Human Neutrophils Do Not Degrade Major Basement Membrane Components During Chemotactic Migration

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At sites of inflammation, circulating neutrophils (PMNs) migrate through microvessel walls into the subendothelial interstitium. While endothelial passage is mediated by adhesion proteins, including those of the integrin, selectin and immunoglobulin superfamily classes, the mechanisms used to cross the subendothelial basement membrane (BM) are unclear. Studies examining tumour cell invasion and lymphocyte extravasation suggest several possible mechanisms, including proteolysis. Different cells, however, may use different mechanisms to effect passage. To examine neutrophil–basement membrane interactions in more detail, human PMNs were embedded within reconstituted BM (Matrigel) and used in migration assays. The integrity of the gel following migration was assessed by assaying for the release of incorporated radiolabelled products and by immunoblotting for specific matrix molecule epitopes. PMNs migrated through Matrigel in response to the chemotactic peptide FMLP. Degradation products of laminin, heparan sulphate proteoglycan or of gelatin, however, were not detected. In contrast, phorbol ester, which triggers activation without migration, released ~40% of incorporated HSPG, 30% of gelatin and 20% of laminin as intact molecules or degraded fragments. Electron microscopy of migrating cells demonstrated pseudopodia associated with channels within the Matrigel. Although the serine proteinase inhibitor DFP, plasma and a specific anti-neutrophil elastase IgG blocked degradation, these agents failed to inhibit migration. Migration was inhibited, however, when the Matrigel concentration was increased to 10 mg/ml. Thus, although PMNs will degrade matrix components they do not do so during migration, and proteolytic remodelling of the BM is not a pre-requisite for neutrophil passage. Copyright © 1997 Elsevier Science Ltd

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neutrophils then pass between endothelial cells and subsequently cross the underlying basement membrane. Precisely how neutrophils penetrate the subendothelial basement membrane, however, is not clear.

There are numerous recent reports examining the mechanisms used by migrating cells to cross basement membranes. These studies have investigated the degree to which proteolysis is involved in the migration of a variety of cell types (Alexander and Werb, 1992; Melchiori et al., 1992; Ratner et al., 1992; Carnegie and Cabaca, 1993; Kariko et al., 1993; Hu et al., 1994; Leppert et al., 1995; Xia et al., 1996; Zempo et al., 1996) across basement membranes. While some have correlated an increased expression of matrix metalloproteinases with migration, others (Ratner et al., 1992; Carnegie and Cabaca, 1993; Leppert et al., 1995) highlight differences in the migratory capacity of different sub-populations of cells and also in their expression of proteinases involved in degrading basement membrane components during extravasation. That activated neutrophils are capable of secreting potent, basement membrane-degrading proteases and oxygen metabolites has been shown previously in a number of studies (reviewed by Weiss, 1989), and conceivably these substances might also play a role during neutrophil emigration. On the other hand, wholesale destruction of vascular basement membranes is ordinarily not observed during neutrophil extravasation in vivo, except during severe inflammatory episodes (Weiss, 1989). In addition, plasma antiproteinases probably blunt the degradative activities of any enzymes secreted by cells trafficking through vessel walls. In some circumstances, however, neutrophils have been shown to form with their targets close, tightly protected microdomains, which are shielded from antiproteinases (Weiss and Regiani, 1984; Wright and Silverstein, 1984; Heiple et al., 1990; Rice and Weiss, 1990). Consequently, there appear to be two possible mechanisms whereby neutrophils could cross vascular basement membranes: (1) focal proteolysis of the matrix; and/or (2) non-proteolytic formation by neutrophils of transient passageways or channels through the matrix. Subsequently, the trans-basement membrane migratory paths taken by PMNs could be repaired by the endothelium (Huber and Weiss, 1989). Alternatively, the paths may simply close behind trafficking neutrophils owing to some bioelastic properties of the matrix itself.

