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Relationship between Gelatinase Secretion and Chemotaxis of Rat Polymorphonuclear Leukocytes

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In response to chemoattractants, rat polymorphonuclear leukocytes (PMNs) secreted latent gelatinase, which was predominant among PMN neutral proteinases such as collagenase, elastase and cathepsin G. The PMNs highly responsive to chemoattractants migrated earlier than other PMNs and secreted large proportions of their latent gelatinase, though their cellular content of gelationase was at a normal level. The gelatinase species secreted from PMNs were metalloproteinases and have the same molecular weights (96 and 230 kilodaltons) as those of proteinases stored in granules of PMNs. After treatment with an activator, the secreted gelatinase degraded bovine type IV collagen. From these results we hypothesize that there is a population of PMNs that actively migrates and secretes latent gelatinase in response to chemoattractants. The PMNs probably reach the vascular site near the inflammatory lesion faster than other PMNs and secrete large amounts of gelatinase to degrade basement membranes, resulting in the formation of a pathway for extravascular emigration of other PMNs.

Keywords——polymorphonuclear leukocyte; chemotaxis; gelatinase; enzyme secretion; type IV collagen

Introduction

In response the various inflammatory stimuli, polymorphonuclear leukocytes (PMNs) adhere to endothelial cell surfaces of the post capillary venules near the site of inflammation.[1,2] These PMNs traverse the endothelium through interendothelial junctions, and penetrate the basement membranes.[3] The basement membranes function as barrier when PMNs emigrate to the extravascular space. A major structural protein of basement membrane is type IV collagen which is chemically and genetically distinct from stromal type I and III collagens and cartilage type II collagen.[4] Previously characterized animal collagenases which cleave stromal collagens fail to degrade type IV collagen.[5,6] Recently, Murphy *et al.*[7] reported that type IV collagen was degraded by human neutrophil gelatinase, which has frequently been found to be present in situations where collagenases have been identified.[8,9] If the enzymatic degradation of basement membrane is an essential step in the emigration of PMNs to extravascular space, an excellent candidate for the key enzyme is gelatinase secreted from activated PMNs. The present paper describes an investigation of the relationship between PMN chemotaxis and gelatinase release, and discusses the role of gelatinase in the process of PMN emigration across the basement membrane under inflammatory conditions.

Materials and Methods

Preparation of PMNs——Rat peritoneal exudate PMNs were obtained 16 h after an intraperitoneal injection of 40 ml of 1% (w/v) casein in Ca^{2+}-free Krebs–Ringer bicarbonate solution.[10] Rat peripheral PMNs were prepared as described below. Venous blood was collected in a 50 ml polyethylene tube containing 1 ml of heparin solution (0.5% heparin in phosphate-buffered saline (PBS)). The blood was layered on an equal volume of the Mono-poly resolving

medium[11] (Flow Laboratories Ltd.), to which Ficoll (20 mg/ml) had been added. The tube was centrifuged at $1600 \times g$ for 30 min in a swinging bucket rotor at room temperature. After the centrifugation three cell fractions were obtained; mononuclear cells at the plasma-medium interface, PMNs below the interface and red blood cells as a bottom pellet. The PMN fraction was recovered and washed gently with RPMI-1640 medium.[12] The purity of the PMN preparation was about 99%.

Chemotaxis Assay——PMN chemotaxis *in vitro* was evaluated by means of a procedure using a new disposable Boyden chamber.[10] Briefly, 0.44 ml of PMN suspension (1×10^7 cells/ml of RPMI-1640 medium) was applied to the upper compartment, while the lower compartment contained 0.44 ml of 2% (v/v) zymosan-activated serum (ZAS) or 3.3×10^{-8} M *N*-formyl-L-Met-L-Leu-L-Phe (FMLP) in RPMI-1640 medium containing 0.2% bovine serum albumin. 2% (v/v) ZAS or 3.3×10^{-8} M of FMLP in RPMI-1640 medium containing 0.2% bovine serum albumin. The upper and lower compartments were separated by a polycarbonate filter (10 μm thickness) with pores 2 μm in diameter. The Boyden chamber was incubated at 37 °C in a humidified atmosphere containing 5% CO_2. After incubation the medium in the lower compartment was recovered and then centrifuged at $700 \times g$ for 5 min at 4 °C. The supernatant was used for assays of various enzyme activities, while the PMNs in the pellet were resuspended in PBS containing 0.05% ethylenediaminetetraacetic acid (EDTA), and counted in a Coulter counter. Migration rate of PMNs was calculated as follows;

