Mechanisms of Neutrophil Transmigration Across Renal Proximal Tubular HK-2 Cells

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Key Words

Transepithelial migration • TNF α • LPS • IL-8 • HK-2 • Neutrophils

Abstract

Background: Adhesion of intratubular leukocytes to proximal tubules in biopsies of patients with rapidly progressive glomerulonephritis and the appearance of leukocytes in the urine in interstitial nephritis suggest interactions between leukocytes and tubular epithelia in renal diseases. The aim of this study was to investigate the effect of cytokines and endotoxin on leukocyte migration through proximal tubular epithelial cells and also to determine the role of the transmembrane adhesion molecules ICAM-1 and CD47 in this process. **Methods:** Experiments determined transepithelial migration (TEM) of PMN (polymorphonuclear) leukocytes through monolayers of HK-2. Expression of ICAM-1 and CD47 was assessed via confocal immunofluorescence, FACS analysis and western blotting. The effect of antibodies against ICAM-1 and CD47 on TEM was examined. Furthermore measurements of cytokine release (IL-6 and IL-8) were performed. **Results:** Preincubation

of HK-2 cells with either TNF α or LPS resulted in stimulation of PMN migration through monolayers of HK-2 cells. There was no preferred direction of transmigration. ICAM-1 was expressed by HK-2 cells and expression was increased after 4 h stimulation with TNF α or LPS. Application of ICAM-1 antibodies inhibited TEM. CD47 was expressed in both HK-2 cells and PMN. CD47 antibodies inhibited predominantly basolateral-to-apical TEM. HK-2 cells released IL-8 and IL-6 preferably into the apical compartment. Additionally, we showed that fMLP induced transmigration through monolayers of HK-2 cells was associated with significant increased CD47 expression on PMN cell surfaces. **Conclusions:** Inflammatory mediators stimulate TEM of PMN through monolayers of HK-2 cells without a clearly discernible preference of direction. Mechanisms involved in TEM stimulated by cytokines or endotoxin appear to be mainly changes in surface receptor densities of HK-2 cells with ICAM-1 and CD47 playing an essential role.

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Introduction

Several kidney diseases appear to be associated with migration of leukocytes across renal tubular epithelia. The appearance of leukocytes in the urine during interstitial nephritis and other kidney diseases suggests transmigration from basolateral to the apical side of the tubulus. Furthermore, intratubular leukocytes were shown to adhere to ICAM-1 positive proximal tubular epithelial cells in biopsies of patients with rapidly progressive glomerulonephritis (GN), suggesting the possibility of migration of leukocytes even from tubular space into the renal interstitium [1, 2].

Although transmigration of leukocytes through epithelial layers is well characterised in intestinal cells [3, 4] much less is known about these processes in the kidney. Transmigration of either PMN or exudate peritoneal cells through MDCK cell monolayers, a canine renal cell line exhibiting many features of distal tubular cells [5], was examined but required a chemo attractant concentration gradient [6, 7]. We have previously demonstrated that inflammatory mediators stimulate PMN adherence to monolayers of LLC-PK1 cells and subsequently their transepithelial migration (TEM) [8]. Basolateral-to-apical migration was the favoured direction, most likely augmented by IL-8 associated haptotactic PMN stimulation [8]. Due to the use of cell types of different species (porcine epithelial cells and human PMN) a detailed investigation of underlying mechanisms was not possible.

Generally, transmigration is a well-orchestrated process, closely regulated by integrins and members of the immunoglobulin gene superfamily (IgSF), which is one of the major families of cell surface adhesion molecules comprising ICAM, PECAM and VCAM [9]. A pivotal role for the $\beta 1$ and $\beta 2$ - integrins, interacting with their epithelial ligands ICAM-1 and VCAM, has been described [10, 11]. ICAM-1 expression is upregulated in different kidney disorders associated with inflammation such as acute transplant rejection, rapid progressive glomerulonephritis or acute renal failure due to ischemia and/or reperfusion [12-15]. Thus ICAM-1 is potentially a crucial factor in transepithelial migration of leukocytes [16]. However, other IgSF members may also participate in this process. One of the candidate proteins in postadhesive stages of PMN migration across epithelia is the widely expressed CD47 (integrin-associated protein/IAP). Originally, CD47, a transmembrane glycoprotein, was identified in association with $\alpha v \beta 3$ integrins. CD47 plays a immunoregulatory role, as demonstrated in CD47

knock-out mice that have a defect in PMN recruitment and therefore suffer from increased susceptibility of infection [17].