We have previously reported that phorbol ester (PMA)-activated neutrophils cause the proteolytic release of polypeptide laminin fragments from the reconstituted basement membrane, Matrigel (Heck et al., 1990; Steadman et al., 1993). This proteolysis was shown to be dependent specifically on the release of the serine proteinases elastase and cathepsin G from the azurophilic (primary) lysosomal granules. In addition, these neutrophil enzymes generated laminin fragments that bound to neutrophils and stimulated cell movement and chemotaxis (Steadman et al., 1993). The present study was designed to determine, using sensitive immunochemical and ultrastructural techniques, whether matrix degradation was an essential feature of the PMNs migratory response to chemotactic agents.

**MATERIALS AND METHODS**

**Reagents**

Affinity purified sheep anti-laminin IgG was prepared and characterized as previously described (Abrahamson and Caulfield, 1982), as was human neutrophil elastase (HNE) (Heck et al., 1985). Phorbol 12-myristate 13-acetate (PMA) and formyl-methionyl-leucyl-phenylalanine (FMLP) (Sigma Chemical Co, St Louis, MO) were stored in dimethyl sulphoxide at -20°C and diluted in Hank's balanced salt solution (HBSS) immediately before use. Diisopropyl fluorophosphate (DFP) (Sigma) was stored under nitrogen in 10 μl aliquots at -70°C until use.

Laminin was purified from the mouse Engelbreth–Holm–Swarm (EHS) tumour by salt extraction, ion exchange chromatography and gel filtration (Timpl et al., 1979; Abrahamson and Caulfield, 1987). Derivatization of laminin with 125I-Na (carrier-free) (Amersham, Arlington Heights, IL) was carried out by the lactoperoxidase method as described previously (Steadman et al., 1993) to a specific activity of between 1.5 x 10^5 and 5.8 x 10^5 mCi/μg. Protein-bound 125I was separated from free iodide by chromatography on Sephadex G25 (Pharmacia, Piscataway, NJ) and frozen in PBS.

Gelatin was prepared from rat-tail collagen and labelled with 14C to a specific activity of 0.045 μCi/mg as described previously (Causten and Barret, 1979; Steadman et al., 1988).
Human neutrophils

Basement membrane-HSPG was prepared from human mesangial cells, metabolically labelled in culture with 35S-sulphate and purified as described previously (Thomas et al., 1994, 1995).

Matrigel (Collaborative Research, Bedford, MA) was kept frozen until use and polymerized by incubation at 37°C for 60 min. Matrigel has laminin (35%), type IV collagen (2.8%), HSPG (22%) and entactin (10%) as its major constituents. Radiolabelled laminin, gelatin or HSPG were added to the unpolymerized Matrigel at concentrations that represented approximately 5% of the total concentration of each component, according to the manufacturer's published composition data. After gelling, the overlying medium was removed and the gels washed extensively. Analysis of the washes demonstrated that 80–85% of added laminin, 72–85% of added gelatin and 93–98% of added HSPG were stably incorporated into the final gels.

Human neutrophil isolation

Human neutrophils were obtained from heparinized blood of healthy volunteers by dextran sedimentation and Ficoll-Paque isopycnic centrifugation as described by Büyum (1968). Cells were washed in ice-cold PBS and used immediately.

Neutrophil chemotaxis through Matrigel

Chemotaxis was performed in a 30-well plate apparatus (purchased from Guy Duremberg, Pasadena, CA). Chemotactic agents in HBSS containing 1% BSA (HBSS/BSA) or buffer alone were added to the lower well of the plate and a Nucleopore, 12 mm diameter polycarbonate filter (3.0 μm pore size) (Costar Corporation, Cambridge, MA), which had previously been treated for 1 hr at 37°C in HBSS/BSA, was lowered over the well. The upper section of the plate was then assembled and 300 μl of neutrophils at 1.5 × 10⁶ ml⁻¹ in HBSS/BSA were added to the upper well. In experiments to assess the migration of neutrophils through Matrigel, 20 μl of Matrigel at the appropriate concentration in HBSS was allowed to polymerize on the filter by incubation at 37°C for 1 hr before adding PMN. The plate was incubated at 37°C for a further 2 hr, the filters removed, fixed in 3.5% paraformaldehyde and dehydrated in a series of ethanol dilutions. The filters were then cleared with xylene and stained with haematoxylin. PMNs that had traversed the filters were counted adherent to the underside in five high power fields (hpf) (400 × ) for each of the duplicate wells, and the mean and SEM for each treatment calculated.

