Regulation of polymorphonuclear leukocyte apoptosis: role of lung endothelium-epithelium bilayer transmigration

Maowen Hu, Xinchun Lin, Qiaoting Du, Edmund J. Miller, Ping Wang, and H. Hank Simms

Division of Surgical Research, Department of Surgery, North Shore University Hospital and Long Island Jewish Medical Center, Manhasset, New York

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Hu, Maowen, Xinchun Lin, Qiaoting Du, Edmund J. Miller, Ping Wang, and H. Hank Simms. Regulation of polymorphonuclear leukocyte apoptosis: role of lung endothelium-epithelium bilayer transmigration. Am J Physiol Lung Cell Mol Physiol 288:L266–L274, 2005. First published October 8, 2004; doi:10.1152/ajplung.00209.2004.—Delayed polymorphonuclear leukocyte (PMN) apoptosis exacerbates acute lung injury. To reach the alveolar spaces, PMNs must migrate across both pulmonary endothelial and epithelial cell layers. We hypothesized that transmigration across the endothelium-epithelium bilayer suppresses PMN apoptosis and sought to elucidate the underlying mechanisms. PMNs freshly isolated from normal volunteers were allowed to migrate across polycarbonate membranes alone or membranes coated with a bilayer of human lung endothelial and epithelial cells. After migration toward different chemoattractants (IL-8, formyl-Met-Leu-Phe, or leukotriene B4), PMN apoptosis and caspase activities were assessed by annexin V, histology, and enzymatic assays, respectively. Messenger RNA and specific protein expression in three receptor ligand-mediated, apoptosis-inducing pathways (Fas, TNF-α, and TNF-related apoptosis-inducing ligand) were further examined by gene array, RT-PCR, flow cytometry, and Western blot analyses. The data demonstrated that transbilayer migration suppressed PMN apoptosis, and this effect was not chemoattractant type specific. Kinetic analyses further showed that the delay of apoptosis was sustained to at least 18 h. Transbilayer migration caused significant decreases in caspase (-3, -8, and -9) activities. The changes in apoptosis-related gene expression support the survival role of transbilayer migration. Furthermore, the reduced apoptosis was correlated with downregulation of Fas ligand and TNF receptor 1 expression. Our data reveal that migration across a lung endothelium-epithelium bilayer suppresses PMN apoptosis. The decreased activity and/or expression of proapoptotic proteins may provide possible targets for the regulation of inappropriate delay in PMN apoptosis during lung inflammation and injury.

APOPTOSIS OF POLYMORPHONUCLEAR LEUKOCYTES (PMNs) is a key event in the orderly resolution of human inflammatory responses. It leads to functional downregulation (39) as well as the recognition and clearance of the apoptotic PMNs by macrophages (11, 29). Inappropriate delay of PMN apoptosis, resulting in persistence of PMNs at sites of inflammation, can lead to the overproduction of reactive oxygen intermediates and proteolytic enzymes, causing an exaggeration of the inflammatory response and tissue damage. It has been shown that PMN apoptosis within the lungs is suppressed in patients with acute respiratory distress syndrome (ARDS) (20), and this suppression may play a pivotal role in the onset and progression of ARDS (35). Because ARDS remains a common, devastating clinical problem with an estimated 50% mortality (42), it is very important to study how PMN apoptosis is regulated in terms of understanding the pathobiology of acute lung injury and uncovering potential therapeutic targets.

PMNs die rapidly by a constitutive program of apoptosis both in vitro and in vivo. Although the key molecular control of PMN apoptosis remains to be elucidated, several inflammatory mediators, such as IL-1, IFN-γ, granulocyte/macrophage colony-stimulating factor, IL-8, or LPS have been shown to extend the PMN life span (5, 7, 13, 15, 38). Conversely, PMN apoptosis may be induced by other mediators, such as TNF-α, IL-10, activation of the oxidative burst, or phagocytosis of bacterial species (6, 17, 27, 28). Increased migration of PMNs across the lung endothelium-epithelium bilayer is an early response during acute lung injury. It has been shown that both epithelial and endothelial cells play an active role in PMN activation and migration (2, 31, 34). Previous studies have also suggested that these cells may be important in regulating PMN apoptosis. For example, the activation of cell surface adhesion molecules on the umbilical vein endothelial cells during PMN recruitment modulates apoptosis (33, 37). The life span of PMNs is increased after transmigration across the intestinal epithelium (14). To reach the alveolar spaces, PMNs must migrate across both pulmonary endothelial and epithelial cell layers. Although we have shown that transmigration across a lung epithelial monolayer inhibits PMN apoptosis (12), the effect of migration across the human lung endothelium is unknown. Moreover, only single layer transmigration has been studied in previous work. The impact of migration through a lung endothelium-epithelium bilayer on PMN apoptosis, which is clinically more relevant, remains unclear.

