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An *In Vitro* Assay for Study of Neutrophil Migration Through Interstitial Matrix Using BD Falcon™ Cell Culture Inserts

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Introduction

Understanding the mechanisms by which cells migrate through the interstitium is important in determining the role of inflammatory cells in disease processes. We have developed an *in vitro* model for the study of inflammatory cell migration through interstitial extracellular matrix (ECM) produced on a 3.0 μm BD Falcon™ Cell Culture Insert.

Materials and Methods

R22 Cell Culture and ECM Production

R22 rat aortic smooth muscle cells are cultured as previously described.¹ Trypsinized R22 cells are suspended in MEM supplemented with fetal bovine serum, tryptose phosphate broth, and cefotaxime and streptomycin. Cells are plated onto untreated BD Falcon Cell Culture Inserts with 3.0 μm Polyethylene terephthalate (PET) membranes (0.8×10^6 pores 1 cm^2) (*Figure 1A*) at a density of approximately 2.5×10^4 cells/ cm^2 . The inserts are placed in BD Falcon multiwell plates, medium is added to the wells to maintain a level equal to that in the insert and the cultures are incubated at 37°C in 5% CO_2 . The cells are fed every four days and upon reaching confluence are given daily supplements of 50 $\mu\text{g}/\text{ml}$ ascorbic acid² for eight to ten days, until sufficient matrix is formed for use. Radiolabeled ECM is produced by culturing R22 cells in medium containing ³⁵S methionine and ³H proline.

To prepare the ECM-coated membranes for use with inflammatory cells, the R22 cells are lysed with 25 mM NH_4OH and the inserts are then washed three times with sterile water and once with Dulbecco's phosphate-buffered saline (PBS) containing 0.02% NaN_3 . The PBS is removed and the inserts are stored at 4°C until use. Immediately prior to use, the inserts are washed three times with appropriate buffer or media to remove any residual NaN_3 and rehydrate the ECM.

Neutrophil Isolation and Migration

Neutrophils (PMNs) are isolated by differential centrifugation of human donor blood through Polyprep-Lymphoprep gradients. The cells are washed and held on ice in buffer or medium (RPMI) until use. Chemo-attractant (100 nM FNLP in 1% BSA) is added to the medium in the well under the ECM-membrane unit. The neutrophils are added to the top compartment at approximately 1×10^6 cells/ cm^2 . The units are then incubated at 37°C in 5% CO_2 for one to two hours.

Microscopy

Samples were examined using light, thin section transmission electron (TEM) and scanning electron (SEM) microscopy. Light microscopy samples were fixed in 3.7% formaldehyde, rinsed in buffer and embedded in LR White resin. Sections were cut, stained with toluidine blue and examined on a Zeiss light microscope. TEM samples were rinsed, fixed in 2% glutaraldehyde in cacodylate buffer, post fixed in OsO_4 , dehydrated and

embedded in LR white. Thin sections were stained with uranyl acetate and lead citrate and examined on a JEOL 1200 EX electron microscope. SEM samples were fixed in 2% glutaraldehyde, critical point dried and gold-palladium coated. They were examined on a JEOL 5300 scanning microscope.

Radiolabel Assay

Labeled ECM-membrane units are prepared and isolated neutrophils are introduced as described herein. At appropriate time points the medium is removed from both the insert and the underlying well and pooled. The ECM remaining in the inserts is lysed by the addition of 2 M NaOH. The samples are spun down to remove cells and debris and the supernatants are saved for scintillation counting. The effects of various substances (for example, $\alpha 1$ -PI, an elastase inhibitor) can be assessed by adding them to inserts along with the neutrophils and incubation medium.

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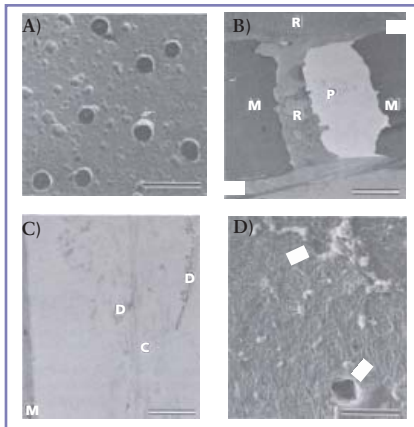


Figure 1: (A) SEM of 3.0 μm pore membrane from BD Falcon Cell Culture Insert. Bar = 10 μm. (B) TEM of confluent R22 cells and matrix on 3.0 μm pore membrane insert. Arrows = ECM, R = R22 cell, M = membrane, P = pore (Note: the gap in the pore between the sample and the edge of the membrane is due to sectioning artifact). Bar = 3 μm. (C) TEM of ECM on Cyclopore membrane after R22 cell lysis. M = membrane, C = collagen fibrils, D = residual R22 cell debris. Bar = 1 μm. (D) SEM of matrix on 3.0 μm pore membrane. Arrows = pore locations. Bar = 5 μm.

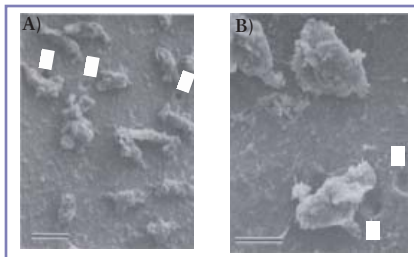


Figure 2: SEMs of activated PMNs on ECM-membrane inserts. Arrows = pore locations. Bars = (A) 10 μm and (B) 5 μm.