In additional studies, polycarbonate culture inserts (12 mm diameter, 3 μm pore size) (Millipore Corporation, Bedford, MA) were coated with the appropriate concentration of Matrigel and, after polymerization, 100 μl of neutrophils (10⁷ cells/ml) were added. The inserts were then placed in the wells of a 24-well culture plate and either a chemotactic agent (FMLP) or a degranulation stimulus (PMA) was added beneath the filter. Incubations were performed at 37°C for times of up to 7 hr and ended by removing supernates and fixing the inserts in 1.6% paraformaldehyde and 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. The samples were then dehydrated and embedded in Polybed 812. Ultra-thin cross-sections of the filters were doubly stained with uranyl acetate and lead citrate and examined in a Hitachi H6000 electron microscope.

Superoxide generation by Matrigel-embedded neutrophils

Neutrophils were suspended in Matrigel (3 mg/ml) to a final concentration of 1 × 10⁷ cells/ml and 250 μl aliquots of this suspension were placed in 1.5 ml microcentrifuge tubes. After polymerization at 37°C for 1 hr, the neutrophil/Matrigel mixture was pelleted by brief centrifugation (1000 g for 30 sec) and the pellet washed with HBSS at 37°C. Ferricytochrome C (Sigma; 100 μg in 100 μl HBSS/BSA) was added to each tube, followed by a range of doses of FMLP or PMA in 200 μl HBSS/BSA. Tubes were incubated at 37°C for up to 2 hr. Controls consisted of neutrophil/Matrigel preparations in the absence of added test stimulants. Incubations were terminated by centrifugation at 4000 g for 1 min and supernates were measured colorimetrically at A₅₅₀ nm. The production of superoxide (determined as reduced ferricytochrome C in the supernates) was expressed as mmoles 0₂⁻ per minute per 10⁶ PMN using a molar extinction coefficient of 28 000. The supernates and remaining pellets, from both stimulated and unstimulated cells, were then analysed for laminin, gelatin or HSPG breakdown products. In certain experiments, 100 μl of undiluted plasma or plasma diluted in HBSS/BSA were added to the pellet for 30 min prior to the
Supernates from the modified Boyden chambers containing Matrigel-coated polycarbonate filters were electrophoresed in 4-15% polyacrylamide gels and transferred to nitrocellulose. The transferred proteins were detected by incubation with rat anti-mouse EHS tumor laminin mAb 5D3 and detected by binding to the complete transfer of prestained high molecular weight standards (Bio-Rad Laboratories, Richmond, CA). After blocking for 1 hr at room temperature in 1% BSA, 5% normal goat serum and 5% non-fat dry milk in PBS, the nitrocellulose was reacted with goat anti-rat IgG-horseradish peroxidase and processed with 0.05% 3,3'-diaminobenzidine tetrahydrochloride.

Table 1. Neutrophil-induced matrix molecule release from Matrigel-coated, Boyden chamber filters

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>V-gelatin</th>
<th>S-HSPG</th>
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<tr>
<td>FMLP</td>
<td>10^-6 M</td>
<td>10^-7 M</td>
</tr>
<tr>
<td>PMA (10^-6 M)</td>
<td>10^-5 M</td>
<td>10^-4 M</td>
</tr>
<tr>
<td>PMA + plasma (1:8 dilution)</td>
<td>10^-3 M</td>
<td>10^-2 M</td>
</tr>
<tr>
<td>PMA + 1 mM DFP</td>
<td>10^-1 M</td>
<td>10^-0 M</td>
</tr>
<tr>
<td>PMA + 100 g/ml anti-HNE antibody</td>
<td>10^+1 M</td>
<td>10^+2 M</td>
</tr>
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</table>