Like other cell populations (24), PMNs appear to possess three receptor ligand-mediated, apoptosis-inducing pathways [Fas, TNF-α, and TNF-related apoptosis-inducing ligand (TRAIL)] belonging to the TNF receptor (TNF-R) family. Fas and Fas ligand (FasL) are both constitutively expressed on the cell surface of PMNs and therefore can induce cell death by both an autocrine or paracrine mechanism (16), although previous work suggested that spontaneous PMN cell death is not dependent on Fas-mediated signaling (3, 8). PMNs express TNF-R1 and TNF-R2, and TNF-α has a biphasic effect on PMN apoptosis, depending on the incubation time and the dose (23). The TRAIL apoptotic system has also been implicated in the regulation of immune function, and TRAIL-R2 is considered the major receptor that can initiate PMN apoptosis (26).
We hypothesize that transbilayer migration can modulate PMN apoptosis. To mimic the blood-alveolar space barrier, we constructed a bilayer using microvascular endothelial and epithelial cells both derived from human lung and examined the degree of apoptosis in PMNs following transbilayer migration. In our model system, we further studied the effect of transbilayer migration on three death receptor pathways and the downstream caspase activation. Our results reveal the role of transbilayer migration on regulating PMN apoptosis and may provide insights into possible targets for the regulation of PMN apoptosis during lung inflammation and injury.

MATERIALS AND METHODS

Reagents. Recombinant human IL-8 was obtained from Pepro Tech (Rocky Hill, NJ). Ficoll-Hypaque, blue dextran, and formyl-Met-Leu-Phe (fMLP) were obtained from Sigma (St. Louis, MO). Leukotriene B4 (LTB4) was obtained from Calbiochem (San Diego, CA). Antibodies against TNF-α, TNF-R1, TNF-R2, and actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Fas, polyclonal antibody and monoclonal antibodies to Fas (FITC) and TRAIL-R2 (FITC) were obtained from Alexis (San Diego, CA). Anti-FasL polyclonal antibody against TRAIL was purchased from Upstate Cell Sciences. Anti-FasL was fractionated on Ficoll-Hypaque and dextran gradients to separate the cells in a physiologically relevant “vascular-to-alveolar” size; Corning, Corning, NY) was inverted, and A549 cells (2 × 10^6) were seeded in a bilayer construction, and the integrity of each monolayer was also examined using the same methods used for the bilayer.

PMN transmigration. Isolated PMNs (3 × 10^6 cells) were added into the upper chamber of the transwell and allowed to migrate across the membrane alone or the membrane coated with a bilayer. IL-8, fMLP, or LTB4 was added into the lower chamber as a chemoattractant. The migration time and the concentration of each reagent were specified in each experiment. In the dose-response experiments, a series of concentrations (10^-10, 10^-9, and 10^-8 M) was used. In the kinetic analyses, we removed the bilayer after a 4-6 h migration and continued PMN culture for an additional 2, 8, or 12 h to reach total incubation times of 6, 12, or 18 h, respectively.

Assessment of apoptosis by cellular morphology and annexin V assay. After migration, PMNs in the lower chamber were collected for apoptosis analysis. For morphology examination, cells were stained using the PROTOCOL method. Each experimental condition was analyzed by direct counting of apoptotic PMNs after staining. PMNs were determined to be apoptotic if they demonstrated a rounded morphology with concomitant dark, pyknotic nuclei. The minimum number of cells counted was 400 per condition. The results were confirmed by annexin V-phycocerythin (PE) and 7-aminactinomycin D double staining using the annexin V-PE apoptosis detection kit I (BD, San Diego, CA) following the manufacturer’s instructions. At least 30,000 PMNs per condition were analyzed via flow cytometry.

