Migration of leukocytes across an endothelium-epithelium bilayer as a model of renal interstitial inflammation

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In a variety of chronic renal diseases, the severity of tubulointerstitial inflammation is an important histological parameter that correlates well with progression and impairment of renal function (25). Recent data from animal studies could also demonstrate the involvement of leukocytes in experimental ischemic and septic acute renal failure (5, 25).

Activation of endothelial cells by inflammatory mediators promotes leukocyte infiltration by increased cellular adhesion molecule expression, vascular permeability, and production of chemoattractants (6, 12). Proximal tubular epithelial cells have been shown to act as immune cells and actively participate in the orchestration of inflammatory events (25).

Proximal tubular epithelial cells and the peritubular capillary system are in very close proximity. The onset and progression of impaired renal function is generally based on the defective interplay between tubular and vascular renal compartments. Previous studies have suggested that endothelial and epithelial cells in the kidney form a complex network of interactions. For example, Kim et al. (13) demonstrated that, under hypoxic conditions, proximal tubular epithelial cells induce vascular endothelial growth factor-dependent angiogenesis in cultured endothelial cells. Furthermore, endothelial cells seem to regulate sodium transport in proximal tubular epithelial cells as shown by Linas and Repine (16). Despite the growing body of knowledge on migration across monolayers of endothelial or epithelial cells, possible interaction between these cell types and the resulting modulation of leukocyte migration have not been studied in depth.

Experimental studies suggest that inhibition of tubulointerstitial inflammation may reduce injury in renal diseases (25). There is evidence that the activation of melanocortin receptors may be a new strategy to control inflammatory processes. α-Melanocortin-stimulating hormone (α-MSH), a potent anti-inflammatory peptide, has been shown to be effective in animal models of local and systemic inflammatory disorders, including sepsis syndrome, and inflammatory bowel disease, and acute renal failure (14, 17, 22). The effects of α-MSH are mediated by melanocortin receptors found on macrophages, polymorphonuclear neutrophils (PMN), and renal tubular cells and acts by inhibiting maladaptive activation of genes that cause inflammatory and cytotoxic injury (5, 10). However, the effect of α-MSH on PMN migration is not known.

Therefore, the focus of our study was to establish a model that more closely mimics the tubulointerstitium, culturing monolayers of human renal proximal tubular epithelial cells (HK-2), and human dermal microvascular endothelial cells (HDMEC) on opposite sides of Transwell growth support. In this bilayer model, we could establish enhanced PMN migration and cytokine production after pretreatment with proinflammatory substances [tumor necrosis factor-α (TNF-α) and endotoxin (LPS)]. Furthermore, we could demonstrate a potent anti-inflammatory effect of α-MSH in this model.
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MATERIALS AND METHODS

Cell culture. HK-2 cells (human proximal epithelial cell with functional and morphological characteristics of normal adult proximal tubular cells (PTC); see Ref. 23) were obtained from ATCC (Rockville, MD). Cells were grown in DMEM-Ham’s F-12 (PAA Laboratories, Linz, Austria) supplemented with 10% FCS (Biological Industries, Kibbutz Beit Haemek, Israel), 1-glutamine (2 mM; Biological Industries), penicillin (100 U/ml), and streptomycin (100 µg/ml; Sigma Chemical, Deisenhofen, Germany). For the experiments, the 18th-28th passages of HK-2 cells were used.

HDMEC were obtained from Promocell (Heidelberg, Germany). HDMEC were maintained in MCDB-131 medium containing 1 µg/ml hydrocortisone (Promocell), 10 ng of epidermal growth factor (Promocell), 2 mM l-glutamine (Biological Industries, Kibbutz Beit Haemek, Israel), penicillin (100 U/ml) and streptomycin (100 µg/ml; Sigma Chemical), and 10% FCS at 37°C in a 5% CO2 atmosphere. Passages 4 to 6 were used for experiments.