SEM of three experiments, each using cells from a different donor. Results are expressed as percentage of incorporated material corrected for unstimulated controls.
samples were then subjected to discontinuous gel permeation chromatography on a column of Sepharose CL-6B. Fractions (0.6 ml) were collected, mixed with an equal volume of 70% ethanol, 4 ml of Insta Gel II scintillation fluid (Packard) and their 35S content quantified on a Packard 1900 CVA 1n-Carb liquid scintillation counter.

In separate experiments, without the addition of radiolabelled HSPG, supernates from PMN incubations with FMLP or PMA were analysed using 3–12% gradient SDS-PAGE. The gels were run and proteins transferred as above. The nitrocellulose blots were incubated in primary antibody (polyclonal anti-perlecan core protein, a kind gift of Dr John Hassel, University of Pittsburgh Medical School) then washed and incubated with goat-anti-rabbit IgG-horse-radish peroxidase. Binding was detected using the ECL system (Amersham International, Aylesbury, U.K.) according to the manufacturer’s instructions.

**Neutrophil binding to Matrigel**

Binding experiments were carried out to analyse the interaction of PMN with Matrigel. The wells of 24-well plates (Corning) were coated with 100 μl of 30% v/v Matrigel (diluted with HBSS) at 37°C for 1 hr and washed with 500 μl HBSS/BSA for an additional hour. Aliquots of neutrophils (100 μl containing 1 x 10^6 cells) were then added to each well and the plates incubated at 37°C for 30 min in the presence or absence of FMLP and PMA. The wells were then flooded with warm HBSS/BSA to remove non-adherent cells. The adherent cells were lysed in 250 μl of 1.0 M citric acid, pH 4.0, containing 1% v/v Triton X-100. Peroxidase substrate (250 μl of 1% v/v H_2O_2 and 4% w/v 2-2’-azino-di-(3-ethyl-benzathiazolin-sulphonate) in the lysis buffer was added and the plates left at room temperature for 15 min before stopping the reaction with 5 M perchloric acid. Reaction mixtures were pipetted into 1.5 ml microcentrifuge tubes, debris pelleted at 11 000 g for 1 min, and the A_{415} nm of the supernate measured spectroscopically. This absorbance was then compared to a standard curve established by lysing known numbers of cells from the same neutrophil preparation in each experiment. There were no measurable changes observed in the intracellular peroxidase content of adherent cells incubated on the Matrigel substrate. There was also no detectable peroxidase released by PMN prior to the addition of lysis buffer during any of the incubations employed in these experiments.

**Lactate dehydrogenase assay**

Duplicate 100 μl samples of PMN supernate were incubated with 20 μl nicotinamide adenine

Fig. 2. Western blot with anti-laminin mAb 5D3 of supernates from Matrigel-coated filters incubated with PMNs in Boyden chambers, as described in Materials and Methods. (1) Matrigel with unstimulated PMN; (2) Matrigel and PMN stimulated with 5 x 10^{-7} M PMA; (3) Matrigel and PMN stimulated with 5 x 10^{-7} M FMLP. Arrowheads indicate laminin breakdown products.
dinucleotide ([NADH] 2.5 mg/ml in PBS) and 700 μl PBS at room temperature. Aliquots (10 μl) of sodium pyruvate (10 mg/ml in PBS) were added to each tube to start the reaction and absorbancies (340 nm) were read over 12 min at 2 min intervals. After correcting for a reagent blank, lactate dehydrogenase (LDH) activity was calculated from the linear decrease in absorbance, which corresponds to the consumption of NADH. The results were expressed as a percentage of change in absorbance between the PMN supernate sample and that obtained from cells lysed by sonication. Cell damage, expressed as percentage release of LDH, was never more than 6% of the total LDH content of the cells.

