two closely associated immunoreactive bands at approximately 9 and 10 kd following stimulation of HPMC with IL-1β or TNF-α (1000 pg/ml) (Figure 6).

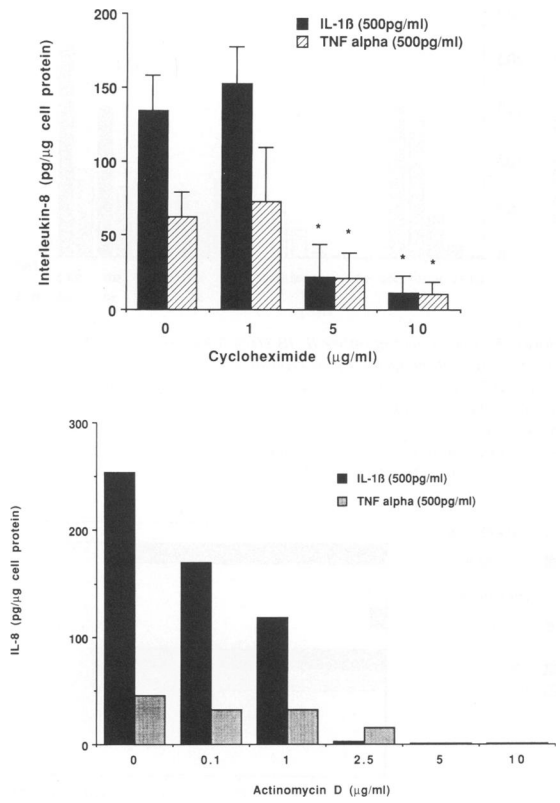

Figure 5. *Dose effect of cycloheximide* (top) *(1–10 µg/ml) or actinomycin D* (bottom) *(0.1–10 µg/ml) on the generation of IL-8 by HPMC stimulated by IL-1β (500 pg/ml) or TNF-α (500 pg/ml). Data presented are the means of IL-8 release expressed as pg/µg cell protein from five separate experiments with HPMC from different donors. *, statistically significant difference ($P < 0.05$) compared to the control IL-1β- or TNF-α-stimulated IL-8 release.*

PCR Analysis of HPMC mRNA

HPMC mRNA isolated from control and cytokine-stimulated cells was reverse transcribed and subjected to PCR amplification. These experiments demonstrated the generation of a IL-8-specific single band transcript of 270 base pairs. Treatment of HPMC with IL-1β or TNF-α resulted in mean 2.8-fold and 2.6-fold increases, respectively, in the expression of this specific mRNA signal when compared to the α-actin housekeeping gene transcripts amplified from the same samples (Figure 7).

Cell-Associated IL-8

HPMC monolayers were sonicated following control or cytokine treatment. Control HPMC monolayers contained low levels of cell-associated IL-8 which were increased following IL-1β or TNF-α treatment (Figure 8).

PMØ IL-8 Synthesis

Unstimulated PMØ released 948 ± 179 pg IL-8/10^6 cells in 3 hours; following stimulation with *S. epidermidis* this level was increased to 1199 ± 257 pg IL-8/10^6 cells.

Discussion

The present study demonstrates that HPMC express specific mRNA and synthesize the potent neutrophil chemoattractant IL-8. Treatment of HPMC with IL-1β and TNF-α resulted in a time- and dose-dependent release of immunoreactive IL-8. The identity and molecular size of this molecule were confirmed in Western blot experiments using anti-IL-8 antibody. These experiments demonstrated two closely associated immunoreactive bands between 9 and 10 kd. Whether these two molecular weight forms represent the 72- and 77-amino acid IL-8 variants previously described in endothelial cells and leukocytes[9–11] or are larger variants is currently under investigation. PCR amplification of reverse transcribed HPMC total RNA, using specific IL-8 primers, identified a single 270-base pair transcript which was specifically increased in amount following IL-1β or TNF-α pretreatment.

As has been previously demonstrated for pleural mesothelial cells[28] pretreatment of HPMC monolayers with actinomycin D or cycloheximide resulted in a dose-dependent inhibition of IL-8 synthesis. These data confirm that the control of IL-8 synthesis in response to IL-1β and TNF-α is at least partly dependent on *de novo* protein synthesis and the presence of increased levels of IL-8-specific mRNA. HPMC IL-8 synthesis was not completely blocked by cycloheximide treatment, however. These data suggest that either the dose of cycloheximide was not sufficient to completely block all protein synthesis (higher doses where tested but found to be cytotoxic to growth-arrested HPMC) or preformed IL-8 exists that is associated with the cell; in this respect sonicated unstimulated HPMC contain low but detectable levels of cell-associated IL-8 which were increased following cytokine treatment. To our knowledge this is the first demonstration of cell-associated IL-8 in HPMC; whether this is membrane-bound or true intracellular IL-8 remains to be investigated, as does its function. It has recently been suggested that endothelial cell membrane-bound IL-8 may be important in polymorphonuclear leukocyte adhesion and emigration;[29] whether this is the case in HPMC is unknown.

