Immunology 1995 **84** 227–232

Polarized secretion of interleukin-8 by human mesothelial cells: a role in neutrophil migration

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SUMMARY

We investigated the role of human mesothelium in an *in vitro* model of peritonitis with emphasis on the secretion of the neutrophil chemoattractant interleukin-8 (IL-8) and the migration of polymorphonuclear leucocytes (PMN) across monolayers of peritoneal mesothelial cells. PMN showed minimal migration across non-activated mesothelial monolayers (< 2%). However, migration was induced after mesothelial cell activation by IL-1β (24%) and this induced migration was significantly blocked by antibodies against IL-8 (63% inhibition; $P \leq 0{\cdot}01$). IL-1β-activated mesothelial monolayers were shown to secrete IL-8 in a polarized way, which was preferentially oriented towards the apical side of the monolayer. Our results indicate that the influx of PMN into the peritoneal cavity is, at least in part, controlled by the mesothelial cell layer of the peritoneal membrane.

INTRODUCTION

The abdominal cavity is lined with a monolayer of mesothelial cells resting on a basement membrane. The mesothelium provides a slippery, non-adhesive and non-thrombogenic surface that allows smooth mobility of internal organs.[1] During peritonitis there is a massive influx of polymorphonuclear leucocytes (PMN) from the circulation to the peritoneal fluid. These granulocytes migrate across the mesothelial lining to reach the peritoneal cavity. Recent *in vitro* experiments have shown that mesothelium may actively participate in the neutrophil influx through enhanced expression of the adhesion molecule intercellular adhesion molecule-1 (ICAM-1) on its surface and by secretion of the neutrophil chemoattractant interleukin-8 (IL-8).[2] This investigation was carried out to examine further the functional role of the mesothelial lining in the migration of PMN. The pattern of IL-8 secretion and its influence on migration of PMN across mesothelial monolayers was studied in an *in vitro* model of peritonitis.

Received 31 May 1994; revised 3 October 1994; accepted 27 October 1994.

Abbreviations: CA 125, cancer antigen 125; FMLP, formyl-leucyl-methionyl-phenylalanine; HSA, human serum albumin; ICAM-1, intercellular adhesion molecule-1; IL-1, interleukin-1; 7MCP-1, monocyte chemoattractant protein-1; PMN, polymorphonuclear leucocytes.

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MATERIALS AND METHODS

Mesothelial cell culture

Mesothelial cells (MC) were isolated from human omentum, according to techniques modified from Nicholson *et al.*[3] and Wu *et al.*,[4] as described previously.[5] In brief, small pieces of omentum were removed early in the operative procedure from patients undergoing abdominal surgery for non-infectious conditions. All patients gave informed consent. The omentum was transferred to fluid containing 0·05% (w/v) trypsin–0·02% (w/v) EDTA (Gibco Life Technologies, Paisley, UK). After 15 min the detached MC were pelleted by centrifugation at 1200 rpm for 5 min and resuspended in supplemented M-199 medium (Gibco). MC were grown until confluence in a 37°, fully humidified, 5% CO_2 cabinet in polystryrene culture flasks (75 cm^2; Costar, Cambridge, MA) precoated with fibronectin. The identity of MC was demonstrated by the absence of von Willebrand factor staining[6] and the presence of intracellular cytokeratins by using immunofluorescence with monoclonal antibodies[7] (Dakopatts, Glosstrup, Denmark).