Flow cytometry. PMNs were allowed to migrate across the transwell membrane alone or the membrane coated with a bilayer in the presence of IL-8 (10^-9 M). After migration for 8 h, PMNs were collected and stained with FITC-labeled anti-Fas or anti-TRAIL-R2 monoclonal antibodies. An isotypic nonspecific IgG was used to assess background staining. At least 30,000 PMNs per condition were analyzed via flow cytometry, and the mean fluorescence activity under each experimental condition was compared.

Enzymatic caspase activity assay. Activity of individual caspases in PMNs was measured by assessing cleavage of a tagged caspase-3 [(Asp-Glu-Val-Asp)-AFC (7-amino-4-trifluoromethyl-coumarin)], caspase-8 [(Ile-Glu-Thr-Asp)-AFC (7-amino-4-trifluoromethyl coumarin)], or caspase-9 [(Leu-Glu-His-Asp)-AMC (7-amino-4-methylcoumarin)] substrate peptide using ApoAlert Caspase Fluorescent Assay kits (BD Clontech, Palo Alto, CA). Fluorometric detection was performed at excitation and emission wavelengths of 400 and 505 nm for caspase-3 and caspase-8, or 380 and 460 nm for caspase-9, using a fluorimeter (PerSeptive Biosystems, Framingham, MT).

Gene array procedure and analysis. Total RNA was isolated from PMNs after 8 h of transbilayer or transmembrane migration in the presence of IL-8 (10^-9 M) using an RNeasy Mini kit (Qiagen, Valencia, CA). mRNA was then converted into cDNA followed by aRNA using a MessageAmp aRNA kit (Ambion, Austin, TX) according to the manufacturer’s instructions. To ensure optimal hybridization to the oligonucleotide array, we fragmented 20 µg of aRNA such that the fragments were between 35 and 200 bases in length, by incubating the aRNA at 94°C for 35 min in fragmentation buffer. The sample was then added to a hybridization solution containing 100 mM 4-morpholinepropanesulfonic acid, 1 M NaCl, and 20 mM EDTA in the presence of 0.01% Tween 20. The final concentration of the fragmented aRNA was 0.05 µg/µl. Hybridization was performed for 16 h by incubating 200 µl of the sample to an Affymetrix GeneChip HG-U133A (Santa Clara, CA). Initial microarray was then stained with streptavidin-phycocerythin and scanned at a 6-µm resolution by an Affymetrix laser scanner according to the procedures developed by Affymetrix. First-pass analysis of the scanned data was performed with GeneChip software (version 5.0). These microarrays were normalized using the global scaling method recommended by Affymetrix. We used the Affymetrix Data Mining tool to calculate the gene changes and subsequently NetAffx analysis to identify apoptosis-related genes. Only the genes with at least 1.8-fold changes (P < 0.05) were presented.

RT-PCR analysis. Total RNA was treated with RNase-free DNase I and reversely transcribed using SuperScript Reverse Transcriptase (Life Technologies). PCR was then performed from cDNA using the following primer pair: human FasL, 5'-AGTCTCCCAAGTCCCTGGCTCTG-3' and 5'-GACCCAGAGACCTGACGACTGC-3'. PCR products were loaded on a 1.2% agarose gel and visualized with ethidium bromide.
PMNs were allowed to migrate across the Transwell membrane alone or the membrane coated with a bilayer toward IL-8 (10^{-9} M). After 8 h, the media in the lower chamber were collected, and TNF-α release was measured by ELISA (minimum detectable concentration = 10 pg/ml). Recombinant human TNF-α standards were obtained from R&D Systems and dissolved in 0.1% BSA solution (low endotoxin grade from Sigma). The anti-human TNF MAb was prepared and was a gift from Kevin J. Tracey (North Shore-Long Island Jewish Research Institute, Manhasset, NY).