(number of PMNs collected from the lower compartment/number of PMNs applied in the upper compartment) $\times$ 100 (%)

Enzyme Activity Assay——Gelatinase assay was performed as follows.[13,14] Sample solution (200 μl), 980 μl of the assay buffer (0.05 M Tris–HCl, pH 7.5, containing 0.15 M NaCl, 5 mM $CaCl_2$ and 0.02% NaN_3) and 20 μl of dimethyl sulfoxide (DMSO) or *p*-aminophenylmercuric acetate (APMA) (18 mg/ml of DMSO) were mixed and then the mixture was preincubated at 37 °C for 20 min. Fluorescein isothiocyanate-conjugated gelatin solution (200 μl) was added to the mixture and the whole was incubated for 16 h at 37 °C. At the end of the reaction, dioxane (2.8 ml) was added to precipitate undegraded gelatin molecules. The mixture was cooled in an ice bath for 1 h and centrifuged at $1600 \times g$ for 30 min. Fluorescence intensity of the supernatant was measured at 495 nm for excitation and 520 nm for emission.

Cathepsin G, elastase, collagenase and β-glucuronidase were assayed by using succinyl–Ala–Ala–Pro–Pre–4-methyl coumaryl–7-amide,[15,16] succinyl–Ala–Pro–Ala–4-methyl coumaryl–7-amide,[15,16] FITC-collagen[13,17] and *p*-nitrophenyl-β-D-glucuronide[18] as substrates, respectively.

Zymography of Gelatin-Degrading Proteinase——Visualization in gelatinase activity in sodium dodecyl sulfate (SDS)-substrate gel was performed according to the method of Heussen and Dowdle[19] with some modifications. Separation gel (7.5% acrylamide) contained 0.1% SDS and 0.1% gelatin. Concentrated medium obtained from the Boyden chamber or PMN lysate was mixed with an equal volume of sample buffer (0.25 M Tris–HCl, pH 6.8, containing 10% SDS, 4% sucrose and 0.1% phenol red). After standing at room temperature for 1 h, 5—20 μl of the sample was loaded into wells of 4% acrylamide stacking gel containing 0.1% SDS on a minislab gel apparatus. The gel was run at 20 mA/gel for 2 h at 4 °C. After electrophoresis, the gel was soaked in the assay buffer containing 2.5% Triton X-100 with gentle shaking for 30 min at room temperature. The gel was rinsed and incubated overnight at 37 °C in the assay buffer containing 1% Triton X-100. The gel was stained for 30 min in 0.1% Coomassie brilliant blue R-250 in 50% methanol–10% acetic acid and then destained in 10% methanol–10% acetic acid.

Preparation of PMN Lysate——PMNs were homogenized with a Teflon-glass homogenizer in 0.34 M sucrose containing 3 mM phenylmethylsulfonyl fluoride (PMSF) and 5 mM *N*-ethylmaleimide. Nuclei and cell debris were sedimented at $700 \times g$ for 10 min at 4 °C and the precipitate was re-homogenized with the sucrose solution containing proteinase inhibitors. The pooled supernatant was centrifuged at $27000 \times g$ for 40 min at 4 °C and the precipitate was suspended in the assay buffer containing 3 mM PMSF and 5 mM *N*-ethylmaleimide. Freezing-thawing was repeated 7 times and the suspension was centrifuged at $140000 \times g$ for 1 h at 2 °C. The supernatant was recovered as enzyme solution.