IL-8 assay

MC were subcultured to confluent monolayers on polycarbonate membranes (0·4 μm pore size, 24·5 mm in diameter) of Transwell cell culture chamber inserts (Costar). The filters were precoated with fibronectin prior to addition of the MC. MC monolayers reached confluence in 5 days as determined by light microscopy and by May–Grünwald–Giemsa staining. Inverted monolayers were cultured according to Parkos *et al.*[8] with minor modifications (Fig. 1). For this purpose, a section of a

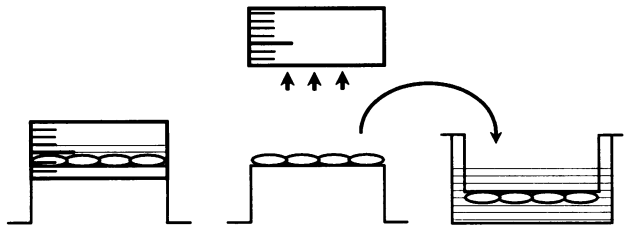

Figure 1. Preparation of an inverted mesothelial cell culture in a Transwell filter system. The insert containing the filter was inverted and a piece of a 50-ml centrifuge tube was tightly, but gently, fixed to the bottom part of the insert. The MC suspension were added to the inverted insert and allowed to attach overnight. The tube prevents leaking of the culture fluid from the inverted filter. The tube was then removed, the insert was placed upright in a 6-well culture dish and MC attached to the underside of the filter were cultured for 5 additional days to reach a confluent mesothelial cell monolayer.

50-ml centrifuge tube (Costar) was gently, but tightly fixed to the inverted bottom part of each insert to prevent leakage of medium or cells. The inverted filters were also routinely treated with fibronectin. MC were added to the inverted inserts and allowed to attach overnight (Fig. 1). Thereafter, the section of a 50-ml centrifuge tube was removed, and the insert was placed upright into 6-well culture dishes. This culture was maintained for 5 days to reach MC confluency prior to use (Fig. 1). Pretreatment of the monolayer with an optimal concentration of human recombinant IL-1β (rIL-1β; 25 U/ml; Genzyme Corporation, Cambridge, MA) did not influence the microscopic morphology of the confluent monolayers. All media were refreshed in the experiments before addition of rIL-1β to one of the compartments. At indicated intervals, samples from both compartments were taken and assayed for IL-8 by enzyme-linked immunsorbent assay (ELISA).[9] The results were expressed as absolute nanograms of IL-8 produced by the monolayer.

Transport of rIL-8 through MC monolayer

Recombinant IL-8 was obtained by transfecting *Escherichia coli* DH5 with the plasmid pMBL11 that contained cDNA encoding the 72-amino acid species of human IL-8 (British Biotechnology Ltd, Oxford, UK).[9] Recombinant IL-8 was added to the apical side of non-activated MC monolayers, cultured on polycarbonate membranes (0·4 μm pore size, 24·5 mm in diameter). Over 6 hr serial samples from the lower compartments were taken and assayed for IL-8 by ELISA.[9]

Granulocyte isolation

Blood was obtained from healthy volunteers. Granulocytes were purified from a buffy coat by density gradient centrifugation over isotonic Percoll as described by Roos & de Boer.[10] Purity of the granulocytes was >98%, with >95% neutrophils. Viability meausred by lactate dehydrogenase (LDH) release[11] was >95%.

Labelling of neutrophils

Freshly purified neutrophils were radiolabelled with ^{51}Cr according to Gallin *et al.*[12] Labelling of PMN with ^{51}Cr has no effect on chemotaxis of the neutrophil[12] and is therefore a standard method in adherence and migration experiments.[13] Briefly, neutrophils (10^7 cells/ml) suspended in 50% (v/v) M-199, 50% (v/v) RPMI-1640 (Gibco), supplemented with 0·1% (v/v) human serum albumin (HSA), were incubated with 10 μCi ^{51} Cr/ml (sodium chromate, 200–500 Ci/g; New England Nuclear, Boston, MA) at 37° for 1 hr with gentle shaking. The cells were subsequently washed and resuspended in 50% (v/v) M-199, 50% (v/v) RPMI-1640, 0·5% (v/v) HSA (incubation medium). Viability after labelling remained >95%.