Isolation of PMN. Human PMN were obtained from the peripheral blood of healthy volunteers (anticoagulated with EDTA) by Lymphoprep density gradient centrifugation, followed by dextran sedimentation and hypotonic lysis of contaminating erythrocytes using aqueadestillata (Mayrrofer Phaumazetutika, Linz, Austria; see Ref. 27). Cell preparation yielded >95% polymorphonuclear cells (by morphology in Giemsa stain) and >99% viability (by trypan dye exclusion).

Renal endothelium-epithelium monolayer and bilayer construction. HK-2 cells were cultured on the bottom side of Transwell growth support, according to Joannidis et al. (11). A sterilized silicone ring was mounted around the inverted Transwell insert, and 7.5 × 10⁴ HK-2 cells in HK-2 medium (as described above) were added to the chamber. The following day, inserts were rehydrated and HDMEC cells in HDMEC medium (as described above) were seeded at 3.3 × 10⁴ cells/well. Bilayers were maintained in HDMEC medium. For the epithelial and endothelial monocultures, the same procedure was carried out with the exception that partner cells were not added. Monocultures and cocultures were maintained for 4/5 days in HDMEC medium.

Morphology. Filter-grown mono- and cocultures of the two cell types utilized were fixed for 15 min by 1% glutaraldehyde in PBS. Filters were washed in PBS and postfixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer, dehydrated in graded series of isopropanol, and embedded in Durcupane ACM (Fluka, Switzerland). Sections were stained by toluidine blue (light microscopy) or by uranylacetate and lead citrate (electron microscopy).

Assessment of coculture integrity. To confirm that the cocultures were confluent, the growth inserts were inspected daily by light microscopy. Additionally, the following measurements were performed.

Measurement of electrical resistance across the monolayer. The functional integrity was tested using a resistance meter (Endohm; World Precision Instruments). After establishment of insert cultures, the electrical resistance was monitored on a daily basis until confluence was achieved. All data were corrected for the resistance measurements across blank collagen-coated inserts that showed a resistance of 14.52 ± 2.31 Ω·cm² (n = 36; data from 6 experiments).

Assessment of the passage of FITC-labeled BSA. In a series of three experiments, 100 µl of phenol red-free RPMI 1640 (Biochrom; Berlin) containing 100 µg/ml FITC-BSA (Sigma, Chemical) were added to the apical compartment, and its passage across the coculture was monitored after 10 min and 4 h by sampling the apical and basolateral supernatant and measuring the fluorescent activity using the Cytoflour 2350 fluorescence system (Millipore). The ratio of fluorescent activity between the two samples was calculated. Filter alone without cells developed ~20% of equilibrium within 10 min, which increased to roughly 50% after 4 h (n = 3). In contrast, when HK-2 cells and HDMEC were cocultured on the growth support inserts, <1% and 5% (n = 3) of equilibrium was measured after 10 min and 4 h, respectively. Bilayers that exhibited an electrical resistance of >16 Ω·cm² showed a permeability of <5% FITC-BSA, indicating that a tight barrier was formed.

Leukocyte transmigration experiments. Preliminary experiments determined both the time courses and the appropriate number of PMN to add to the upper chamber of the Transwell growth arrest. Transmigration experiments were performed as previously described (11). Confluent monolayers or cocultures (composed of confluent HK-2 on the lower and confluent HDMEC cells on the upper side of the Transwell inserts) were washed twice in PBS and incubated with TNF-α (50 ng/ml) or LPS (100 µg/ml) for 4 h. Incubation of the monolayers with the test substances was followed by washing the upper and lower surfaces of the Transwell filter cups two times with PBS and then transferring them to new, clean wells. Next, 0.1 ml of PMN suspension (2.0 × 10⁶ cells/ml) was added to the upper compartments, and PMN were allowed to migrate across the bilayer for 5 h.