**RESULTS**

**Neutrophil migration through polymerized Matrigel**

To examine processes whereby PMNs migrate through basement membranes, we carried out modified Boyden chamber assays in which polycarbonate filters were coated with a reconstituted basement membrane. In response to a 2 hr incubation with 10^{-9} M FMLP, 29 ± 7 PMN/HPF migrated through 3 mg/ml Matrigel films and were counted adherent to the under-sides of the filters. In addition to being visible on the underside of the filters, PMNs were visible within the gels and adherent to the upper gel surface (Fig. 1). Increasing the concentration of Matrigel to 10 mg/ml reduced the numbers of PMN migrating by 85% to 5.1 ± 2.5 (mean ± SEM, n = 3).

To determine whether PMNs migrating under these conditions remodelled the matrix releasing proteolytic cleavage products, ^125^I-laminin, ^14^C-gelatin or ^35^S-HSPG were incorporated into the polymerized Matrigel films and supernates from the incubations were counted for radioisotope release. There was no release of any labelled molecule above background control levels as a result of exposure to FMLP. In contrast, incubation with the degranulation stimulus PMA, under the same conditions, caused significant release of labelled products from Matrigel (Table 1).

To determine whether the laminin or HSPG molecules had been substrates for proteolytic degradation by PMN the supernates were further analysed by SDS-PAGE and immunoblotting with the anti-laminin mAb 5D3. This mAb recognizes an epitope on the long arm of the laminin molecule (Abrahamson et al., 1989), and, after incubation of laminin with elastase purified from neutrophils, mAb 5D3 binds to broad bands of 110–160 and 170–210 kDa (Steadman et al., 1993). PMNs migrating through Matrigel in response to FMLP did not release detectable proteolytic fragments of laminin, whereas PMA treatment did result in laminin degradation and release (Fig. 2).

Similarly, supernates from incubations with Matrigel in which ^35^S HSPG had been incorporated were analysed for potential breakdown products by chromatography on Sepharose CL 6B. ^35^S-labelled products were not released into the supernate as a result of FMLP-stimulated
PMN migration (Table 1). In addition, the elution profile of the 35S-HSPG extracted from Matrigel after 2 hr of PMN migration was the same as that extracted from Matrigel incubated with unstimulated PMN and as that extracted from Matrigel that had been incubated in buffer alone in the absence of neutrophils (Fig. 3a). In contrast, incubation with PMA resulted in the dose-dependent release of intact 35S-HSPG (Table 1) and also of smaller molecules (Fig. 3), which were the result of proteolytic cleavage and which were recognized after SDS-PAGE and immunoblotting by antibodies directed against the core protein (Fig. 4). There was no detectable production of free glycosaminoglycan chains, however, under resting, migrating or PMA-activated conditions.

PMA-stimulated release of labelled molecules was almost completely inhibited by DFP and affinity-purified anti-HNE IgG (Table 1).

If undetectably low levels of proteolysis were occurring during PMN migration through Matrigel, we reasoned that the addition of DFP, anti-HNE IgG, or plasma would inhibit migration. These inhibitors, however, did not affect FMLP-induced PMN migration through Matrigel, and the same numbers of cells were counted in the presence of these agents as were counted in their absence (Table 2). Although FMLP did not stimulate release of

![Western blot with anti-perlecan antibody of supernates from Matrigel-coated filters incubated with PMNs in Boyden chambers as described in Materials and Methods. (1) Matrigel with unstimulated PMN; (2) Matrigel and PMN stimulated with 10^{-7} M FMLP; (3) Matrigel and PMN stimulated with 10^{-6} M FMLP; (4) Matrigel and PMN stimulated with 10^{-7} M PMA. Arrowheads indicate breakdown products of HSPG.](image)