Migration assay

MC were subcultured on normal and inverted polycarbonate membranes (8·0 μm pore size, 24·5 mm in diameter; Costar) as already described. In the conventional configuration PMN were sedimented by gravity to the apical surface of the MC normally facing the abdominal cavity. Inverted filters were used to study migration in the more physiological direction (i.e. submesothelial tissue to abdominal fluid), thus permitting the PMN to approach and adhere to the basolateral side of the MC monolayers. In some experiments the monolayers were preincubated with an optimal concentration of rIL-1β (25 U/ml added to the lower compartment) for 6 hr prior to adding the PMN to the upper compartment. In all experiments the upper compartment of the cell culture chamber inserts was washed twice with incubation medium [50% (v/v) M-199, 50% (v/v) RPMI-1640 (Gibco) supplemented with 0·5% (v/v) HSA], prewarmed to 37°, before adding the PMN. However, the fluid in the lower compartment was not replaced unless otherwise indicated. ^{51}Cr-labelled neutrophils (1×10^6 cells/ml), prewarmed to 37°, were then added to the upper compartments. When indicated, mAb against IL-8 (mAb IL-8/6)[9] and in some experiments C5a (10^{-8} M; Sigma Diagnostics, St Louis, MO) or FMLP (10^{-8} M; Sigma) were added to the lower compartment 5 min prior to adding the PMN. The chambers were incubated in a 5% CO_2 incubator at 37° for 30 min. After incubation, the fluid from both compartments was collected. The radioactivity present in the fluid as well as on the filter, cut out of its cylindric container, was determined in a gamma-counter. Recovery was always >92%. The radioactivity measured on the filter was taken to represent PMN adhesion. Light microscopic examination of the MC monolayers showed that most of the neutrophils were located at the margins of the MC. The extent of migration was calculated from the radioactivity found in the fluid sampled from the lower compartment. The results were expressed as percentages of the total radioactivity, i.e. PMN added to the chambers.

Measurement of chemotaxis

The Boyden chamber (48-well chemotaxis chamber; Neuro Probe Inc., Cabin John, MD) was used for measurement of PMN chemotaxis. The Boyden chamber contained a 8·0-μm migration filter on top of an 0·45-μm stop filter. Chemotactic stimulus, added to the lower compartment of the Boyden chamber, consisted of the supernatant of the basolateral or apical compartment of rIL-1β-prestimulated mesothelial cell monolayers cultured on 0·4 μm filters, as described above. Recombinant IL-1β was added to the apical side of the monolayer. Culture medium and C5a (10^{-8} M; Sigma), diluted in culture medium, served as controls. When indicated, mAb against IL-8 (mAb IL-8/6)[9] was added to the apical supernatant 5 min prior to adding the PMN. PMN were suspended in medium containing 132 mM NaCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$,

6 mM KCl, 1·2 mM KH_2PO_4, 20 mM HEPES, 5·5 mM glucose, and 0·5% (w/v) HSA (CLB, Amsterdam, the Netherlands), pH 7·4, supplemented with heparin (10 IU/ml). 10^5 PMN were added to the upper compartment of the Boyden chamber and incubated for 1·5 hr. Thereafter, the 8·0-μm Boyden chamber filters were stained, dehydrated with xylol and the number of PMN was counted with a microscope at 400 × magnificiation. Chemotaxis was defined as the mean of the number of PMN counted in five microscopic fields.

Transmesothelial electrical resistance measurement

The confluency and integrity of the mesothelial monolayer was tested by measuring the transmesothelial electrical resistance[14,15] (Millicel-ERS, Millipore Corporation, Bedford, MA) on both non-stimulated and prestimulated MC monolayers. MC were subcultured on normal and inverted polycarbonate membranes (8·0 μm pore size, 24·5 mm in diameter; Costar) as described above. The monolayers were cultured for 5 days to reach confluence. In some experiments the monolayers were preincubated with rIL-1β (25 U/ml, added to the apical side of the monolayers) for 6 hr as described earlier. In all experiments the medium of both compartments of the cell culture chamber inserts was replaced by medium M-199 without serum before measurement of the electrical resistance. Filters without MC monolayers served as controls. After the initial measurements the confluency of the MC monolayers was deliberately disrupted by replacing the medium with PBS containing EDTA (2 mM; Boehringer Mannheim GmbH, Mannheim, Germany). After incubation for 30 min in a 37°, 5% CO_2 cabinet, the electrical resistance was again measured. Filters without MC monolayers served as controls.