The aim of the present study was to investigate the mechanisms of transepithelial migration further in an in vitro model using both, human proximal tubular cells and human PMN. The role of adhesion molecules as well as interleukin secretion in the process of transepithelial migration of leukocytes was examined.

Materials and Methods

Cell culture

HK-2 cells (human renal epithelial cell line with features of proximal tubular cells) [18] obtained from the American Type Culture Collection (ATCC, Rockville, USA) were used in passages 20-33. HK-2 cells cultured in Dulbecco's modified Eagle's medium (DMEM/HAM'S F-12, PAA Laboratories, Linz, Austria) supplemented with 10% fetal calf serum (FCS, Biological Industries, Kibbutz Beit Haemek, Israel), L-glutamine (2 mM, Biological Industries, Kibbutz Beit Haemek, Israel), penicillin (100 U/ml) and streptomycin (100 μ g/ml), both Sigma, Chemical Corp., Deisenhofen, Germany.

Isolation of polymorphonuclear neutrophils (PMN)

Human PMN were obtained from peripheral blood of healthy volunteers (anticoagulated with EDTA) after discontinuous density gradient centrifugation on Percoll by dextran sedimentation and centrifugation through a layer of Ficoll-Hypaque, followed by hypotonic lysis of contaminating erythrocytes using bi-distilled water (Fresenius Kabi, Austria) [19]. Cell preparations yielded > 95% PMN (by morphology in Giemsa stains) and > 99% viability (by trypan dye exclusion).

Culture of HK-2 cells on Transwell® tissue culture plates

6 mm polyester filter inserts with 3 μ m pores (Transwell® -Clear 3472, Costar, Cambridge, MA) were prepared by overnight incubation in 10% FCS containing medium prior to seeding cells. 2.0×10^5 HK-2 cells/insert were seeded onto either non-inverted (apical-basolateral migration) or inverted (basolateral-apical) inserts. Inverted filters were reverted on the day after seeding. HK-2 formed a tight permeability barrier within 3-5 days. Medium was exchanged for fresh medium three times a week.

Test of HK-2 monolayer permeability

Confluence of the HK-2 monolayers was verified microscopically and functional integrity (tightness) by daily measurement of transepithelial resistance with Endohm 6 TM/EVOM TM system (World Precision Instruments, Sarasota, FL). Cells were considered to have reached confluence when they exhibited a transepithelial resistance (TER) higher than 17 Ω cm². In preliminary experiments we tested the monolayer permeability using FITC labeled albumin which was added to the upper compartment of the growth supports. 50 μ l samples

were taken from the upper compartment and from the lower chamber beneath the HK-2 monolayers growing on the growth and fluorescence was measured with CytoFluor 2350 fluorescence measurement system (Millipore Corp) with excitation at 485 ± 20 nm and emission at 530 ± 25 nm. The ratio of fluorescence activity between the two samples was calculated. Without HK-2 monolayers about 25% of equilibrium developed within 10 minutes, which increased to roughly 50% after 4 hours. When HK-2 cells exhibited a transepithelial resistance higher than $15 \Omega \text{ cm}^2 < 1\%$ and 5% of equilibrium was measured after 10 minutes and 4 hours, respectively, indicating that a tight barrier was formed. To avoid the possibility that HK-2 cells formed a second layer on the underside of the growth supports, experiments were performed immediately after cells had reached confluence.

Assay of neutrophil transmigration through renal epithelial monolayers (Transepithelial migration (TEM))

In preliminary studies kinetic and dose response experiments of PMN adhesion to tubular cells were performed. The maximal response could be observed after 4 hours. Therefore it was chosen as incubation period for further experiments. For "apical-to-basolateral" TEM experiments [20, 21] confluent HK-2 monolayers grown on Transwell™ growth supports were washed twice with PBS, incubated with TNF α (50 ng/ml) or LPS (100 $\mu\text{g/ml}$) for 4 hours or with medium alone (control). Incubation of the monolayers with the test substances was followed by washing the upper and lower surfaces of the Transwell™ growth supports twice with PBS and then transferring them to new, clean wells (lower compartments). Thereafter 0.1 ml of PMN suspension (2×10^6 cells/ml) was added to the upper compartments and PMN were allowed to migrate across the epithelial monolayers for 5 hours.