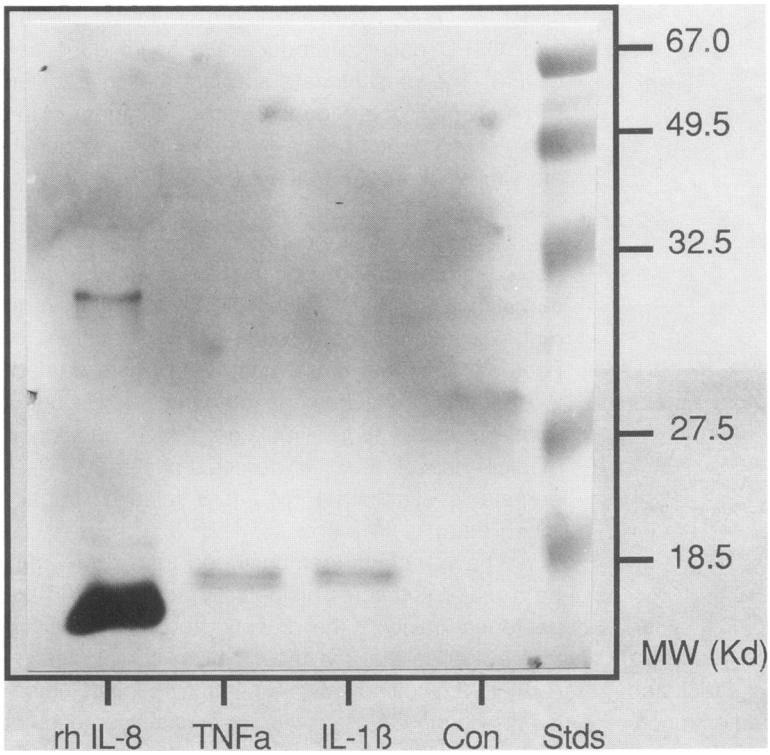

Figure 6. *Western blot analysis of 10× concentrated HPMC 18-hour supernatants and recombinant IL-8 using polyclonal sheep anti-human IL-8 antibody. HPMC were either unstimulated or stimulated with IL-1β (1000 pg/ml) or TNF-α (1000 pg/ml). Data are from a single representative experiment of three performed with HPMC prepared from separate omental specimens.*

The induction of IL-8 by other cytokines, particularly IL-1β and TNF-α, has been previously described in many cell types including fibroblasts, keratinocytes, endothelial cells, glomerular mesangial cells, and lung mesothelial cells.[7,27,28,30–34] The present study extends these findings to HPMC and confirms that the effect of IL-1β and TNF-α on IL-8 synthesis occurs as a result of increased levels of IL-8-specific mRNA. That these cytokine effects were specific was demonstrated in antibody inhibition experiments where anti-cytokine antibodies reduced the respective cytokine-induced IL-8 levels to those present in unstimulated controls. Anti-IL-6 antibody did not affect either IL-1β- or TNF-α-induced IL-8 release.

Combined IL-1β and TNF-α stimulation of HPMC resulted in a synergistic increase in IL-8 synthesis which was significantly greater than the additive values of the two cytokines alone. Although IL-1β and TNF-α bind to separate membrane receptors they share many biological activities in common.[35,36] Previous reports have also demonstrated