RESULTS

Interleukin-8 secretion

The concentration of IL-8 was measured in the fluid of both compartments of the Transwell system. The mesothelial cell monolayer forms a barrier between these compartments, which enabled us to measure differences in apical and basolateral secretion. In the absence of deliberate stimulation, mesothelial cell monolayers secreted only low levels, i.e. less than 2 ng, of IL-8 (Fig. 2). After the addition of rIL-1β to the apical surface of the monolayer, i.e. in the upper compartment, the mesothelial cells within 6 hr secreted large amounts of IL-8 in a polarized way: more IL-8 was found in the upper compartment than in the lower compartment of the system ($P \leq 0{\cdot}05$; Fig. 2). Surprisingly, when rIL-1β was introduced to the basolateral side of the monolayer, i.e. added to the lower compartment, IL-8 was also preferentially secreted into the upper compartment ($P \leq 0{\cdot}05$; Fig. 2). Mesothelial cells, thus, seem to create a gradient of IL-8 towards the apical side of the monolayer, irrespective of the direction of the inducing stimulus. To test this hypothesis further, inverted monolayers, in which the MC were adherent to the lower side of the filter, were likewise stimulated. Again, MC IL-8 secretion was found to be polarized towards the apical side of the monolayer ($P \leq 0{\cdot}05$; Fig. 2).

The transport of exogenous rIL-8 was tested through non-activated mesothelial monolayers, cultured on normal filters (0·4 μm pore size, 24·5 mm in diameter) to study the potential

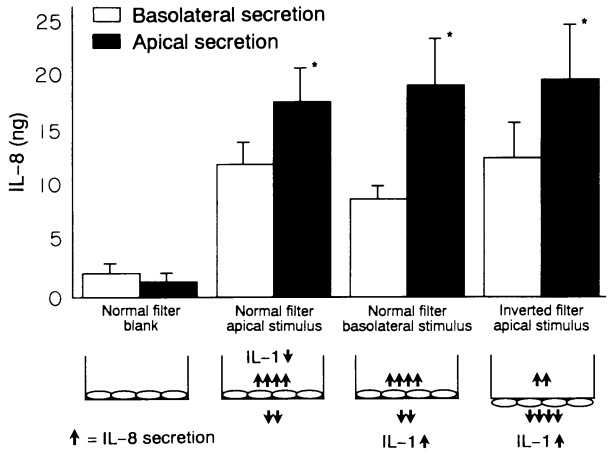

Figure 2. Secretion of IL-8 by confluent monolayers of MC. Normal and inverted 0·4 μm filters were used. MC were stimulated by addition of rIL-1β (25 U/ml) to the apical or basolateral side of the monolayer and the IL-8 secretion was measured after 6 hr. Results are expressed as the mean ± SEM of eight separate experiments. *Apical concentration significantly higher than the basolateral concentration of IL-8 ($P \leq 0{\cdot}05$, two-tailed paired Student's *t*-test).

confounding influence of active or passive back-diffusion of secreted IL-8 through the monolayers. Within 6 hr part of the rIL-8 added to the upper compartment was detected in the lower compartment, indicating that active or passive transport of IL-8 through the monolayer did occur (Fig. 3).

Diffusion of rIL-8 through a filter (0·4 μm, 24·5 mm in diameter) without a MC monolayer was tested to control for the possible influence of the design of the Transwell system. The Transwell filter itself formed no major barrier to rIL-8 diffusion, nor was coating of the filter with fibronectin of influence (data not shown).