Transmigration of PMN was quantified as described previously [9] with some modification. Briefly, migration of PMN was stopped by washing and thereby removing non-migrated PMN from the upper surfaces of the monolayers. Thereafter, the tissue culture plates bearing the Transwell™ growth supports were centrifuged in a Beckman GPR centrifuge (1800 rpm, 10 min) in order to pellet migrated PMN on the bottom of the lower compartments. PMN were resuspended in HBSS (Gibco, Invitrogen™) and loaded with 2 μM 2', 7'-bis-(2-carboxyethyl)-5, 6-carboxyfluorescein-acetoxymethylester (BCECF AM; Molecular Probes Inc., Eugene, Oregon, USA). After washing twice with HBSS, the number of migrated cells was determined by measuring the fluorescence of BCECF AM-loaded cells using a microtitre plate reader (Cytofluor®, PerSeptive Biosystems, USA). Fluorescence was read at 530 ± 25 nm. Each independent experiment was performed in duplicate. Data are expressed as "Transmigration Index", which represents the ratio of PMN migration across stimulated monolayers divided by the number of PMN migrated through unstimulated epithelial monolayers.

To investigate transmigration for the "basolateral-to-apical" direction the same procedure was performed with the cells cultivated on the underside of the Transwell™ growth supports (i.e. cells were cultured first on inverted filters, which

were subsequently reverted) [8].

Cytokine release from HK-2 cells

Supernatants were collected from the apical and basolateral compartment after 4 hours of incubation with TNF α (50 ng/ml) or LPS (100 $\mu\text{g/ml}$) and assayed for IL-8 (Biosource, Nivelles, Belgium) and IL-6 (R&D Systems Inc. USA) by ELISA.

Immunofluorescence Microscopy

For immunofluorescence staining HK-2 cells were grown on culture slides (BD Falcon™). After incubation with mouse anti-human ICAM (R&D Systems, Minneapolis, MN) or mouse anti-human CD47 (Research Diagnostics, INC) cells were fixed 20 min at room temperature in 3.6 % paraformaldehyde (Merck, Darmstadt, Germany) and washed with PBS containing 0.2 % bovine serum albumin (BSA, Sigma Aldrich, St. Louis, MO). Cells were stained with secondary goat anti-mouse coupled to Alexa-488. Nuclei were stained with DAPI (Molecular Probes, Oregon, USA). Images were captured with a Zeiss Axioskope 2 (Zeiss, Göttingen, Germany) and Axiovision Rel 4.3. software using identical acquisition parameters per experiment. Images were then processed and overlaid using Adobe Photoshop 7.0 software (Adobe Systems, San Jose, CA, USA).

For confocal images cells were grown on culture slides (BD Falcon™). Confocal images were acquired using a Zeiss Laser Scan Microscope (LSM 410 invert). Slides were excited at the 488-nm wavelength of the argon laser passing a Plan-Apochromat 100/1.4 NA oil objective.

FACS analysis of surface molecule expression on HK-2 cells and PMN

HK-2 cells were treated with trypsin and a total of 5×10^5 cells were washed twice in Dulbecco's phosphate buffered saline, containing 0.5% bovine serum albumin and incubated with 150 $\mu\text{g/ml}$ human IgG for 20 minutes at 4°C. After pelleting, cells were incubated with 10 $\mu\text{g/ml}$ anti ICAM-1, VCAM-1, (both from R&D Systems, Minneapolis, MN) or CD47 (Research Diagnostics, INC) antibodies or the respective isotype matched control IgG (eBioscience, San Diego, CA) for 30 minutes at 4°C. After washing, cells were incubated with 10 $\mu\text{g/ml}$ biotinylated mouse anti-mouse IgG (eBioscience, San Diego, CA) for another 30 minutes. Then cells were washed twice with PBS and subsequently incubated with a 1:25 dilution of streptavidin-PE® (Phycoerythrin) (Becton Dickinson, San Jose, CA). Cells were washed twice and then immediately analyzed on a FACS with Cellquest software (Beckton-Dickinson FACScan, San Jose, CA).