PMN contents of the cell layers and lower compartments were quantified by assaying the azurophil granule marker myeloperoxidase (MPO) as described previously (20) with slight modifications. Briefly, inserts containing the cocultures were washed four times with Hanks’ balanced salt solution (HBSS) to remove nonmigrated PMN. HBSS (0.6 ml) containing 10% Triton X-100 was added to the lower compartment to release the MPO from the neutrophils. The pH was adjusted with 100 µl of 1 M citrate buffer, pH 4.2. Color development was assayed at 405 nm on a microtiter plate reader (Labsystems Bioscan; Biochromatic) after mixing equal parts of sample and a solution containing 1 mM 2,2’-azino-di-(3-ethyl)-dithiozalumin sulfonic acid (Sigma Chemical) and 10 mM H₂O₂ (Sigma Chemical) in 100 mM citrate buffer, pH 4.2. After 5 min, the reaction was terminated by the addition of SDS to a final concentration of 0.5%. The assay was standardized with known numbers of the same PMN used in each individual experiment and was linear in the range used (0–10⁶ cells). MPO activity was negligible in lysates of cocultures unexposed to PMN.

In a subset of experiments, the bilayers were preincubated with various concentrations of α-MSH (10⁻⁷ to 10⁻¹² M) for 4–24 h. Each independent experiment was performed in duplicate. Because of the variations between the individual sets of experiments, data are expressed as “migration index,” which represents the ratio of PMN migration across stimulated cocultures and through unstimulated coculture in each experiment.

Cytokine release measurements. Supernatants were collected from the upper (endothelial) and lower (epithelial) compartment after 4 h of incubation with TNF-α (50 ng/ml) or LPS (100 µg/ml). Interleukin (IL)-8 (R&D Systems) and IL-6 (R&D Systems) were assayed by ELISA, according to the manufacturer’s instructions. The absorbance was measured with a microplate reader (Labsystems Multiscan Bichromatic).

Statistical analysis. The data are expressed as means ± SE. Unless indicated differently, each independent experiment in transmigration experiments was performed with cells from a different donor. Differences between means were tested for statistical significance. Student’s t-test or Mann-Whitney test was applied as appropriate. If ratios of stimulated migration were compared with controls (i.e., index = “1”) one-sample t-test for difference from a hypothetical mean (i.e., 1) was applied. If several experiments were compared against each other, one-way ANOVA followed by Bonferroni test for selected pairs was performed. P < 0.05 was considered statistically significant. GraphPad PRISM version 3.03 (GraphPad Software) was used for statistical analysis.
RESULTS

Morphology of mono- and coculture systems. Morphological examination of the mono- and coculture systems by light and electron microscopy provided some interesting findings (Fig. 1). HK-2 cells when grown in monoculture for 5 days exhibited basolateral extrusions through the filter pores, which spread across the surface of the underneath of the filter (Fig. 1). However, the HK-2 cells did not fully traverse the filter and remained in a polarized monolayer at the seeded side. Conversely, HDMEC cells traversed the filter and grew on both sides in a double monolayer (Fig. 1). When both cell types were cultured in coculture, there were less HK-2 extrusions through the filter, and the HDMEC remained on the seeded side (Fig. 1, E and F). In the coculture situation, there was a clear separation of the epithelial and endothelial monolayer.

Electrical resistance measurement of the bilayer model. We investigated the time course of barrier formation in the mono- and coculture systems (Fig. 2). HDMEC in monoculture exhibited a low electrical resistance throughout the experiment.

Fig. 1. Morphological examination of HK-2, human dermal microvascular endothelial cell (HDMEC) monocultures, and cocultures. Cells were seeded in polyester Costar Transwell supports with 3.0-μM pores as described. After 4–5 days of culture, the membranes were fixed and sectioned. A, C, and E are light microscopy images; B, D, and F are transmission electron microscopy images. A and B are HK-2 monolayers, C and D are HDMEC monolayers, and E and F are cocultures. Inset in F shows a Weibel-Palade body, a typical feature of the vascular endothelium. HK-2 cells in monoculture do not completely cross the filter membrane but exhibit cytoplasmic extrusions through the pores (A and B). HDMEC cells do completely cross the membrane and grow on both sides of the filter (C and D). When both cells are cultured together, there is less HK-2 extrusion in the membrane, and HDMEC remain on the side of the filter they were seeded (E and F).
formyl-Met-Leu-Phe (fMLP) (10 nM) toward a concentration gradient of the chemoattractant and LPS (100 μg/ml) of the bilayer model. Pretreatment with TNF-α (50 ng/ml) and LPS (100 μg/ml; Fig. 3) significantly increased the baseline transmigration across the cocultures by a factor of 2.76 ± 0.64 and 3.5 ± 0.58, respectively. A similar increase in transmigration index was observed when PMN were allowed to migrate toward a concentration gradient of the chemoattractant N-formyl-Met-Leu-Phe (fMLP) (10⁻⁶ M) in the epithelial compartment (migration index 2.51 ± 0.63).