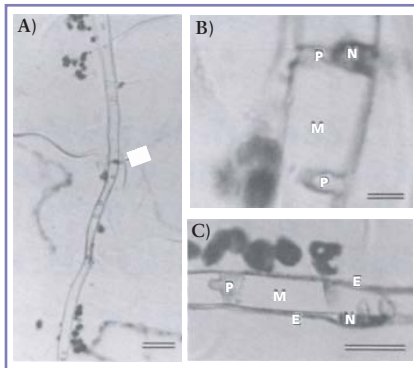


Figure 3: Light micrographs of trypan blue stained ECM-membranes with activated PMNs. Panel (B) is an enlargement of the area marked on (A) with the arrow. In panel (C) note the PMN "N" on the bottom side of the insert between the membrane "M" and the ECM "E". In all panels: P = pore, N = PMN, M = membrane, E = ECM. Bars = (A) 40 μm, (B) 5 μm, (C) 20 μm.

Results and Discussion

R22 Cell Culture and ECM-coated Cell Culture Insert Membranes

TEM examination of BD Falcon™ Cell Culture Inserts with confluent R22 cells after eight days of ascorbic acid supplementation reveals that the cells are able to migrate through the pores, attach to the underside of the membrane and produce a matrix layer on both sides of the insert (Figure 1B). The ECM elaborated by R22 cells has striking similarity to interstitial matrix and consists of a highly insoluble mixture of types I and III collagen, elastin, proteoglycans and fibronectin. Collagen fibrils seen in TEM of samples embedded after lysis of R22 cells demonstrate typical interstitial banding patterns (Figure 1C). SEM of ECM-coated membranes show that the R22 produced matrix completely covers the 3.0 μm pores (Figure 1D). The matrix covering the 3.0 μm pores is un-supported by underlying membrane. Because of this, some beam damage occurs at the pore locations during scanning, which shows up as cracking in the ECM layer.

Neutrophil Migration

Isolated human neutrophils in RPMI were applied to the ECM-membrane inserts and chemoattractant agents added to the underlying wells (Figure 2). Even in the absence of a chemotactic stimulus, it has been observed that PMNs undergo a characteristic shape change, adhere to the matrix and become motile. With the addition of an agent such as f-Nle-Leu-Phe (FNLP) microscopic examination of the BD Falcon™ Cell Culture Inserts at various timepoints reveals that the PMNs migrate through the matrix covered pores to the underside of the ECM-membrane (Figures 3A, 3B and 4B). When PMNs are added to the ECM-membrane inserts preincubated with α1-PI, no migration to the underside is seen.

Radiolabel Assay

The release of radioactive counts by PMNs on labeled ECM-membrane inserts under various conditions was examined. One set of inserts was pre-treated for 60 minutes with α1-PI, an elastase inhibitor. PMNs were added to both treated and untreated units in the presence and absence of FNLP. After a two-hour incubation at 37°C in 5% CO₂, the samples were analysed by scintillation counting (Figure 5).

More counts are released in the presence of chemoattractant, indicating a greater amount of matrix degradation. The addition of α1-PI to the system not only interferes with migration, but also suppresses the level of degradation as evidenced by a decrease in the release of soluble counts.

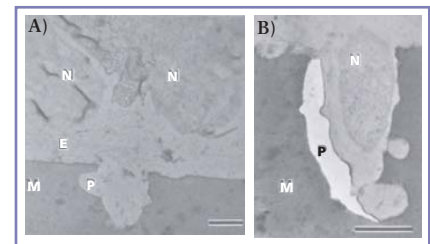


Figure 4: TEMs of activated PMNs on ECM-membrane. (A) Neutrophils adjacent to a pore. (B) Neutrophil in pore. In both panels: N = PMN, E = ECM, P = pore, M = membrane. Bars = 2 μm.

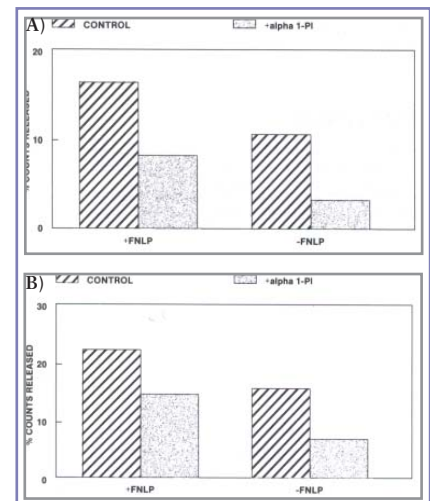


Figure 5: Comparison of the release of radioactive counts by PMNs on labeled ECM-membrane inserts. (A) Percent of total ³H counts released by cell mediated digestion during two-hour incubation. (B) Percent of total ³⁵S counts released by cell mediated digestion during two-hour incubation.

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Conclusion

BD Falcon™ Cell Culture Inserts with 3 µm pore PET membranes are a suitable substrate for the production of an *in vitro* ECM model. R22 cells attach and grow with minimal difficulty and produce continuous sheets of interstitial-like ECM. Isolated human PMNs activate and migrate both on and through the ECM-membrane inserts in response to chemoattractants. Cellular behavior and interactions with the matrix can be assessed by various means, including different forms of microscopy and radiolabel analyses. This ECM-coated membrane insert system provides a flexible tool for the study of the migratory processes of inflammatory cells.

References

1. Roemer, E.J. and Simon, S.R., *BD Falcon Cell Culture Inserts as a support substrate for an in vitro extracellular matrix system*. BD Biosciences Technical Bulletin #402.
2. DeClerck, Y.A. and Jones, P.A., *The effects of ascorbic acid on the nature and production of collagen and elastin by rat smooth muscle cells*. *J. Biochem.* **186**:217 (1980).

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