Materials——Special reagents were obtained from the following sources: APMA, PMSF, FMLP (Sigma), type IV collagen of bovine anterior lens capsule (Nitta Gelatin Co., Ltd., Osaka); *p*-nitrophenyl-β-D-glucuronide (Boehringer Mannheim); elastatinal, chymostatin, succinyl–Ala–Pro–Ala–4-methyl coumaryl–7-amide, succinyl–Ala–Ala–Pro–Phe–4-methyl coumaryl–7-amide (Osaka Peptide Institute); gelatin (Difco).

Results

Proteinase Secreted from PMNs in Response to FMLP

PMNs secrete various proteinases upon stimulation by chemoattractants. The PMNs secreted cathepsin G, elastase and collagenase by in amounts equivalent to 7, 7 and 39% of the stored enzymes, respectively. On the other hand, approximately 86% of stored gelatinase was

TABLE I. Proteinases Secreted from PMNs in Response to FMLP

Enzyme	Enzyme activity (F.I.)		Medium/total (%)
	Cells	Medium	
Gelatinase	5±1	30±2	86
Cathepsin G	95±8	7±1	7
Elastase	28±2	2±1	7
Collagenase	19±7	12±2	39

PMNs were incubated with FMLP (3.3×10^{-8} M) for 80 min at 37°C and then centrifuged. The supernatant and pellet of PMNs were separated and the proteinase activities of the supernatant were determined. PMNs pellets were homogenized in gelatinase assay buffer containing 0.05% Triton X-100. Proteinase activities of the supernatant obtained after centrifugation ($27000 \times g$ for 30 min at 4°C) were measured. In the case of gelatinase assay, 1 mM elastational and 0.2 mM chymostatin were added to the substrate-enzyme mixture to block elastase and cathepsin G, which have an ability to hydrolyze gelatin.

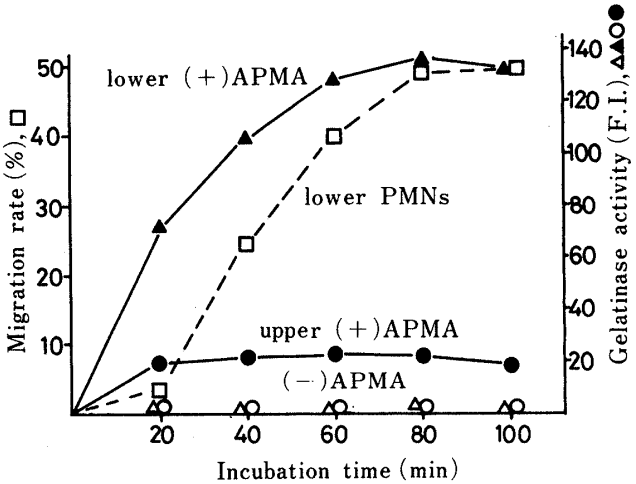

Fig. 1. Rat PMN Chemotaxis (□) and Change in Gelatinase Activity of the Media in the Upper (○, ●) and Lower (△, ▲) Compartments of the Boyden Chamber during Chemotaxis

Active and latent gelatinase activities are expressed by open and closed symbols, respectively. Elicited PMNs were used in this experiment.