Compared to controls (3.46 ± 1.54), HK-2 cells in monoculture exhibited a time-dependent increase in electrical resistance. There was no significant difference in electrical resistance between HK-2 monolayer and HDMEC-HK-2 coculture, indicating that permeability of tight junctions in HK-2 cells was not influenced by coculture conditions.

**Transmigration across the bilayer model.** Sequential migration of PMN across HDMEC and HK-2 cells was studied on the bilayer model. Pretreatment with TNF-α (50 ng/ml) and LPS (100 μg/ml; Fig. 3) significantly increased the baseline transmigration across the cocultures by a factor of 2.76 ± 0.64 and 3.5 ± 0.58, respectively. A similar increase in transmigration index was observed when PMN were allowed to migrate toward a concentration gradient of the chemoattractant N-formyl-Met-Leu-Phe (fMLP) (10⁻⁶ M) in the epithelial compartment (migration index 2.51 ± 0.63).

Comparison of transmigration through monolayers and bilayers. PMN migration across the bilayers showed different behavior from migration across single monolayers. As shown in Fig. 4, LPS (100 μg/ml)-stimulated transmigration of PMN across HK-2 cell monolayers was significantly lower (66.7%) when compared with migration across the HDMEC monolayer. PMN migration was less efficient across the bilayers than across the endothelial monolayers (HDMEC, 52.76%) and did not significantly differ from migration across single epithelial monolayers (HK-2).

IL-6 and IL-8 secretion of endothelial monolayers (HDMEC), epithelial monolayers (HK-2), and epithelial-endothelial coculture (bilayer). We investigated cytokine production in the mono- and coculture models. Supernatants of bilayers and of mono- and coculture models. Supernatants of bilayers and of HDMEC-HK-2 coculture model were collected. IL-6 and IL-8 were analyzed by ELISA (Fig. 5). HDMEC monolayers produced and secreted low levels of both IL-6 and IL-8 in the upper (21.45 ± 11.63 and 370.82 ± 168.82 pg) and in the lower (88.8 ± 9.74 and 870.29 ± 509.4 pg) compartment. HK-2 cells secreted higher amounts of IL-6 (179.55 ± 25.72 pg in the upper and 545.29 pg in the lower compartment, respectively) and also IL-8 (930.8 ± 151.4 pg in the upper and 4,009.4 ± 545.29 pg in the lower compartment, respectively). In the coculture system, similar amounts of cytokines were produced as in the HK-2 monoculture, indicating mainly epithelial cytokine contribution (Fig. 5, A and B).

Time- and dose-dependent effect of α-MSH on PMN migration. The effect of α-MSH on PMN migration across the coculture bilayer model was investigated. α-MSH resulted in a decreased PMN migration across the unstimulated coculture bilayer and resulted in a dose-dependent decrease in PMN migration across LPS-stimulated cells (Fig. 6A). α-MSH showed a maximal reversal of LPS-induced PMN migration at 9 h (Fig. 6B). There was no significant reversal of α-MSH on PMN migration in TNF-α-stimulated cells (Fig. 6C).

**Effects of α-MSH on cytokine release.** α-MSH did not have a significant effect on cytokine production when cells were unstimulated. However, α-MSH at 10⁻⁷ M did result in a decrease in IL-6 release from LPS-stimulated cocultures by act.
32.3% (Fig. 7A). IL-8 secretion was not significantly affected by α-MSH (Fig. 7B).