**Results**

**Assessment of PMN resistance after transbilayer migration to further apoptotic stimuli.** PMNs were allowed to migrate across the transwell membrane alone or the membrane coated with a bilayer toward IL-8 (10^{-9} M). After 4 h of migration, PMNs in the lower chamber were collected and washed twice to remove residual IL-8. PMNs were then incubated with agonistic anti-Fas MAb (200 ng/ml) or a control isotypic MAb (200 ng/ml) for an additional 4 h. The status of cell apoptosis was analyzed by annexin V assay as described previously. The same experiments were also performed with TNF-α (20 ng/ml) (23).

**Statistical analysis.** Results were expressed as means ± SD. Data were analyzed with Student’s unpaired t-test for Figs. 5 and 6 and one-way ANOVA with Tukey’s test for the remaining figures. Differences at P < 0.05 were considered to be significant.

**PMN apoptosis was suppressed following migration through the lung endothelium-epithelium bilayer.** To mimic the in vivo PMN migration route in the lung, we constructed a bilayer model by seeding the HMVEC-L cells on the vascular side and the A549 cells on the alveolar side of the transwell membrane. After 6 days of culture, cells became morphologically confluent on both sides compared with 2 days of culture (Fig. 1A). The bilayer also excluded dextran blue (mol wt 2,000,000) and a control isotypic MAb (200 ng/ml) for an additional 4 h. The status of cell apoptosis was analyzed by annexin V assay as described previously. The same experiments were also performed with TNF-α (20 ng/ml) (23).

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model, and a bilayer model can be established after 9 days of culture. Using this model, we first studied the effect of IL-8 (10⁻⁹ M)-induced transbilayer migration in an endothelium-epithelium direction on PMN apoptosis. As assessed by morphological examination, fewer apoptotic PMNs with dark and pyknotic nuclei (i.e., apoptotic bodies) were present in the cells after transbilayer migration (Fig. 2A). Annexin V assay confirmed that apoptosis was significantly reduced at 8 h in PMNs migrating across the bilayer compared with the values in PMNs crossing the membrane alone (from 48 to 26%, P < 0.05; Fig. 2B). To determine whether the reduced PMN apoptosis was limited to IL-8 alone, PMNs were allowed to migrate toward other chemoattractants, such as fMLP (10⁻⁹ M) and LTB₄ (10⁻⁹ M). As with IL-8, fMLP- or LTB₄-mediated transbilayer migration also caused a significant reduction in PMN apoptosis at 8 h (fMLP from 45 to 21%, P < 0.05 and LTB₄ from 46 to 25%, P < 0.05 in Fig. 2B).

No synergistic or additive effect was identified on PMN apoptosis following transbilayer migration. We have recently shown that transepithelial migration inhibits PMN apoptosis (12). To further determine the contribution of the additional endothelial monolayer and whether migration across an endothelial or epithelial monolayer or both is really necessary for the inhibition of PMN apoptosis, we performed experiments wherein PMNs were allowed to migrate across just an epithelial layer or an endothelial layer or a bilayer toward graded concentrations (from 10⁻¹⁰ to 10⁻⁸ M) of IL-8, fMLP, or LTB₄. We chose these concentrations because for IL-8 and LTB₄, they are within the physiological range during acute lung injury (21). In each case, the percentage of apoptotic cells was also compared with the cells not exposed to any of the chemoattractants. Although the number of the randomly migrated cells was much lower than those in the presence of a chemoattractant, there were enough cells to evaluate apoptosis. As shown in Fig. 3, similarly with transepithelial and transbilayer migration, transendothelial migration significantly inhibited PMN apoptosis. The inhibitory efficacy was not affected
by the type or the concentration of the chemoattractant, and more importantly, the antiapoptotic effect also occurred in the absence of a chemoattractant. Interestingly, there was no additive or synergistic effect comparing transbilayer migration with transmonolayer (endothelium or epithelium) migration. Although the effect of the transepithelial monolayer seems more potent, it was not significantly different from that of the other two conditions.