Table 2. Effects of proteinase inhibitors on PMN chemotaxis to 10^{-9} M FMLP

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>PMNs traversing filter per HPF*</th>
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<tr>
<td>0</td>
<td>32.6 ± 1.9</td>
</tr>
<tr>
<td>DFP</td>
<td>29.9 ± 2.8</td>
</tr>
<tr>
<td>0</td>
<td>18.4 ± 1.0</td>
</tr>
<tr>
<td>Anti-HNE</td>
<td>19.4 ± 0.7</td>
</tr>
<tr>
<td>0</td>
<td>21.3 ± 0.9</td>
</tr>
<tr>
<td>Plasma</td>
<td>18.6 ± 1.2</td>
</tr>
</tbody>
</table>

(1:8 dilution in NBSS/BSA). *Each pair of experiments was carried out at different times. SEM from triplicate trials, each using cells from a different donor.

![Time course of superoxide generation by neutrophils embedded in Matrigel. Cells were incubated with 5 x 10^{-7} M PMA, 10^{-6} M FMLP or buffer alone for the times shown. Results are expressed as nanomoles of superoxide per minute per 10^6 PMN calculated from the accumulation of reduced ferricytochrome C (mean ± SEM of three experiments, each using cells from a different donor).](image)
radiolabelled material by PMN migrating through or embedded within Matrigel, these cells nevertheless underwent a respiratory burst in response to FMLP, albeit to a lesser degree than that generated by PMA (Fig. 5). The time to reach maximum superoxide generation was also longer in response to FMLP than that for PMA. Maximum superoxide generation by Matrigel-embedded PMN was achieved 5–15 min after PMA addition, whereas maximal levels for FMLP were not reached until ~30 min (Fig. 5).

**Neutrophil binding to Matrigel**

To test whether human PMN bound to components contained within polymerized Matrigel and whether this binding was affected by incubation with PMA or FMLP, binding assays...

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**Fig. 6.** Transmission electron micrographs of cross-section of 30% Matrigel-coated filter and PMN incubated for 2 hr with FMLP in the lower chamber. (a) Cells are adherent to the top of the Matrigel surface (upper left of figure) and several neutrophils have also penetrated deep within the Matrigel. Note the clear zones (arrows) within the gel, which apparently represent channels created by the migrating cells. **Magnification × 3300.** (b) Higher power micrograph showing PMN migrating through Matrigel. Note the extended pseudopod (arrow) and its associated channel in the matrix. **Magnification × 12 600.**
using the activity of endogenous neutrophil myeloperoxidase were carried out in 24-well plates. When added at 37°C to plates coated with polymerized Matrigel, PMN bound and binding was increased by incubation with \(10^{-4}\) M PMA by 63% (range 50–77%), and with \(10^{-7}\) M FMLP by 58% (range 50–64%).

**Matrigel morphology during PMN transmigration**

Electron microscopic examination of modified Boyden chamber culture inserts showed that, when stimulated by FMLP, PMN readily penetrated into 3 mg/ml Matrigel films and usually were associated with matrix-free furrows or channels (Fig. 6a and b). No such channels were observed in Matrigel-coated filters incubated in the absence of PMNs (not shown). Although addition of inhibitors or anti-HNE failed to affect migration of PMN through Matrigel, increasing the concentration of the Matrigel itself markedly reduced the numbers of cells penetrating the gel and entering the filters. When undiluted Matrigel (10 mg/ml) was used to coat filters, for example, the number of cells migrating fell from 59.6 ± 10.4 in the absence of matrigel to 5.1 ± 2.5 in the presence of 10 mg/ml matrigel. Cells incubated on undiluted Matrigel were seen adherent to the upper gel surface, but neither cells nor channels were observed within the Matrigel itself (not shown).

**DISCUSSION**

In the present study, we examined neutrophil activation and trafficking within a versatile *in vitro* model of basement membrane. Our results demonstrate that PMNs migrating through Matrigel in a chemotactic FMLP gradient did not degrade the selected matrix components. In contrast, upon stimulation with PMA, neutrophils readily degraded Matrigel-incorporated laminin, gelatin and HSPG.