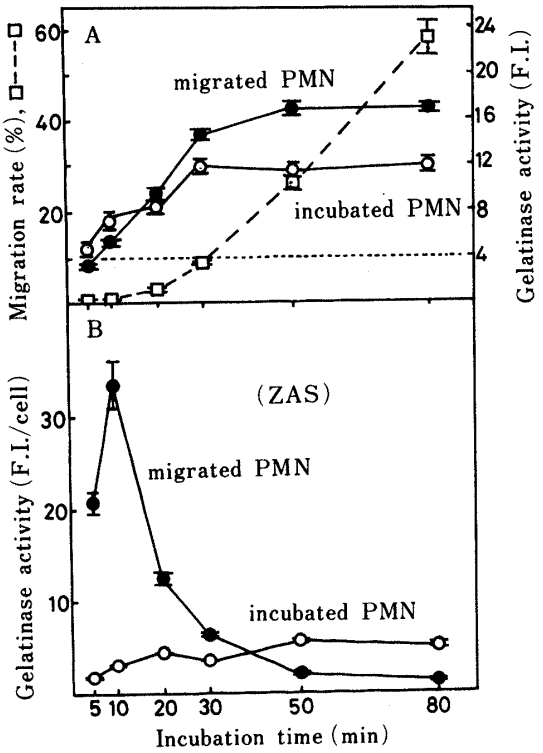

Fig. 2. A Comparison between the Gelatinase Secretion from PMNs Simply Incubated in 2% ZAS and That from the PMNs That Migrated into the Lower Compartment Containing 2% ZAS

(A) PMN suspension (0.44 ml: 4.4×10^5 cells corresponding to 10% migration rate) was simply incubated in 2% ZAS. On the other hand, PMN chemotaxis assay (□) was performed as described under Materials and Methods. Gelatinase activities in the lower compartment of the Boyden chamber and in a medium of simply incubated PMNs were measured after activation with 1 mM APMA.

(B) Gelatinase activities in the media per cell were calculated. Gelatinase activity was divided by the number of the migrated PMNs (●) or the incubated PMNs (○) at each point.

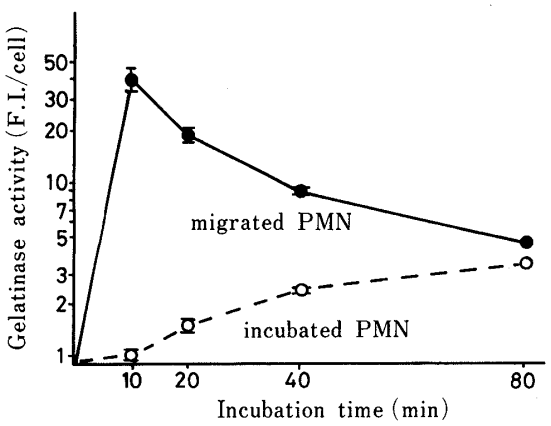

Fig. 3. Gelatinase Secretion from Peripheral PMNs during *in Vitro* Incubation (○) and Chemotaxis (●)

Experimental conditions are similar to those shown in Fig. 2, with the exception that PMNs used were peripheral ones.

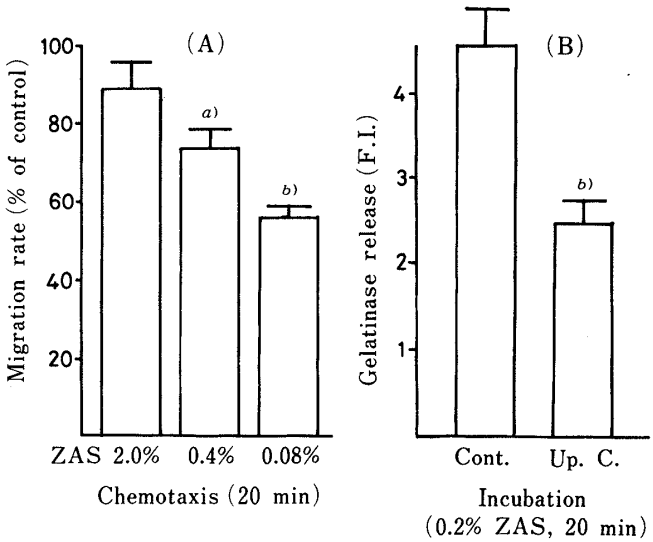

Fig. 4. Chemotactic and Gelatinase Secretion Responses of the PMNs Remaining in the Upper Compartment of the Boyden Chamber