**PMN apoptosis was delayed by transbilayer migration.** To further determine the durability of the antiapoptotic effect, kinetic analyses were performed by measuring the number of apoptotic PMNs over time following transbilayer migration induced by IL-8, fMLP, or LTB4 at a concentration of $10^{-9}$ M. As demonstrated by the annexin V assay, starting from 6 h, the number of apoptotic PMN cells decreased significantly ($\sim 50\%$ relative reduction) after transbilayer migration compared with PMNs crossing the membrane only, and this effect was not chemoattractant specific (Fig. 4). The number of apoptotic cells remained lower after 18 h ($\sim 20\%$ reduction) on IL-8- or LTB4-mediated transbilayer migration (Fig. 4, A and C). The antiapoptotic effect was sustained for at least 12 h for fMLP-initiated transbilayer migration (Fig. 4B). These results were also confirmed by morphology analyses (data not shown). Overall, transbilayer migration both suppressed and delayed PMN apoptosis.

**Expression of apoptosis-related genes supported the survival effect of transbilayer migration on PMNs.** The modulation of PMN apoptosis by multiple biological relevant stimuli has been indicated to occur at the gene expression level (25). To obtain general understanding of which genes may be involved in the survival effect of transbilayer migration on PMNs, we performed gene array analyses using Affymetrix HG-U133A gene chips. Because IL-8-, fMLP-, and LTB4-induced transbilayer migration exerted similar effects on PMN apoptosis, for the remaining studies, we used only IL-8 at a concentration of $10^{-9}$ M. Compared with the genes in PMNs after transmembrane migration, 29 apoptosis-related genes in PMNs after transbilayer migration consistently showed at least 1.8-fold changes with statistical significance ($P < 0.05$) in three individual experiments (Table 1). Among these genes, 24 out of 29 genes were downregulated, whereas only 5 were upregulated. In addition, 23 proapoptotic genes were downregulated and 1 antiapoptotic gene was upregulated after transbilayer migration. Although four proapoptotic genes were increased and one antiapoptotic gene was decreased, the net balance of gene expression changes supported the role of transbilayer migration on the reduction of PMN apoptosis.

**Activities of caspase-3, caspase-8, and caspase-9 were decreased in PMNs after transbilayer migration.** To further investigate the mechanism underlying transbilayer migration-mediated PMN survival, we assessed the activity of three individual caspases (-3, -8, and -9) after transbilayer migration (Table 1), we further investigated the effect of transbilayer migration on three receptor ligand-mediated, apoptosis-inducing pathways. First, we examined the expression of death receptor ligands (FasL, TRAIL, and TNF-α). The decrease of FasL mRNA expression observed in gene array analyses (Table 1) was confirmed by RT-PCR analyses (Fig. 6A). Western blotting assays also detected a corresponding decrease ($\sim 20\%$) in FasL protein expression in PMNs after transbilayer migration (Fig. 6B). However, transbilayer migration did not alter TRAIL protein expression (Fig. 6C). Although a decrease in TNF mRNA expression was found in gene array analyses (Table 1), there were no changes at the cellular protein level as well as in TNF-α secretion (Fig. 6, D and E).

Experiments were also performed to study the expression of death receptors (Fas, TRAIL-R2, TNF-R1, and TNF-R2). In contrast to its ligand (FasL), Fas protein expression was not altered by transbilayer migration (Fig. 7A). TRAIL-R2, the major TRAIL receptor on PMN, was also not changed (Fig. 7A). However, the protein level of TNF-R1 (Fig. 7B), but not TNF-R2 (Fig. 7C), showed a significant decrease in PMNs after transbilayer migration. The correlation of reduced PMN apoptosis with the downregulation of FasL and TNF-R1 sug-
Table 1. Effects of transmigration across a lung endothelium-epithelium bilayer on apoptosis-related genes in PMNs

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<th>Gene</th>
<th>Fold Change</th>
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After 8 h of migration across a bilayer or a membrane alone toward IL-8, total RNA isolated from migrated polymorphonuclear leukocytes (PMNs) was subjected to a gene array analysis using an Affymetrix GeneChip HG-U133A as described previously. These microarrays were normalized using a global scaling method. Affymetrix Data Mining tool and NetAffx analysis were used to identify the apoptosis-related genes that were upregulated (+) or downregulated (−) in PMNs after transbilayer migration compared with transmembrane migration. The genes listed here showed at least 1.8-fold changes consistent in 3 separate experiments (P < 0.05). The arrow represents the predicted effect of the observed gene regulation on apoptosis.

gests possible survival mechanisms involved in the effect of transbilayer migration on PMN apoptosis.