Migration

PMN adhesion and migration across non-activated mesothelial monolayers in normal and inverted filters was minimal (< 2% in 30 min; Table 1). However, migration of PMN across the

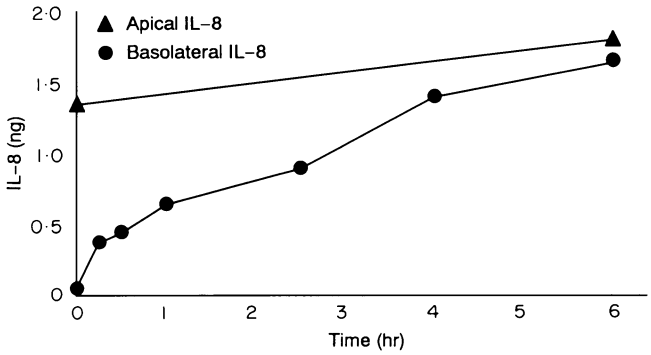

Figure 3. Representative figure of the time–course of rIL-8 transport through a non-stimulated MC monolayer. A normal 0·4-μm filter was used and rIL-8 was added to the apical side of the monolayer at 0 hr. At various times samples of the lower compartment were taken and measured in an IL-8 ELISA.

Table 1. PMN adherence and migration across MC monolayers

	% adhesion	% migration
Normal filters		
Blank	3·0 ± 0·5	1·7 ± 1·1
Apical stimulus	10·9 ± 2·9	12·1 ± 2·7
Inverted filters		
Blank	3·2 ± 0·5	1·8 ± 1·5
Apical stimulus	8·6 ± 1·2	23·7 ± 2·3*†
Apical stimulus‡	20·8 ± 5·4	3·2 ± 1·4
Apical stimulus, mAb IL-8	15·2 ± 2·5	8·8 ± 2·2§
C5A	19·0 ± 4·5	30·6 ± 7·8¶

Mesothelial cells were cultured on a 8·0-μm filters. Neutrophil adherence to and migration across normal and inverted monolayers of MC were measured 30 min after adding the PMN. Results are expressed as the mean ± SEM of four to six experiments performed on separate occasions.

‡Lower compartment was washed twice with incubation medium before adding the PMN. Monoclonal antibody IL-8/6 (1 μg/ml) was added to the lower compartment 5 min prior to adding the PMN. In some experiments neutrophils were attracted to the lower compartment by C5a (10^{-8} M).

*Migration across an inverted filter was significantly higher compared to migration across a normal filter ($P \leq 0{\cdot}01$); †significant increase in migration after stimulation of the MC with rIL-1β, compared to the migration across non-stimulated MC monolayers ($P \leq 0{\cdot}01$); §mAb against IL-8 significantly inhibited migration across stimulated MC monolayers ($P \leq 0{\cdot}01$); ¶C5a induced a significant increase in migration across non-stimulated MC monolayers ($P \leq 0{\cdot}05$). All statistical analyses were performed using the two-tailed unpaired Student's *t*-test.

monolayer was invariably found after MC stimulation with rIL-1β (Table 1). Inversion of the filter resulted in even higher levels of PMN migration (96% increase, $P \leq 0{\cdot}01$; Table 1), which correlated well with the difference in amounts of IL-8 found in the lower compartments, between normal and inverted filters (Fig. 1).

The presumed difference in barrier between normal and inverted filters for neutrophils to reach the lower compartment was determined by using C5a or FMLP as chemoattractant in otherwise unstimulated MC monolayers in PMN adherence to and migration across the filters. There was no difference in neutrophil adherence to and migration across normal or inverted MC monolayers induced by these chemoattractants, indicating no difference in barrier for neutrophils between the two different ways of culturing a confluent MC monolayer on the filters (data not shown).

Increase in adherence to the MC monolayer, but not a significant increase in migration across activated inverted filters was seen when the medium in the lower compartment was replaced with fresh medium before adding the PMN (Table 1). However, when the medium in the lower compartment was left *in situ*, a significant increase in migration was observed ($P \leq 0{\cdot}01$; Table 1).

To delineate the role of MC-excreted IL-8 further, the effect of a mAb against IL-8 (mAb IL-8/6) was tested in the set-up with stimulated inverted filters. Migration of PMN across a monolayer of MC was reduced by 63% ($P \leq 0{\cdot}01$), indicating that IL-8 was the major chemotactic moiety excreted by MC (Table 1).