Western Blot Analysis of ICAM-1 and CD47

HK-2 cells were incubated with TNF α (50 ng/ml) or LPS (100 $\mu\text{g/ml}$) for 4 hours or with medium alone (control). Cells were lysed in lysis buffer for 20 min. Proteins were separated on 7.5% SDS polyacrylamide gels and blotted onto polyvinylidene fluoride membranes. After blocking, blots were incubated overnight at 4° C with either ICAM-1 or CD47 antibodies. Immunoreactivity was determined by using peroxidase-conjugated goat-anti-mouse IgG and the enhanced chemiluminescence reaction (Amersham). Western blot bands

Fig. 1. Transmigration (TEM) of PMN across HK-2 cells. HK-2 monolayers were stimulated from both sides for 4 h with TNF α (50 ng/ml) or LPS (100 μ g/ml). Medium served as control. After washing twice, 2×10^5 PMN were allowed to transmigrate for 5 hours. Migrated cells in the lower compartment were measured fluorometrically as described. Data are expressed as “transmigration index” which is the ratio between stimulated and basal migration of PMN. Values represent mean $\pm$ SEM, n=6. Migration index of spontaneous migration = 1. * $p < 0.05$ vs. spontaneous transmigration. Statistical analysis: Student’s t-test.

were analysed by Fluor-S MultiImager System and the Quantity One Software (BioRad Laboratories).

Effects of ICAM-1 and CD 47 antibodies on transmigration

To investigate a possible influence of ICAM-1 on PMN transmigration through HK-2 monolayers, TEM was measured in the presence of a blocking 10 μ g/ml ICAM-1 antibodies (R&D Systems, Minneapolis, MN) or 10 μ g/ml CD47 antibodies (Research Diagnostics, INC), respectively added to the upper compartment. After preincubation of HK-2 cells with TNF α (50 ng/ml), LPS (100 μ g/ml) or medium (control) for 4 hours cells were washed and the respective antibody was added to the upper compartment for 30 minutes. Cells were washed again and PMN transmigration was measured, as described above.

To discriminate between inhibitory effects of CD47 antibodies on epithelia and PMN, a separate set of experiments was performed where TEM was measured in the continued presence of the CD47 antibodies throughout the migration period, i.e. without washing before transmigration.

Influence of TEM on CD47 expression on PMN

Epithelial cells were cultured to confluence on Transwell™ filters. PMN were placed in the upper chamber of the Transwell™ and induced to migrate into the fMLP (1 μ g/ml) or IL-8 (10^{-8} M) containing lower chamber. To assay PMN surface CD47 expression during transmigration, PMN that remained in the upper chamber or had migrated into the lower chamber of Transwells™ were cooled on ice followed by antibody labelling and flow cytometry as described above.

Statistics

The data are presented as mean $\pm$ standard error of the mean (SEM) of at least three experiments. Where PMN were used, each independent experiment was performed with cells from a different donor. Differences between the means were tested for statistical significance with Student’s t-test or Wilcoxon Signed Rank, as appropriate. P values of less than 0.05 were considered significant.

Results

Transepithelial Migration

A) Apical-to-basolateral migration. The number of PMN that had migrated across the unstimulated HK-2 monolayers in apical-to-basolateral direction was 11200 ± 3200 (mean $\pm$ SEM, n = 6) which correspond to 5.6% of the PMN added to the upper compartment. Both incubation with LPS and TNF α resulted in an increase in transmigration index by a factor of 4.1 ± 0.70 and 3.4 ± 0.62 ($p < 0.05$), respectively (figure 1).

B) Basolateral-to-apical migration. PMN migration across unstimulated HK-2 monolayers in basolateral-to-apical direction was 17300 ± 2750 (mean $\pm$ SEM, n = 9) which corresponds to 8.7% of the PMN added to the upper compartment. Preincubation with TNF α (50 ng/ml) resulted in a TEM index of 2.6 ± 0.88 ($p < 0.05$). LPS (100 μ g/ml) increased transmigration by a factor of 4.8 ± 0.57 ($p < 0.05$, figure 1). Transmigration indexes determined for basolateral-to-apical direction did not show statistically significant differences from those measured for apical-to-basal direction.