Transbilayer migration does not confer PMNs a resistance to further apoptotic stimuli such as anti-Fas MAb and TNF-α. To investigate whether PMNs after transbilayer migration exhibit relative resistance to further apoptotic-inducing reagents, we added anti-Fas MAb or TNF-α to PMNs after transbilayer migration and measured the degree of apoptosis after 4 h of incubation. As demonstrated by annexin V assay, in spite of the antiapoptotic effects, transbilayer migration did not confer resistance to further apoptotic stimuli, and an appropriate increase in PMN apoptosis was observed (Fig. 8, A and B).

DISCUSSION

Although several inflammatory stimuli have been shown to control PMN apoptosis, the role of migration across the lung endothelium-epithelium bilayer on the PMN apoptotic program remains to be determined. To address this question, we used an in vitro model that mimics PMN transbilayer migration into the lung alveolar spaces and found that this migration process prolonged the PMN life span. Supported by several lines of evidence, our data suggest that suppression of PMN apoptosis is mediated by the interaction of PMNs with the bilayer during migration. First, IL-8, fMLP, and LTB₄ are three independent chemoattractants with different effects on PMN apoptosis (10). However, transbilayer migration induced comparable suppression and delay in PMN apoptosis regardless of the chemoattractant used (Fig. 2). Second, since we used transmembrane migration as a control, the only difference in our model system was the migration across an additional endothelium-epithelium bilayer. Thus the survival effect is most likely attributed to the transbilayer migration. Finally, the antiapoptotic effect is chemoattractant independent because it also occurs in the absence of a chemoattractant (Fig. 3).

Overall, our data suggest that the lung endothelium-epithelium bilayer is not a passive barrier but actively contributes to the modulation of PMN apoptosis.

Inhibition of PMN apoptosis can result in the persistence of PMNs at sites of inflammation and can lead to the overproduction of harmful substances, such as reactive oxygen intermediates, causing an exaggeration of the inflammatory response and tissue damage. It has been shown that suppression of PMN apoptosis may play a pivotal role in the onset and progression of ARDS (35). A recent paper from Taneja et al. (32) indicates that delay of PMN apoptosis can exacerbate inflammation in sepsis patients. Although we did not measure the functional change in this paper, our previous work has shown that the superoxide production increased in PMNs following transepithelial migration (12).

As important components of the blood-gas barrier, human lung endothelium and lung vascular endothelium are both known
to play an active role in PMN activation and migration (2, 31, 34). We and others (12, 14) have shown that transepithelial migration inhibits PMN apoptosis. However, modulating PMN apoptosis previously was only obtained in human umbilical vascular endothelial cells (33, 37), not in HMVEC-L cells, the primary cell line, and derived specifically from human lung microvasculatures. In this paper, we observed a similar inhibitory effect on PMN apoptosis following migration across a single endothelial layer compared with migration across a single epithelial layer, and the inhibition induced by migration across a bilayer did not differ from migration across either monolayer (Fig. 3). These results are in a manner analogous to the migration data obtained in a similar transmigration model for PMNs, combining human umbilical vein endothelial cells with the A549 lung epithelial cell line (4). In this model, the migration across each monolayer was increased, but this increase was not further enhanced by migration across the bilayer. Our data suggest that migration across either monolayer is sufficient to inhibit PMN apoptosis. However, it does not exclude the possibility that mechanisms responsible for the inhibitory effect contributed by each layer are different. For example, unlike our previous work with the transepithelial monolayer model (12), we did not identify an increase in antiapoptotic gene Mcl-1 or 14-3-3-ζ at either the mRNA or protein level in PMNs after transbilayer migration (data not shown).