Addition of the chemoattractant C5a to the lower compartment resulted in extensive adherence and high levels of migration of PMN in otherwise unstimulated inverted filters (Table 1).

Monoclonal antibody IL-8/6 did not influence the adherence to and migration of PMN across non-activated mesothelial monolayers (data not shown). Furthermore, the adherence and migration of PMN induced by C5a was also not affected by mAb IL-8/6 (data not shown).

Chemotaxis of mesothelial supernatant

The difference in PMN chemotaxis between basolateral and apical supernatant, obtained from rIL-1β-prestimulated MC monolayers cultured on 0·4 μm filters was shown in the Boyden chamber (Table 2). Supernatant obtained from the basolateral compartment of prestimulated MC monolayers induced significantly less chemotaxis compared to supernatant from the apical compartment ($P \leq 0{\cdot}05$, Table 2). It was again shown in the Boyden chamber that IL-8 was the major chemoattractant secreted by stimulated MC monolayers, as mAb IL-8 added to apical supernatant reduced the chemotaxis of PMN with 74·8% ($P \leq 0{\cdot}01$; Table 2).

Transmesothelial electrical resistance

To establish the confluency and integrity of the MC monolayers used in our experiments, the transmesothelial electric resistance of typical monolayers was repeatedly measured. Mesothelial monolayers after 5 days of culture were shown to give a significant increase in electrical resistance ($P \leq 0{\cdot}01$), compared to bare filters without an MC monolayer (Table 3). Neither culture of the MC on inverted filters nor prestimulation

Table 2. Boyden chamber measurement of PMN chemotaxis

Chemotactic stimulus	No. of PMN migrated
Culture medium	12·8 ± 2·2
Basolateral supernatant	58·0 ± 9·2
Apical supernatant	90·5 ± 0·9*
Apical supernatant, mAb IL-8	22·8 ± 7·6†
C5a	180·8 ± 45·0

Supernatant was obtained from the basolateral or apical compartment of rIL-β-prestimulated mesothelial cell monolayers, cultured on 0·4-μm filters. rIL-1β was added to the apical side of the monolayer. Culture medium and C5a diluted in culture medium served as negative and positive controls, respectively. Results are expressed as the mean ± SEM of four experiments performed on separate occasions. All stimuli gave a significant increase in PMN chemotaxis compared to incubation medium ($P \leq 0{\cdot}01$).

*Number of PMN significantly higher compared to basolateral supernatant ($P \leq 0{\cdot}05$); †significant inhibition of mAb against IL-8 on PMN chemotaxis added to apical supernatant ($P \leq 0{\cdot}01$). All statistic analyses were performed with the two-tailed paired Student's *t*-test.

Table 3. Measurement of transmesothelial electrical resistance

	Normal filter	Inverted filter
Filter without MC monolayer	104·6 ± 2·5	104·6 ± 2·5
Filter with MC monolayer	149·8 ± 3·6*	157·0 ± 2·7*
No stimulus		
Apical stimulus of IL-1β	153·0 ± 8·1*	154·6 ± 6·8*

Mesothelial cells were cultured on 8·0 μm filters. Transmesothelial electrical resistance was measured 6 hr after adding rIL-1β to the apical side of the monolayer. Results are expressed in Ohms as the mean ± SEM of five separate experiments.

*Electrical resistance was significantly higher compared to a control filter without a MC monolayer ($P \leq 0{\cdot}01$, two-tailed paired Student's *t*-test).

of the MC with an optimal concentration of IL-1β altered the electrical resistance (Table 3).

After 30 min incubation with PBS/EDTA, the electrical resistance of all filters had fallen to the same level as the control filter without an MC monolayer (data not shown).