The PMNs that had remained in the upper compartment (Up.C.) after a 40-min incubation for chemotaxis to 2% ZAS were collected and washed three times with fresh medium, and then (A) applied to a Boyden chamber for chemotaxis (20 min incubation) to low concentrations (0.08, 0.4 or 2.0%) of ZAS, or (B) incubated *in vitro* with 0.2% ZAS. Control cells (Cont.) were the peritoneal exudate PMNs that had been incubated at 37 °C for 40 min in the medium. Values are significantly different from the control; *a*) $p<0.01$, *b*) $p<0.001$.

secreted from FMLP-activated PMNs (Table I).

Gelatinase Secretion during PMN Chemotaxis

PMN chemotaxis toward the 2% ZAS in the lower compartment and the gelatinase activity in the upper and the lower compartments of the Boyden chamber were determined (Fig. 1). The latent gelatinase level in the lower compartment increased with the PMN chemotaxis, whereas that in the upper compartment remained at a low level throughout the incubation period. No active gelatinase was detected in media of both compartments, indicating that rat PMNs secrete a latent gelatinase that can be activated by a latent gelatinase activator, APMA.

PMNs incubated with 2% ZAS *in vitro* also secrete a latent gelatinase. Gelatinase secretion from the PMNs simply incubated with 2% ZAS was compared with that from PMNs that migrated into the lower compartment of the Boyden chamber (Fig. 2A). Although the migration rate was very low during the early period (0—30 min), the gelatinase level of the lower compartment was similar to that secreted from the PMNs simply incubated with ZAS (Fig. 2A). Gelatinase activity per cell was calculated to clarify the ability of PMNs to secrete latent gelatinase during the course of incubation (Fig. 2B). The PMNs that migrated into the lower compartment during the early period (0—30 min) of incubation secreted a large amount of gelatinase. The gelatinase activity per migrated cell reached a maximum at 10 min and then rapidly declined, whereas the value per incubated cell slightly increased (Fig. 2B). Similar results were also obtained when FMLP was used instead of ZAS as a chemoattractant (data not shown). In addition, peripheral non-activated PMNs gave similar results (Fig. 3) to those shown in Fig. 2B.

Gelatinase Secretion and Enzyme Contents of PMNs Remaining in the Upper Compartment of the Boyden Chamber

The PMNs that remained in the upper compartment of the Boyden chamber after a 40-min incubation had less ability to show chemotaxis and to secrete gelatinase in response to low concentrations of ZAS (Fig. 4). In order to get information about the levels of granule enzymes stored in the PMNs which possess high abilities of chemotaxis and gelatinase secretion, granule enzyme levels of the PMNs which remained in the upper compartment were compared with those of the PMNs before chemotaxis assay. As shown in Table II, no significant difference in the levels of gelatinase, cathepsin G, elastase and glucuronidase was found between the PMNs before chemotaxis and the PMNs that remained in the upper compartment after chemotaxis.

PMN Gelatinase Species

SDS–gelatin polyacrylamide gel electrophoresis (PAGE) was performed to investigate

TABLE II. Contents of Enzymes in the PMNs Remaining in the Upper Compartment of the Boyden Chamber

Enzyme	Control PMN	U.C. PMN	
Gelatinase	27.4 ±1.0	29.2 ±1.1	N.S.
Cathepsin G	78.8 ±7.1	69.4 ±5.3	N.S.
Elastase	25.8 ±8.5	24.5 ±3.9	N.S.
β-Glucuronidase	0.84±0.01	0.84±0.01	N.S.

Peritoneal exudate PMNs (Control PMNs) and PMNs remaining in the upper compartment after a 40-min incubation for chemotaxis (U.C. PMN) were homogenized in the gelatinase assay buffer containing 0.05% Triton X-100 with a Teflon homogenizer. The homogenates were centrifuged at 27000 × *g* for 30 min at 4 °C, and then each enzyme activity in aliquots of the supernatant was measured. N.S., statistically not significant.