The relevant balance of antiapoptotic and proapoptotic genes is thought to control the commitment of a cell into apoptosis or survival (9). To characterize the mechanisms involved in the antiapoptotic effect of transbilayer migration, we compared the expression of apoptosis-related genes in PMNs after transbilayer migration with that in PMNs after transmembrane migration (Table 1). The changes in 24 out of 29 genes was consistent with the survival effect of transbilayer migration, suggesting that the net balance of gene expression changes would potentially lead to a suppression of PMN apoptosis. We found more genes downregulated than those upregulated at the

**Fig. 6.** Fas ligand (FasL), TNF-related apoptosis-inducing ligand (TRAIL), and TNF-α expression in PMNs. PMNs were allowed to migrate across a transwell membrane (TM) or a bilayer (TB) toward IL-8 at 10^{-9} M. After 8 h of transmigration, PMNs were collected and prepared for either protein detection or gene expression measurement. A: representative RT-PCR for FasL mRNA from 2 separate experiments. Equal cDNA loading was verified by amplifying actin mRNA. B and C: representative Western blots of FasL and TRAIL from at least 3 separate experiments. The resultant blots were quantitated using densitometry. D: representative Western blot of cellular expression of TNF-α. Actin protein expression was detected as a control. E: ELISA for TNF-α secreted into the media. *p < 0.05 compared with transmembrane migration (n = 4).

**Fig. 7.** Fas, TNF-related apoptosis-inducing ligand receptor 2 (TRAIL-R2), and TNF receptor 1 (TNF-R1) expression in PMNs. PMNs were allowed to migrate across a transwell membrane alone (TM) or a bilayer (TB) toward IL-8 at 10^{-9} M. A: after 8 h of transmigration, PMNs were analyzed for Fas and TRAIL-R2 expression by flow cytometry using specific MAb to Fas or to TRAIL-2. PMNs were also stained with an isotypic control IgG. Bars represent the average of mean fluorescence intensity from 4 independent experiments. Protein expression of TNF-R1 (B) and TNF-R2 (C) was detected via Western blotting assays from at least 3 separate experiments. The resultant blots were quantitated using densitometry. *p < 0.05 compared with PMNs after transmembrane migration.
The death receptor-mediated (extrinsic) pathway is initiated upon binding death receptors to their ligands, which belong to the TNF-R family (18). In our experiments, we found that the gene expression of several members in this pathway was downregulated by transbilayer migration (Table 1). We also identified a decrease in activity of caspase-8, the upstream initiator activated by the onset of Fas and TNF pathways (40). These findings led us to further investigate the effect of transbilayer migration on death receptors and their ligands, especially at the protein level. We identified that both TNF-R1 and FasL expression was downregulated by transbilayer migration, and this downregulation was associated with the suppression of PMN apoptosis (Figs. 6B and 7B). Consistent with our study, previous work has shown that FasL, not Fas, is downregulated in PMNs after transepithelial migration (14). The reduction in the cell surface expression of TNF receptors, but not Fas, has also been observed in exuded PMNs (30).

In our model system, we did not find a relative resistance of PMNs to a further apoptotic stimulus with anti-Fas MAb after transbilayer migration (Fig. 8). This suggests that downstream signaling of this pathway is still intact despite the downregulation of its ligand (Fasl, Fig. 6B). The fact that transbilayer migration did not confer PMN resistant to TNF-α may be because TNF-α-mediated apoptosis can be initiated by both TNF-R1 and TNF-R2, and TNF-α may induce apoptosis via a caspase-independent route (19).

In summary, the present study is the first report showing that migration across human pulmonary endothelial-epithelial bilayers can actively modulate PMN apoptosis. In addition, this study provides several important findings. First, transbilayer migration regulates PMN apoptosis at both gene and protein levels. Second, the decreased activities of caspases, and the downregulation of FasL and TNF-R1, may contribute to the survival effect of transbilayer migration on PMNs. Finally, transbilayer migration does not confer a resistance to further apoptotic stimuli (anti-Fas MAb and TNF-α), and an appropriate increase in PMN apoptosis is observed. Delayed PMN apoptosis has also been identified in human subjects and animal models by comparison of circulating PMNs with those isolated from bronchoalveolar lavage specimens (20, 37), suggesting that our syngeneic bilayer model closely mimics the biology/physiology of the human lungs. However, we acknowledge that factors such as isolation-induced PMN priming (36, 41) and cell lines instead of primary cells used in the experiments may interfere with the results, and caution should be taken with direct application of the data to the in vivo situations. Nonetheless, our results with the bilayer model may provide new insight in the molecular basis of PMN apoptosis regulation. This model also adds new aspects to the research on PMN apoptosis in lung inflammatory disorders by providing an extra level of complexity.

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