Epithelial cytokine production

IL-8 and IL-6 production of HK-2 cells was determined by measuring respective concentrations in the supernatants of apical and basolateral compartment. Measurable levels of IL-8 could be detected after 4 hours of incubation with medium alone. Incubation with TNF α (50 ng/ml) or LPS (100 μ g/ml) for 4 hours further increased the IL-8 production by a factor of 20 and 11, respectively. IL-8 was secreted preferentially into the apical compartment. Apical concentration was approximately 3 fold higher than in the basolateral

Fig. 2. Cytokine secretion by HK-2 cells. IL-8 (A) and IL-6 (B) concentrations were determined in supernatants from apical or basolateral compartments. TNF α (50 ng/ml), LPS (100 μ g/ml) or medium (=control) was added to both compartments for 4 hours. Values represent mean $\pm$ SEM, n=3, * p<0.05 apical versus basolateral compartment, #p<0.05 compared to control in the apical compartment, statistical analysis: Wilcoxon Signed Rank.

Table 1. Surface expression of ICAM-1 and CD47 on HK-2 cells. HK-2 cells were exposed to medium, TNF α (50 ng/ml) or LPS (100 μ g/ml) for 4 hours to investigate the expression of ICAM-1 (n=4) and CD47 (n=3) by FACS-Analysis. An isotype matched mouse IgG was used as control (incubated with medium). Results are given as mean fluorescence intensity (MFI) $\pm$ SEM, * p < 0.05 compared to control (medium), statistical analysis: Student's t-test.

	Mean fluorescence intensity (MFI) $\pm$ SEM			
	isotype control	control	TNF α	LPS
ICAM-1	9.28 $\pm$ 1.23	49.02 $\pm$ 13.87	83.66 $\pm$ 21.08*	72.02 $\pm$ 20.82*
CD47	3.72 $\pm$ 0.99	11.56 $\pm$ 1.86	13.21 $\pm$ 5.71	16.45 $\pm$ 2.53

compartment under unstimulated conditions and increased to 4 times after stimulation with TNF α or LPS (figure 2A).

IL-6 was also detectable in considerable amounts under basal conditions. Stimulation with TNF α and LPS resulted in a roughly twofold increase of IL-6 in the apical compartment whereas the increase in the basolateral compartment did not reach statistical significance. Apical concentrations were consistently higher than those measured in the basolateral compartment after stimulation with TNF α or LPS (163 % and 154%, respectively) (figure 2B).

Immunofluorescence microscopy

Expression of ICAM-1 and CD47 in HK-2 was evaluated by indirect immunofluorescence. As shown in figure 3 treatment of HK-2 cells with TNF α and LPS resulted in an increased ICAM-1 expression compared to control. CD47 expression could be detected in unstimulated HK-2 cells. Incubation with TNF (50 ng/ml) and LPS (100 ng/ml) did not alter CD47 expression (figure 3D). Z-scan confocal images demonstrate a prominent ICAM expression on the apical and lateral side of the HK-2 cells (Figure 3B, 3C). CD47 was predominantly localized on the external side of the apical pole. (Figure 3E, 3F).

Fig. 3. Expression of ICAM-1 on HK-2 cells: (A) Immunofluorescence staining of ICAM-1 on HK-2 cells after incubation with LPS (100 µg/ml), TNF α (50 ng/ml) or medium (=control) for 4 hours. HK-2 cells were stained with mouse anti-ICAM-1 antibodies and secondary Alexa-488-conjugated anti-mouse antibodies. Nuclei were marked with blue fluorescent DAPI. (B) A series of confocal horizontal optical cross-sections is shown, the most left micrograph representing the apical side of HK-2 cells. (C) Vertical slices of ICAM expression (XZ and YZ sections) in a HK-2 cell. Expression of CD47 on HK-2 cells: (D) Immunofluorescence staining of CD47 (A) on HK-2 cells after incubation with LPS (100 µg/ml), TNF α (50 ng/ml) or medium (=control) for 4 hours. (E) Confocal laser scanning of CD47 expression showing xy sections on HK-2 cells. (F) Vertical sections (XZ and YZ sections) of CD47 distribution on HK-2 cells.

Fig. 4. Western Blot analysis of ICAM-1 and CD47. Effect of $\text{TNF}\alpha$ and LPS on ICAM-1 and CD47 expression on HK-2 cells. Representative Western Blots of ICAM-1 and CD47 under control condition and following pretreatment with $\text{TNF}\alpha$ and LPS for 4 hours. Beta Actin served as loading control.

