Chemotactic Response of Monocytes to Thrombin

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ABSTRACT Human α-thrombin, the procoagulant activation product of prothrombin, elicits chemotaxis in human peripheral blood monocytes and several macrophagelike continuous cell lines, most notably J-774.2, but not in human peripheral blood granulocytes. α-Thrombin is effective in stimulating cell movement at concentrations ranging from 10^{-10} to 10^{-6} M but is optimally active at 10^{-8} M. At the latter concentration, the degree of response is equivalent, on a molar basis, to that observed with the peptide formylmethionylleucylphenylalanine, (FMP). In contrast to thrombin, prothrombin produces a minimal chemotactic response in monocytes and J-774.2. Blockade of α-thrombin's active center with diisopropylfluorophosphate (DIP-F) or tryptic proteolysis of the procoagulant exosite (i.e., γ-thrombin) fails to alter chemotactic activity. On the other hand, addition of equimolar amounts of antithrombin III (AT3) to α-thrombin reduces thrombin-mediated chemotaxis by 60%, and increased ratios of AT3 to enzyme completely suppress chemotaxis. We conclude that thrombin is a potent monocyte chemotaxin and that the domains in thrombin involved in stimulating cell movement are distinct from the catalytic site and the fibrin recognition exosite. These chemotactic domains appear to be sequestered in prothrombin and in the thrombin-AT3 complex and, as such, are unavailable to the chemotactic receptor on the monocyte cell membrane.

Thrombin (EC 3.4.21.5) is a well characterized serine esterase generated in the course of initiation of the blood coagulation pathways. In addition to its procoagulant activity, expressed through the conversion of fibrinogen to fibrin, α-thrombin has also been shown to stimulate aggregation and release in blood platelets (1, 2) and to elicit a mitogenic response in a variety of fibroblast cell lines in tissue culture (3, 4).

The migration of leukocytes is a major component of the inflammatory response and is, almost certainly, the result of the directed movement of cells along a chemical gradient generated by events at the lesion site (5). Recognizing the multiplicity of effects that thrombin has on cells, and its ubiquity at sites of tissue injury, we wondered whether the enzyme might also be responsible for recruitment of inflammatory cells. In the present communication, we demonstrate that α-thrombin is a potent chemotaxin for one of the principal inflammatory cells, the monocyte.

MATERIALS AND METHODS

Human Thrombins: Human prothrombin complex and highly purified human α-thrombin were prepared and characterized as described previously (6–8). Specific clotting activity of the α-thrombin preparation was 3,975 U/mg. The γ-thrombin preparation, produced by controlled passage of α-thrombin through trypsin immobilized on agarose (7), was composed of 0.01% α-, 15.7% β-, and 84.3% γ-thrombins. It retained 80% of its esterase activity (active site titration), but possessed only 1.74 U/mg clotting activity. Diisopropylfluorophosphate-treated thrombin (DIP-thrombin) was prepared by treating α-thrombin repeatedly with 0.2 M DIP-F at pH 8.0 for 30 min until clotting activity was diminished to ~0.5 U/mg and active titratable sites were reduced to an undetectable level (9). Dilutions of thrombins used in the chemotaxis assays (see below) were carried out in Eagle's minimal essential medium with L-glutamine (MEM/α-modified), containing 1 mg/ml bovine serum albumin (BSA) and 5 μM polyethylene glycol 6000 (PEG) (10).

Human Peripheral Blood Monocytes: Mononuclear cells and granulocytes were isolated from freshly drawn heparinized blood using a Ficoll-Hypaque gradient as described by Boyum (11). The cells were resuspended in MEM/α-modified containing 1 mg/ml BSA and 5 μM PEG to a final concentration of 2.5×10^6 cells/ml.

Macrophagelike Cell Lines: The cell lines WEHI-3 and J-774.2 were obtained from American Type Culture Collection (Rockville, MD) and U-937 from Dr. Robert Senior (Washington University School of Medicine). Cells were grown in RPMI-1640 medium enriched with 10% fetal calf serum FCS.

Human Antithrombin III (AT3): Human AT3 was kindly provided by Dr. Craig Jackson, Washington University School of Medicine. It was isolated from normal human plasma essentially as previously described (12). An M_r ~58,000 was assumed (13).

Thrombin Characterization: The percentage composition of α-thrombins was determined by labeling with ^{14}C DIP-F and quantitating the relative distribution of radioactivity after gel electrophoresis (7). An M_r ~36,500 was assumed for all thrombin forms (6–8). Active site titrations and clotting assays were performed as previously described (6).

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Chemotaxis: Chemotaxis was determined in modified Boyden chambers (Ahlco Mfg. Co., Inc., Southington, CT) by a double micropore membrane technique (14). The upper membrane was polycarbonate (Nucleopore Corp., Pleasanton, CA), 5-μm pore size (2 μm for granulocytes), and the lower membrane was cellulose pore size 0.45 μm (Millipore Corp., Bedford, MA). Chambers were prepared and run in triplicate. After an appropriate incubation period at 37°C (1 h for granulocytes, 2 h for monocytes, and 4 h for cell lines), the membranes were removed and stained with hematoxylin. The extent of cell movement was determined by counting the number of cells per grid at the interface between the two membranes using an eyepiece reticule under 400× magnification. Five random grids were scored in each slide preparation. The mean number of cells per high power field (HPF) was corrected by subtracting the counts obtained in the absence of any stimulus (negative control). The corrected value, termed net cells/HPF, is presented in the text ± SEM. Formylmethionylleucylphenylalanine (FMP) (Sigma Chemical Co., St. Louis, MO) at 10^{-8} M was used as a positive control (15).