The mechanisms used by circulating neutrophils to penetrate subendothelial basement membranes are not known. Once in the underlying tissues, however, neutrophil inflammatory responses involve the activation of a variety of anti-microbial systems that have the potential to cause extensive tissue injury (Weiss, 1989). These responses include the generation of reactive oxygen species, and the release of several potent proteases and hydrolases (such as elastase and cathepsin G) from lysosomal granules. Except in severe inflammation, however, the subendothelial basement membrane is not degraded. Therefore, despite the ability of PMN proteases to cleave collagen and other extracellular matrix macromolecules, the evidence suggests that neutrophils utilize a non-destructive mechanism to traverse basement membranes.

Receptors for several extracellular matrix proteins have been immunolocalized to the PMN plasma membrane and to the inner membrane of the specific (lysosomal) granules (Singer *et al.*, 1989). These granules fuse with the cell membrane in response to a variety of PMN-activating agents and thus increase cell surface expression of extracellular matrix receptors. In addition, they contain metalloproteinases, such as gelatinase and collagenase, which have been implicated in the migration of some tumour cell lines and of some lymphocyte sub-classes (Alexander and Werb, 1992; Melchiori *et al.*, 1992; Ratner *et al.*, 1992; Carnegie and Cabaca, 1993; Kariko *et al.*, 1993; Hu *et al.*, 1994; Leppert *et al.*, 1995; Xia *et al.*, 1996; Zempo *et al.*, 1996). Our experiments confirmed that PMN bound to Matrigel and that this binding was increased by treating cells with PMA or FMLP. In response to FMLP, however, this seems to have occurred in the absence of proteolysis. This supports observations that PMN will migrate under conditions in which granule exocytosis is inhibited (Kuijpers *et al.*, 1992) and also that cytoplasts of PMN that lack lysosomal granules will also migrate in a chemotactic gradient (Huang *et al.*, 1991).

The release of relatively stable laminin fragments by PMA-stimulated PMN embedded in Matrigel has been described previously (Steadman *et al.*, 1993). Although incubation of Matrigel-embedded PMN with FMLP did not stimulate matrix molecule release or degradation, the cells nevertheless underwent a respiratory burst in response to FMLP. The kinetics of the responses were similar to those reported previously for PMN adherent to surfaces coated either with serum, fibronectin, vitronectin, or laminin (Nathan, 1987).

We have previously demonstrated (Heck *et al.*, 1990; Steadman *et al.*, 1993) that our experimental system allows the measurement of as little as 30 ng of purified elastase, which is equivalent to ~1% of the elastase content of \(1 \times 10^6\) PMN. Thus a positive control, consisting of PMA-stimulated cells, was included in
each experiment to ensure that if a low level of proteolysis should occur during treatment with FMLP, it would be detected. In the present study, we tested the effects of plasma on the digestion of Matrigel-incorporated molecules by activated neutrophils. There are several potent protease inhibitors present in plasma (Weiss, 1989) and some of these, such as α1-antitrypsin, have high binding affinities for individual proteases (Travis and Salvesen, 1983). In the case of HNE, α1-antitrypsin reacts specifically with the enzyme to form a stable, proteolytically inactive complex (Weiss, 1989; Travis and Salvesen, 1983). Also present in plasma are broad spectrum protease inhibitors, such as the amyloid P component and α2-macroglobulin, that would potentially prevent PMN proteases from degrading components of Matrigel. In the present experiments, the addition of plasma caused significant, but nevertheless incomplete, inhibition of the neutrophil degradative capacity. This incomplete inhibition may reflect the formation between PMN and Matrigel of close cell-substrate contacts inaccessible to the proteases (Weitz et al., 1987; Heiple et al., 1990; Wright and Silverstein, 1990).