Epithelial expression of adhesion molecules

Since transmigration of PMN through HK-2 monolayers may be mediated by the binding and activation of adhesion molecules, surface expression of several such proteins were analysed by FACS. ICAM-1 was detected on HK-2 cells under basal conditions. A significant shift in fluorescence was observed in HK-2 cells after incubation with $\text{TNF}\alpha$ and LPS (1.9 and 1.7 fold above control) (Table 1). CD47 was also detected in unstimulated HK-2 cells. The expression of CD47 was only slightly influenced by incubation with $\text{TNF}\alpha$ or LPS (Table 1). Control cells did not express detectable levels of VCAM-1 and P-Selectin. $\text{TNF}\alpha$, but not LPS treatment resulted in VCAM-1 expression, whereas incubation with $\text{TNF}\alpha$ or LPS showed only minor surface expression of P-Selectin on HK-2 cells (data not shown).

$\text{TNF}\alpha$ and LPS induced activation of ICAM-1 and CD47

Western Blots analysis of whole cells extracts revealed slight immunoreactivity bands demonstrating the presents of ICAM-1 and CD47 under unstimulated conditions. Exposure of HK-2 cells with $\text{TNF}\alpha$ or LPS for 4 hours resulted in an increased amount of ICAM-1. Furthermore, also CD47 expression of HK-2 cells could be increased after incubation with both $\text{TNF}\alpha$ and LPS (figure 4).

Fig. 5. Effect of transmigration on CD47 expression by PMN. Flow cytometric determination of PMN cell surface CD47 after transmigration across HK-2 monolayers. HK-2 cells were cultured to confluence on inverted Transwell-filters as described. fMLP ($1\mu\text{M}$) or IL-8 (10^{-8} M) was placed in the lower compartment and PMN (1×10^6) were allowed to migrate across HK-2 monolayers (from basolateral-to-apical direction). After 2 hours PMN were collected from the upper and the lower chamber followed by surface labelling of CD47 for FACS analysis. CD47 expression on PMN under basal conditions (A), CD47 expression after transmigration towards fMLP (B) and IL-8 (C).

Fig. 6. Effect of ICAM-1 antibodies on PMN transmigration (TEM) through TNF α or LPS stimulated HK-2 monolayers. HK-2 cells were either cultured on the bottom or on the top of growth supports as described. Confluent monolayers were incubated with TNF α (50 ng/ml), LPS (100 μ g/ml) for 4 hours. Next, blocking ICAM-1 antibodies were added for 30 min either to the apical (TEM in apical-to-basolateral direction) or to the basolateral membrane (TEM in basolateral-to-apical direction). After washing, PMN were added and allowed to migrate for 5 hours. Migration rates in the absence of antibodies were expressed as 100%. Values represent mean $\pm$ SEM (n=6 for apical-to-basolateral TEM, n=4 for basolateral-to-apical TEM); * p<0.05 vs. incubation without ICAM-1 antibodies (Student's t-test).

Fig. 7. Effect of CD47 antibodies on PMN migration through TNF α or LPS stimulated HK-2 monolayers. Confluent HK-2 monolayers cultivated on transwell growth supports were treated with either TNF α (50 ng/ml) or LPS (100 μ g/ml) for 4 hours. Neutrophils in the presence of CD47 antibodies (50 ng/ml) were added to the basolateral compartment and allowed to transmigrate at 37°C for 5 hours. Medium was used as control. Stimulated migration in the absence of antibodies was expressed as 100%. (A) Transmigration of PMN across HK-2 cells in the presence of CD47 antibodies throughout the whole experiment. (B) Transmigration of PMN across HK-2 cell after preincubation with CD47 antibodies for 30 min and consequent removal of antibody by washing with HBSS before transmigration of PMN. Values represent mean $\pm$ SEM (n=4); * p< 0.05 vs. control (Student's t-test).

Influence of TEM on CD47 expression on PMN
The influence of transmigration through HK-2 monolayers on CD47 expression of PMN was measured by FACS analysis. HK-2 cells were cultured to confluence on Transwell™ growth supports as described. fMLP (1 μ M) or IL-8 (10⁻⁸ M) was placed in the lower compartment and PMN (1x10⁶) were induced to migrate across HK-2 monolayers (basolateral-to-apical direction). CD47 was expressed by PMN constitutively. Whereas fMLP-induced transmigration through epithelial monolayers was associated with significantly increased CD47 expression, IL-8-induced transmigration resulted only in a slight increase of CD47 expression on neutrophils (figure 5).