**DISCUSSION**

A variety of in vitro model systems have been established to investigate the migration of inflammatory cells through cell barriers. These studies mainly concentrate on the migration of leukocytes through single monolayers (endothelial, epithelial, and mesothelial cells; see Refs. 1, 20, and 29) However, the in vivo situation is more intricate. In several organs (lung, intestine, etc.), including the kidney, neutrophils traverse both the endothelial lining of blood vessels and the epithelium during acute inflammation. To reach the epithelial surface, PMN need to cross the vascular wall and pass through the interstitial matrix. This process may have effects on epithelial function mediated by the release of factors and subsequent signaling events (28).

In an attempt to mimic this in vivo situation, we developed a bilayer model that allows communication and interaction of human endothelial and tubular epithelial cells cultured on opposite sides of Transwell growth supports. This model responded with enhanced PMN migration and cytokine produc-
endothelial migration may increase leukocyte motility, facilitating the subsequent passage through the epithelial monolayer. It has been reported that diapedesis through the endothelium leads to changes of the leukocyte physiology. PMN, in contact with inflammatory endothelium, showed reduced apoptotic activity and enhanced phagocytosis (19). We observed that the number of monolayer- and bilayer-associated PMN (PMN that only partly traverse the cell barriers) was comparable, indicating that accumulation between the two cell layers did not occur (data not shown). Additionally, endothelial and epithelial cells may influence each other through the release of soluble factors promoting migration across the bilayer. In the cocultures, there was a higher concentration of IL-6 and IL-8 in the upper (endothelial) compartment compared with endothelial monolayers. Although it would be possible to interpret these findings as enhanced cytokine production by the endothelial cells under the condition of coculture with epithelia, it is most likely that cytokines were released by the basolateral membrane of the epithelial cells and consequently crossed the endothelial cell layer. IL-6 is known to inhibit apoptosis of PMN in vitro (6), whereas IL-8, a potent chemoattractant that can be produced by endothelial and epithelial cells (2), plays a critical role in recruitment of PMN to sites of inflammation (26).

Excessive recruitment of PMN in the tubulointerstitium is associated with tissue damage and deterioration of renal function. Decreased PMN migration into areas of inflammation is considered to exert a beneficial effect. In previous studies, α-MSH, a neuropeptide with broad anti-inflammatory properties, showed renoprotective effects in animal models of ischemia and endotoxemia (22). In this study, we demonstrate that α-MSH inhibits PMN migration across a human endothelial/tubular epithelial bilayer in a time- and dose-dependent manner after stimulation with LPS. In contrast, the inhibitory effect of α-MSH on TNF-α-stimulated migration was only weak and did not reach statistical significance. Why the inhibitory effect of α-MSH was more pronounced after LPS stimulation remains unclear. Scholzen et al. (24) demonstrated that α-MSH reduced, via a not yet identified mechanism, the inflammatory response of various stimuli, including LPS, TNF-α, and IL-1. Although α-MSH acts as a potent inhibitor of LPS-activated nuclear factor-κB, the effect of α-MSH on TNF-α-treated endothelial cells could be mimicked by the protein kinase A activator forskolin. This indicates that α-MSH may exert its effect by interfering with several signal transduction pathways, e.g., via elevation of cAMP (4), protein kinase C (3), or intracellular calcium release (8).

The inhibitory effect of α-MSH peaked at 9 h when added simultaneously with LPS to the bilayer. PMN express the MC-1 receptor and α-MSH reduces chemotaxis (4). One could speculate that the reduced chemotactic activity might be responsible for the observed reduction of PMN migration. However, application of α-MSH after stimulation with LPS resulted in a minor inhibition of migration. This suggests α-MSH may affect both the PMN and the endothelial cells. Although an anti-inflammatory and cytoprotective effect of α-MSH have been reported also for tubular epithelial cells (10, 14), transmigration across HK-2 monolayers was not influenced by α-MSH (data not shown), making their importance in α-MSH-inhibited migration unlikely.
Our data show sequential migration of PMN across endothelial and renal tubular epithelial cells. Transmigration is stimulated by TNF-α and LPS. Anti-inflammatory effects of α-MSH could be verified on this bilayer model after incubation stimulated by TNF-α and LPS. Anti-inflammatory drugs protect against renal injury after ischemia-reperfusion injury. 

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REFERENCES