DISCUSSION

The entry of neutrophils into tissues or body cavities is a prerequisite for an early and adequate inactivation of invading micro-organisms. Results of prevous studies indicate that the presence of a gradient of chemotactic mediators generated at the site of inflammation, e.g. formylmethionyl peptides of bacterial origin, the anaphylatoxin C5a, lipid-derived mediators such as platelet-activating factor (PAF), or IL-8, is of utmost importance for transendothelial migration of PMN.[13,16] Our results indicate that in the peritoneal cavity the mesothelium itself, once activated, is able to create a functional chemotactic gradient of IL-8 in the physiologically relevant direction.

The apical surface of peritoneal mesothelium facing the abdominal cavity is profusely carpeted with microvilli.[17] This extension of apical surface compared to the basolateral surface of mesothelium might cause the difference in apical versus basolateral secretion of IL-8. Furthermore, mesothelium is known to possess sophisticated junctional complexes that might allow a gradient of IL-8 to be maintained.[17] Measurements of the transmesothelial electrical resistance proved indeed that confluent MC monolayers create a tightly joined layer of cells in which the polarized secretion of cytokines and transmesothelial migration of PMN can be studied. However, in spite of its polarized secretion we also found some passive or active transport of IL-8 across non-stimulated MC monolayers. This may be because part of the apically secreted IL-8 is secondarily transported or passively diffused to the basolateral side of the monolayer. The small molecular size of the cytokine IL-8 (8000 MW) probably facilitates the diffusion through the MC monolayer. It is known from patients on chronic peritoneal dialysis and from studies in experimental animals that the peritoneal clearance of molecules decreases as the molecular size of the inoculum increases.[18,19] This concurs with the results from a previous study[5] in which a greater proportional difference between preferential apical against basolateral secretion was found by activated MC monolayers for the tumour marker, cancer antigen 125 (CA 125; 220 000 MW).

Electron microscopic EM analysis of MC has revealed an abundance of rough endoplasmic reticulum, well-developed Golgi complexes and large nuclei showing predominance of euchromatin over heterochromatin,[17] indicating that mesothelium is capable of protein synthesis and secretory activity. Recently, activated mesothelial cells were shown to produce several cytokines, including granulocyte colony-stimulating factor (CSF), monocyte chemotactic protein-1 (MCP-1), IL-6,[20] IL-8[21–23] and transforming growth factor-β (TGF-β).[24]

We now report that MC are capable of secreting at least one such cytokine, IL-8, in a polarized fashion, which allows it to be functionally active and physiologically relevant. Indeed, high levels of IL-8 can be found in dialysate samples from patients on peritoneal dialysis during episodes of bacterial peritonitis.[25,26] The functional activity of the mesothelial IL-8 gradient was shown in our study by different PMN migration experiments. Apical-derived supernatant from stimulated MC monolayers induced higher chemotactic activity compared to basolateral supernatant. Even more important than the absolute difference in chemotactic potency between the two sides of the cell layer is probably the presence of the gradient itself. Neutrophil movements are fuelled by the presence of a chemotactic gradient of mediators, e.g. IL-8, generated at the site of inflammation.[16]

The pattern of polarized secretion of IL-8 as we found, which is apically directed irrespective of the site of the inducing stimulus, is not unique. Recently, the same polarized type of secretion was found for CA 125 by MC[5] and for the cytokine MCP-1 by rat type 2 alveolar epithelial cells.[27] IL-8 was also found to be secreted in a polarized fashion by other cell types. McCormick *et al.*[28] studied the IL-8 secretion of activated T84 epithelial cells in the Transwell system and found that IL-8 was preferentially secreted to the basolateral side of the monolayer, indicating that the Transwell filter forms no barrier for IL-8 diffusion.

The secretion of other products by MC, or of cytokines by other human cell types, may likewise be secreted in a polarized fashion, as we described for IL-8 by MC. This would concur with the physiological cascade of inflammatory events as observed in nature.

ACKNOWLEDGMENTS

The authors thank Margreet Hart for providing monoclonal antibodies against human IL-8, and Dr. Peter Kr. von dem Borne for technical assistance. This work was supported by the Dutch Kidney Foundation Grant No. 92.1265.

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