Role of adhesion molecules in transepithelial migration of PMN

A) Effects of ICAM-1 antibodies on transmigration. Apical addition of ICAM-1 antibodies before apical-to-basolateral transmigration had a significant effect on transmigration. TEM stimulated by preincubation with either TNF α (50 ng/ml) or LPS (100

µg/ml) for 4 hours was significantly inhibited by 89% and 90,1%, respectively after apical incubation with an ICAM-1 antibody (figure 6).

Basolateral addition of ICAM-1 antibodies before basolateral-to-apical transmigration was less effective, inhibiting LPS induced TEM by 65% ($p < 0.05$) and TNF α induced TEM by 60% (n.s.) (figure 6).

B) Effects of CD47 antibodies on transmigration.

Basolateral addition of CD47 antibodies prior to basolateral-to-apical transmigration inhibited both TNF α and LPS stimulated TEM (figure 7A) by 73% and 82%, respectively ($p < 0.05$). Preincubation of HK-2 monolayers with CD47 antibodies did not result in significant inhibition of stimulated TEM (figure 7 B). No inhibitory effect of CD47 antibodies on apical-to-basolateral transmigration could be observed (data not shown).

Discussion

Clinical findings suggest that interactions between leukocytes and renal epithelia play a significant role in acute and chronic inflammatory processes of the kidney [22-24]. Leukocyte infiltration is a characteristic feature of interstitial inflammation leading to stimulation of proximal renal tubular epithelia which in turn become involved in the inflammatory cascade and may have a proactive and perhaps independent role in amplification of renal injury [25, 26]. To further investigate the interaction between these two cell types we examined the effects of cytokine or endotoxin stimulation on a human renal cell line (HK-2 cells) [27]. Following stimulation we observed predominantly apical release of the cytokines IL-8 and IL-6 (figure 2) as well as transepithelial migration of PMN (figure 1). In addition we could demonstrate an essential involvement of ICAM-1 and CD47 in transmigration through HK-2 monolayers.

Preincubation with TNF α and LPS stimulates transmigration of PMN through HK-2-cell monolayers. Although this has been reported for non human renal epithelial cell lines such as MDCK cells or LLC-PK1 cells [8, 28, 29], the mechanisms involved in this process are not yet fully elucidated. Based on our previous work we investigated the role of cytokines in TEM. HK-2 cells responded to incubation with TNF α or LPS by releasing the cytokines IL-6 and, to a higher extend, IL-8. Enhanced release of these cytokines may have contributed to the observed higher migratory response. IL-6 is known to enhance survival of PMN in vitro [30] and has been shown to decrease cell-cell association of carcinoma cells

[31]. IL-8 is a potent PMN chemoattractant produced by epithelial and endothelial cells [8, 32, 33]. There appeared to be a preference for apical secretion of both cytokines by HK2 cells after stimulation with TNF α or LPS. A similar observation was made for human peritoneal mesothelial cells [34]. If the observed concentration gradient between the apical and basolateral compartment had been the major driving force for transepithelial migration, one would have expected a higher rate for basolateral-to-apical migration in comparison to the opposite direction. However, this was not the case in our experiments, where migration rates in both directions were of similar magnitude. This is in contrast to other reports where transmigration in intestinal epithelia showed quantitative differences clearly preferring basolateral-to-apical direction [35]. We found the same preference for direction of TEM in a previous study using a heterogeneous system of human neutrophils and porcine LLC-PK1 cells [8]. A possible explanation for this discrepancy might be differences in the arrangement of tight junctions. HK-2 cells exhibit a much lower transepithelial resistance than LLC-PK1 ($15 \pm 2 \Omega \text{ cm}^2$ vs. $>95 \Omega \text{ cm}^2$, respectively; personal observation) or intestinal cells. More leaky junctional complexes in HK-2 cells as compared to LLC-PK1 cells would presumably confer less resistance to PMN migration in apical-to-basolateral direction. Another explanation for different behaviour in transmigration could be reduced compatibility between necessary adhesion molecules when using cells from different species as was the case when we used LLC-PK1 cells [8]. A third explanation could be the formation of cell layers on both sides of the growth supports [36]. We did, however, carefully exclude this possibility by checking transepithelial resistances daily and performing the experiments on the first day when HK-2 cells reached confluency. In any case in our human model there exists an IL-8 gradient which does not significantly alter the direction of migration.