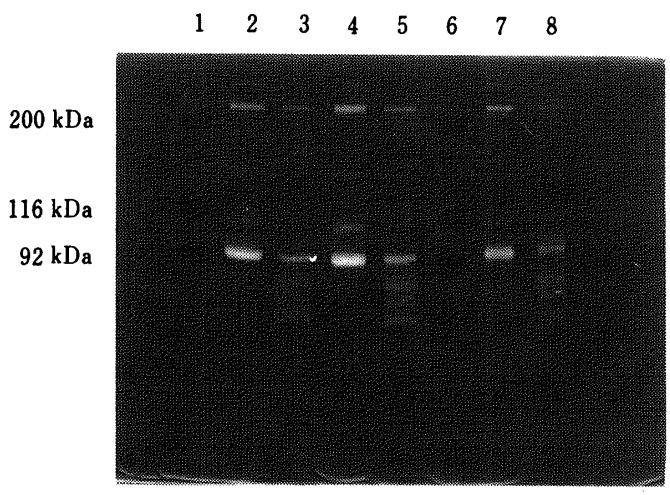

Fig. 5. Zymography of Gelatinase Secreted from PMNs and Stored in PMNs

PMNs were incubated with FMLP (3.3×10^{-8} M) for 20 min or 40 min and then incubation medium was obtained. Homogenate of PMNs was obtained as described under Materials and Methods. Lanes 1 and 6, molecular markers; lane 2, 20-min incubation medium; lane 3, 20-min incubation medium treated with APMA; lane 4, 40-min incubation medium; lane 5, 40-min incubation medium treated with APMA; lane 7, PMN homogenate; lane 8, PMN homogenate treated with APMA.

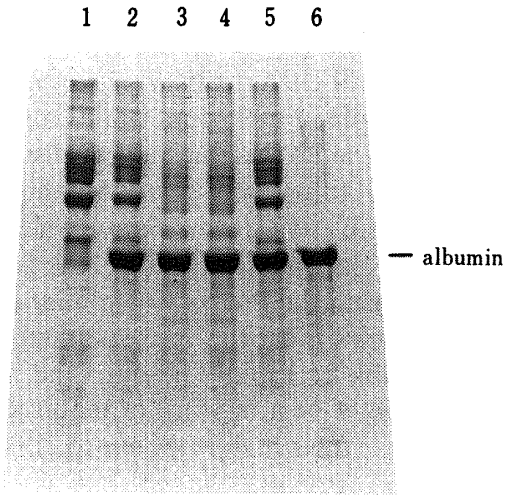

Fig. 6. Degradation of Soluble Type IV Collagen by PMN Incubation Medium

Type IV collagen was incubated for 2 d at 35 °C with untreated medium (lane 2), the medium activated with APMA (lane 3), PMSF-treated activated medium (lane 4) or *o*-phenanthroline-treated activated medium (lane 5). Type IV collagen alone (lane 1), PMN incubation medium alone (lane 6). The separation gel contained a linear gradient of acrylamide concentration from 5 to 20%.

the species and the molecular weight of gelatinase in PMN lysate and in the medium of the lower compartment after a 20-min or 40-min incubation. Latent gelatinase was activated during SDS–gelatin PAGE and appeared as clear zones of proteolysis against a blue background of undegraded gelatin. The zymograms of the three samples were similar to one another (Fig. 5). Latent gelatinase contained two major species with apparent molecular weights of 96 and 230 kilodaltons (kDa) and three minor zones of 110, 135 and 270 kDa (Fig. 5). When the samples were treated with APMA, all of the clear bands became faint, but three additional gelatinase species with lower molecular weights of 86, 82 and 78 kDa appeared. These gelatinase activities were completely inhibited by incubation with 1 mM *o*-phenanthroline but not with 1 mM PMSF (data not shown). These results indicate that the gelatinases secreted from rat PMNs after stimulation with chemoattractants were metalloproteinases with the same molecular weights as those stored in granules of PMNs.