RESULTS

Purified human α-thrombin promotes a dose-dependent migration of human peripheral blood monocytes at concentrations ranging from 10^{-6} to 10^{-10} M, with optimal movement occurring at 10^{-8} M (Fig. 1, Table I). By contrast, over the same concentration range, prothrombin elicits minimal migration of monocytes (Fig. 1). In most experiments, α-thrombin is equivalent, on a molar basis, to FMP in its ability to stimulate monocyte migration. However, whereas FMP is chemotactic for neutrophils, α-thrombin is ineffective even at doses as high as 10^{-6} M (data not shown).

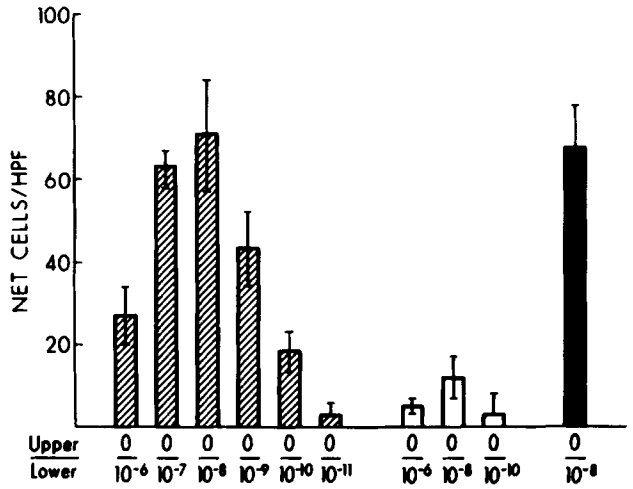

FIGURE 1 Stimulated migration of human monocytes as a function of α-thrombin dose. The chemotactic activity of prothrombin and FMP are shown for comparison. Each bar represents the mean of triplicates ± SEM.

"Checkerboard analysis" establishes that monocyte response to thrombin represents gradient-directed migration (chemotaxis) rather than enhanced random cell movement (chemokinesis). Only when the thrombin concentration in the lower chamber exceeds that of the upper (cell-containing) chamber does net movement of cells toward the lower chamber occur (Table I).

In addition to monocytes, thrombin stimulates chemotaxis in several macrophagelike cell lines, including WEHI-3, U-937, and J-774.2. Data comparing the chemotactic responses of this latter cell line with peripheral blood monocytes are shown in Table II. It is of interest that the dose of α-thrombin required for optimally stimulating cell movement in J-774.2 is the same as it is for blood monocytes, i.e., 10^{-8} M. Similarly, neither blockage of the enzymatic center (DIP-thrombin) nor modification of the procoagulant exosite (γ-thrombin) inhibits the ability of thrombin to stimulate migration of either cell type. Thus, the ability of α-thrombin to promote chemotaxis in monocytes or J-774.2 is independent of the enzyme's esterolytic and procoagulant capabilities. Curiously, DIP-thrombin consistently elicits a 35–45% greater response in monocytes than either α-thrombin or γ-thrombin, when all are compared at what was determined to be their optimal dose. On the other hand, both the optimal dose required for cell migration and the number of cells exhibiting a chemotactic response are approximately equal for all three thrombin preparations in studies with J-774.2. This difference in the behavior of the two cell types can be explained if it is assumed that (*a*) a larger segment of the monocyte population responds to DIP-thrombin rather than to α-thrombin and that (*b*) this difference in responsitivity does not exist in the more uniform J-774.2 cell line. Population heterogeneity has already been documented for monocytes (16), but whether it obtains for chemotaxis remains to be established.

Because enzymatic inactivation of thrombin does not inhibit its ability to act as a chemotaxin, the question arises as to whether thrombin bound in a complex with a physiologic inhibitor would retain its chemotactic activity. To test this possibility, equimolar amounts of thrombin and purified human antithrombin III (AT3) were added to the lower compartments of Boyden chambers in a medium containing 0.5 U/ml heparin as a catalyst (Organon Diagnostics, West Orange, NJ). Cells were placed in the upper compartments with medium alone. As shown in Fig. 2, AT3, in equimolar amounts with α-thrombin, reduced the chemotactic activity of the enzyme by 63%. Addition of 10-fold excess AT3 reduced the chemotactic activity to control levels (data not shown). By contrast, the chemotactic activity of DIP-thrombin was unaffected by AT3. Moreover, neither AT3 nor heparin alone exhibited significant activity as chemotaxins.