As described for leukocyte movement across endothelia [37] PMN migration across epithelial monolayers is likely a multistep process requiring engagement and disengagement of several receptor-ligand complexes between PMN and epithelial cells. There appears to be a dependency of PMN migration on ICAM-1, as shown for mesothelial [38] and airway epithelial cells. [39]. TNF α and LPS have been shown to induce ICAM-1 expression in tubular epithelial cells [40, 41]. In FACS and immunofluorescence experiments we demonstrated an increased expression of ICAM-1 by HK-2 cells following 4 hours of incubation with either

TNF α or LPS (figure 3 and table 1). Upregulation of ICAM-1 expression on HK-2 cells after stimulation with TNF α has previously been reported [42] and could partly explain higher transmigration rates observed after stimulation. This is supported by the observation that preincubation with ICAM-1 antibodies were able to prevent migration almost completely (figure 6). This inhibitory effect was more pronounced for apical-to-basolateral TEM than vice versa. Previous studies of renal biopsies could demonstrate, that ICAM-1 is expressed mainly on the apical membrane in renal epithelial cells [43]. We found an apical expression of ICAM-1 on HK-2 cells and could demonstrate a functional role of ICAM-1 for apical-to-basolateral transmigration. This seems not to be the case for the basolateral-to-apical direction. The predominant apical expression of ICAM-1 on HK-2 cells (figure 3) is probably why apical addition of antibody gives a more potent inhibition. We also investigated other adhesion molecules thought to be involved in transmigration [44, 45] such as VCAM-1 and P-Selectin (data not shown) but failed to detect significant expression on the surface of HK-2 cells. Thus, a role for these adhesion molecules in TEM is unlikely.

Finally we examined the role of CD47 in TEM. CD47 is a multiple, membrane-spanning IgG member and it is universally expressed on most cell types [46]. Since previous studies with CD47-deficient mice demonstrated an impaired host defence associated with a defect in PMN accumulation at the infection site, it has been suggested that CD47 may play a role in regulating leukocyte migration during the inflammatory response [47]. Our experiments support the importance of CD47 in PMN trafficking also in renal epithelia. In addition to the expected expression of CD47 on PMN, we could also detect significant CD47 expression on the apical and lateral surface of HK-2 cells (figure 3 and table 1). Furthermore, transmigration experiments performed in the presence of CD47 inhibitory antibodies showed approximately 80% reduction in the basolateral-to-apical migratory response. Migration in the other direction however could only be moderately inhibited. The inhibitory effect on migration by CD47 antibodies appears to be exerted mainly on PMN since application of antibody on epithelia alone did not have any effect. Using immunofluorescence microscopy and FACS analysis we showed that apical CD47 expression is not influenced by TNF α and LPS. The reason for the differences of

inhibition of migration in the two opposite directions investigated is, at the moment, unclear. One possible explanation could be a polarised distribution of CD47 on HK-2 cells which we can not exclude. Due to methodical limits we could visualise only apical expression of CD47. In contrast to our experiments with renal epithelia Liu et al. reported that anti-CD47 treatment resulted in a significant delay but not in complete inhibition [48]. We did observe an inhibitory effect of CD47 antibodies even after 5 hours of TEM. Although differences in the kinetics of cell migration through various epithelial monolayers can not be excluded, the contrasting results of Liu and co-workers [49] are most likely due to an experimental design different from ours. They were using the chemoattractant fMLP as stimulating factor which results in upregulation of CD47 on PMN as proven by our FACS experiments. In contrast, CD47 expression was neither found to be upregulated in PMN nor in HK-2 cells upon stimulation with IL-8, TNF α or LPS. Similar data in intestinal epithelia suggest that expression of CD47 on both epithelia and PMN may be important in the regulation of transmigration [50, 51]. We postulate that CD 47 expression on PMN is required only for basolateral to apical migration. This is supported by our migration and FACS analysis and also by the fact that integrins are expressed on the basolateral side of epithelial cell [8]. So, blocking of the CD47 may prevent CD47-integrin docking and thus basolateral-to-apical migration.

In conclusion, the present investigation demonstrates for the first time transmigration of PMN through confluent HK-2 cell monolayers. Transmigration occurs without use of a chemotactic gradient by priming epithelial cells with TNF α or LPS. Furthermore a preferred direction of TEM was not detected. ICAM-1 expression on HK-2 cells appears to play a significant role in apical-to-basolateral migration whereas CD47 expression does not. Basolateral-to-apical PMN migration is critically dependent on the expression of CD47 in PMN. Thus there appear to be distinct processes involved in recruitment of PMN from either side of the renal epithelium.

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