Type IV Collagen Degradation by PMN Gelatinase

PMNs were incubated with 10^{-7} M FMLP for 2 h at 37 °C to obtain a latent gelatinase-rich medium. Then the medium was further incubated with bovine lens capsular type IV collagen, and the degradation of type IV collagen was analyzed by SDS–PAGE (Fig. 6). The APMA-treated medium degraded type IV collagen. This degradation of type IV collagen was inhibited by *o*-phenanthroline but not by PMSF, suggesting that type IV collagen was degraded by APMA-activated gelatinase in the PMN incubation medium.

Discussion

Rat PMNs secreted a latent gelatinase in response to chemoattractants such as ZAS and FMLP (Figs. 1 and 2). This latent gelatinase was predominant among the secreted PMN neutral proteinases such as collagenase, elastase and cathepsin G (Table I). Dewald *et al.*[20] also reported that gelatinase secretion is highly responsive to secretory stimuli and gelatinase is released efficiently from PMN in comparison with the release of β-glucuronidase (contained in azurophilic granules) and vitamin B_{12}-binding protein (contained in specific granules).

In PMN chemotaxis *in vitro* the number of PMNs in the lower compartment increased after a lag phase of 20 min, while latent gelatinase secretion occurred without a lag time (Figs. 1 and 2). The PMNs that migrated within 20 min secreted a large amount of gelatinase (Figs. 2B and 3). Diffusion of gelatinase through the filter from the upper to the lower compartment was negligible within at 30 min when PMNs were incubated at 37 °C with 2% ZAS in the upper compartment. Therefore, the gelatinase activity detected in the lower compartment was secreted from the PMNs which migrated into the lower compartment. Peripheral PMNs also gave results similar to those obtained with the elicited PMNs (Fig. 3). Highly matured PMNs may be able to rapidly migrate and actively secrete gelatinase. Both elicited and peripheral PMNs might be a mixture of matured and prematured PMNs. If highly matured peripheral PMNs lose their high response to chemoattractants on the way to the peritoneal cavity from the blood stream, elicited but unmatured PMNs may be matured in the cavity by inflammatory stimuli. Therefore, peripheral and elicited PMNs would exhibit similar patterns (Fig. 2 and 3).

On the other hand, the PMNs that remained in the upper compartment of the Boyden chamber after a 40-min incubation had significantly low abilities to secrete latent gelatinase and to migrate towards low concentrations of a chemoattractant (Fig. 4). These results suggest that the PMNs that begin chemotaxis earlier than other PMNs secrete large portions of their latent gelatinase stored, though their gelatinase content is at a normal level (Table II).

There are several reports on subpopulations of PMNs.[21,22] Harvath and Leonard[23] described two PMN subpopulations, one which migrates to various chemoattractants and the

other which fails to migrate. Goldman and Goetzl[24] also mentioned two classes of PMNs having different affinity and number of leukotriene B_4 receptors. The PMNs that begin to migrate early and secrete large portions of their gelatinase may be one subpopulation having higher affinity and a larger number of receptors for chemoattractants than other PMN subpopulations.

Murphy *et al.*[7] reported that gelatinase of human PMNs has an ability to degrade type IV collagen. Type IV collagen is a major component of basement membrane, acting as a barrier when PMNs migrate to the extravascular space from the vascular lumen. In close agreement with the results of Murphy *et al.*,[7] we found that the gelatinase secreted from rat PMNs in response to chemoattractant degrades type IV collagen (Fig. 6).

From these results we hypothesize that there is a population of PMNs highly responsive to chemoattractants and actively secreting their latent gelatinase. These PMNs probably reach the vascular system near the inflammatory lesion faster than other PMNs and secrete a large amount of gelatinase to degrade the basement membrane, resulting in the formation of a migration pathway for other PMNs.

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