Electron microscopic examination of the Matrigel containing trafficking PMNs showed channels or pathways within the matrix, and these were often associated with the pseudopodia of migrating cells. How these channels were formed is not known. Despite a lack of evidence for proteolytic damage, the possibility still remains that the channels were created by focal proteolysis of the matrix at levels undetected by our assays and insensitive to inhibitors of HNE. Alternatively, migrating cells may have created the channels by a non-degradative mechanism involving extensions of pseudopodia that effectively swept the Matrigel aside. We favour this alternative, because increasing the density of the polymerized Matrigel decreased the numbers of PMNs migrating through the gel and at a concentration of 10 mg/ml protein (undiluted Matrigel) there was almost complete inhibition of both migration and channel formation. Furthermore, under these conditions, there was still no detectable proteolysis. Migration blockade was not, however, a result of failure of FMLP to diffuse through undiluted Matrigel, because even under these conditions PMN adherent to the gel surface still underwent a respiratory burst in response to FMLP (data not shown).

Scanning electron microscopy has previously been used to assess the passage of PMNs through amniotic tissues in vitro (Bakowski and Tschesche, 1992). These studies concluded that basement membrane proteolysis was involved during transit across amniotic basement membranes, but non-degradative, mechanical forces exerted by penetrating PMN were not ruled out. However, in these same studies, little or no degradation of stromal type I collagen fibres was observed. Because both type IV and type I collagenases are released by the same activators of PMN, these results are difficult to interpret. None the less, TIMP inhibited basement membrane passage without affecting migration through stromal collagen type I. In contrast, however, others have shown in different models that several different inhibitors failed to block the migration of PMN through extracellular matrices (Huber and Weiss, 1989; Huber et al., 1991) and that HNE inhibitors had no effect on diapedesis. To address the possibility that very low levels of localized proteolysis were occurring that were not affected by large molecular weight inhibitors, we included the low molecular weight protease inhibitor DFP, as well as anti-HNE IgGs, and assayed their effects on PMN migration through Matrigel. These substances, which were effective inhibitors of PMA-stimulated degradation, did not affect migration. This is an important point in two ways. First, an absence of degradation of matrix during migration was confirmed. Second, many PMN enzymes are secreted in latent form. Although others have shown previously that latent (and activated) plasminogen activators are not involved in PMN transmigration (Furie et al., 1987; Huber and Weiss, 1989), we reasoned that latent metalloproteinases, which are activated by HNE, might play a role in Matrigel transmigration. However, the inclusion of serine protease inhibitors again had no effect. Thus, we neither detected proteolysis during migration nor suppressed migration through the use of proteinase inhibitors at concentrations that blocked PMA-induced laminin degradation.

Although the absolute amounts of the various proteins in different basement membranes in vivo are unknown, those matrices underlying the endothelium of the microvasculature, which are typical sites of leukocyte extravasation, are
ultrastructurally the thinnest and least electron dense. In contrast, the much thicker and denser renal glomerular basement membrane, which is frequently a site of PMN adherence during immune damage (Davies et al., 1978; Pipoly and Crouch, 1987; Vissers and Winterbourn, 1988; Leardkamolkarn et al., 1990; Baricos and Shah, 1991), is almost never actually crossed by the cells, even during intense inflammatory episodes. We speculate therefore that inflammatory mediators, together with what appear to be variable biophysical properties of basement membranes at different sites, may jointly regulate PMN trafficking.

In summary, changes induced in the matrix during PMN migration did not result from extensive proteolysis of the principal matrix constituents (laminin, collagens and proteoglycans). Our findings suggest, therefore, that cells responding to a chemotactic signal travel through extracellular matrices using a non-degradative mechanism. Whether the distinct channels seen associated with cells migrating in Matrigel also occur in vivo needs to be determined. Nevertheless, other studies have shown that functional endothelial cells are needed to preserve basement membrane barrier function in vitro (Huber and Weiss, 1989), and perhaps, in vivo, a function of these cells is the repair of channels created by migrating leukocytes.

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