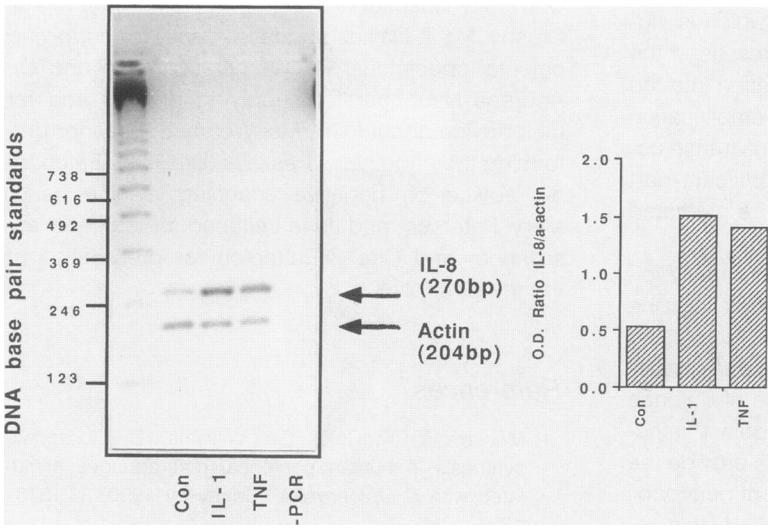

Figure 7. Left. *transcription of IL-8 induced upon stimulation of HPMC with IL-1β and TNF-α. HPMC were incubated for 6 hours with control medium* **(con),** *IL-1β (1000 pg/ml,* **(IL-1)** *or TNF-α (1000 pg/ml,* **TNF).** *The RNA was extracted and PCR was performed with primers specific for the IL-8 gene (270-bp fragment) and α-actin (204-bp fragment) as described in Materials and Methods. A portion of the PCR for IL-8 and α-actin were mixed and electrophoresed. Is DNA molecular weight standards. The products were subsequently stained with ethidium bromide, photographed (the negative of which is presented), and compared to DNA standards. In addition the specificity of the reaction was confirmed in tubes where no cDNA was present* **(−PCR).** **Right.** *densitometric scanning of the negative of the left-hand side of the figure expressed in arbitrary absorbance units of bands obtained as above. The data presented are the mean ratio (absorbance IL-8/absorbance α-actin) of three separate RT/PCR experiments performed with cells from different donors.*

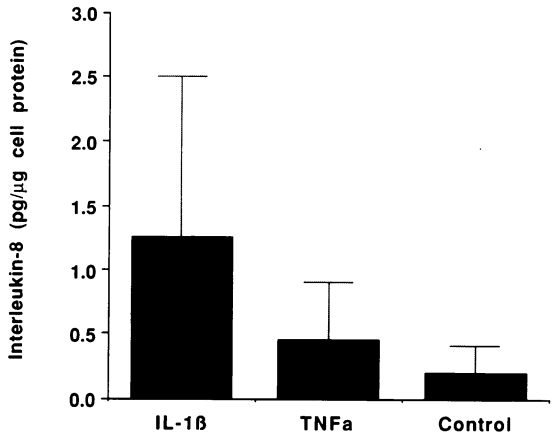

Figure 8. *Cell-associated IL-8 from sonicated HPMC. Cells were either unstimulated or exposed to IL-1β (500 pg/ml) or TNF-α (500 pg/ml) for 24 hours. Data are the mean of IL-8 measured from three experiments performed with HPMC prepared from separate omental specimens.*

that these two cytokines can act together in a synergistic fashion.[37-39] Occupancy of both receptors appears necessary for this synergistic effect and suggests that the cellular signal transduction mechanisms activated by IL-1β and TNF-α may be modulated following combined stimulation such that the degree of cell activation is up-regulated. The precise manner by which these events occur in HPMC, however, remains to be determined. To our knowledge this is the first report of a synergistic induction of IL-8 in any cell type and suggests that in the peritoneal cavity the rapid infiltration of neutrophils following infection may be at least partly the result of increased IL-8 secretion by HPMC.

Of particular relevance to peritoneal inflammation are the studies which demonstrate increases in neutrophil infiltration *in vivo* following IL-1β and TNF-α injection.[12,37,38] These data suggest that combined IL-1β and TNF-α treatment (neither of which is significantly chemotactic for neutrophils) results in the synergistic increase in neutrophil infiltration into the peritoneal cavity,[12] indicating that the amplification of the signals necessary for neutrophil infiltration occurs within the peritoneum itself. A significant part of this chemotactic activity appears to be mediated by increased levels of IL-8.[24]

During peritonitis bacterial infection is rapidly followed by a massive influx of neutrophils into the peritoneal cavity.[40] The cellular signals controlling these events are poorly understood. A small population of resident PMØ form the first line of defence in protecting the peritoneal cavity against infection,[40] and it is assumed that these cells provide the initial chemotactic signals for subsequent neutrophil accumulation following bacterial insult. In this respect we have demonstrated that PMØ isolated from CAPD patients produce significant quantities of IL-8. The present data suggest that in addition HPMC (which occur in much larger numbers in the noninfected peritoneal cavity than PMØ and generate similar quantities of IL-8 on a cell-for-cell basis) can also contribute significantly to pro-inflammatory events, in particular neutrophil recruitment, in the peritoneal cavity. Whether this is the result of indirect stimulation by PMØ-derived cytokines or via direct interaction of the mesothelium with bacteria or bacterial products is currently being investigated. We have recently demonstrated that both control and *S. epidermidis*-stimulated PMØ-conditioned medium as well as *S. epidermidis* bacteria-free supernatants stimulate HPMC IL-8 release (unpublished data).

We have recently demonstrated that HPMC may be a major source of biologically active IL-6 in the peritoneal cavity;[15] the present study provides further evidence that the mesothelium contributes directly to a cytokine network controlling inflammatory events during CAPD, in this case via its secretion of IL-8. The potential secretion of HPMC IL-8 during the onset of peritonitis and the synergistic amplification by inflammatory cytokines might be an important part of a complex cytokine pro/anti-inflammatory network operating to control intraperitoneal inflammatory events during CAPD peritonitis.

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