TABLE I

Monocyte Chemotaxis to α-Thrombin *

		α-Thrombin upper compartment (M)				
		0	10^{-12}	10^{-10}	10^{-8}	10^{-6}
				M		
α-Thrombin, lower compartment, (M)	10^{-12}	12 ± 0.8	1.8 ± 0.4	3.2 ± 1.0	−2.4 ± 1.2	3.1 ± 0.4
	10^{-10}	32 ± 5.3	30 ± 5.3	−1.4 ± 2.3	−4.2 ± 1.6	−6.8 ± 1.4
	10^{-8}	130 ± 11	99 ± 8.4	45 ± 3.4	−4.1 ± 0.3	8.2 ± 4.1
	10^{-6}	90 ± 8.1	130 ± 5.2	44 ± 2.3	39 ± 1.8	−1.2 ± 0.5

* Results are net cell movement/HPF ± SEM. n = 12.

TABLE II

Comparative Chemotactic Responses of Monocytes and Macrophages to Thrombins

Cell type	Chemotaxin	Dose*	Net cell migration/HPF‡
		M	
Monocyte	α-thrombin	10^{-8}	120 ± 10
	γ-thrombin	10^{-7}	140 ± 12
	DIP-thrombin	10^{-10}	170 ± 9
	FMP	10^{-8}	110 ± 8
J-774.2	α-thrombin	10^{-8}	93 ± 2.8
	γ-thrombin	10^{-8}	99 ± 18
	DIP-thrombin	10^{-8}	101 ± 12
	FMP	10^{-8}	61 ± 5

* Doses tested produced optimal cell migration as determined by dose-response curves.

‡ Representative experiment. Results shown are means of triplicates. Essentially identical results have been found in three separate experiments.

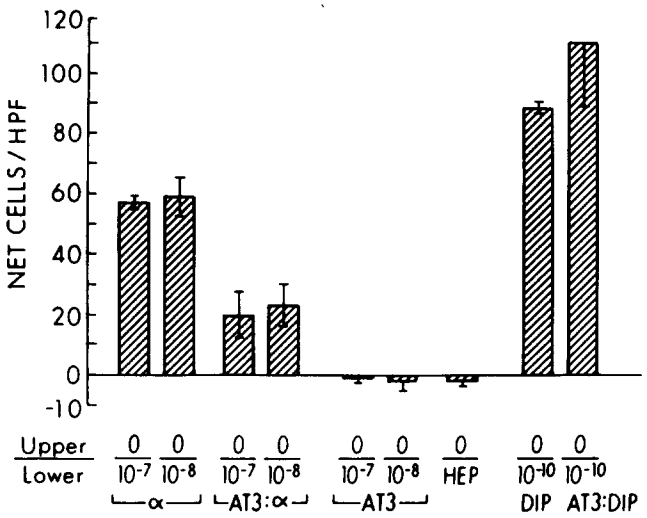

FIGURE 2 Effect of addition of equimolar amounts of AT3 on the chemotactic activity of α-thrombin (α) and DIP-thrombin (DIP) for monocytes. Concentrations (M) of test substances are indicated. AT3 and heparin (HEP) alone (0.5 U/ml) are shown as controls. Each bar represents the mean of triplicates ± SEM.

DISCUSSION

Human peripheral blood monocytes respond chemotactically to purified, enzymatically active α-thrombin. This response is evident at thrombin concentrations ranging from 10^{-10} to 10^{-6} M but is most pronounced at 10^{-8} M. On a molar basis, α-thrombin is as potent a monocyte chemotaxin as FMP at its optimal concentration (10^{-8} M).

In contrast to α-thrombin, prothrombin displays little chemotactic activity. This is somewhat surprising because both of the major cleavage products of prothrombin activation, α-thrombin and fragment 1.2 (17), are chemotactic for monocytes (18). However, these results parallel observations made on the chemotactic activity of proteolytic fragments derived from C3 and C5 that are potent chemotaxins in contrast to the intact parent molecules (19). Thus, the tertiary structure of prothrombin, like that of C3 and C5, must be such that it either precludes recognition of its chemotactically active constituents or, assuming receptor occupancy, negates the triggering of secondary events essential for cell movement. A similar mechanism may be invoked to explain the loss of chemotactic activity of α-thrombin when the latter is complexed with AT3. Because AT3 is a relatively large molecule (M_r ~58,000) as compared with thrombin (M_r ~36,500), we propose that the site or sites on thrombin required for chemotaxis are sequestered coincident to complex formation and, as in the case of prothrombin, are rendered inaccessible for reaction with putative receptors on the monocyte cell membrane.

The ability of thrombin to function as a chemotaxin for human peripheral blood monocytes stands in sharp contrast with other known stimulatory effects of esterases on cells. For these latter events, including the initiation of mitotic activity in fibroblasts by thrombin (3, 4), and stimulation of cell mobility in neutrophils by kallikrein and plasminogen activator (20), retention of esterolytic activity is an absolute requirement. Finally, the present studies provide evidence for yet another important role for thrombin in the biology of wound repair. Because significant amounts of free, enzymatically active thrombin are known to accumulate within fibrin clots (21), the ability of thrombin to stimulate monocyte movement may provide a mechanism whereby one type of inflammatory cell, the monocyte, is recruited to the site of tissue injury.

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