ASSEMBLY OF THE PROTHROMBIN ACTIVATOR COMPLEX ON RABBIT ALVEOLAR MACROPHAGE HIGH-AFFINITY FACTOR Xa RECEPTORS

A Kinetic Study

By MARIA P. McGEE and HENRY ROTHBERGER

From the Section of Rheumatology, Department of Medicine, Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, North Carolina 27103

Enzymatic conversion of prothrombin to thrombin is a central event in coagulation. For prothrombin conversion to occur at biologically significant rates, assembly of the "prothrombinase" complex is required (1). This complex includes specific enzyme (factor Xa), a nonenzyme glycoprotein cofactor (factor Va), calcium ions, and negatively charged phospholipids in a membrane configuration (surface cofactor) (2, 3). As estimated from in vitro kinetic studies, lack of any of the latter individual components greatly decreases rates of thrombin formation to values inadequate for hemostasis (4). During assembly of the prothrombinase complex, factor Xa binds to a site in the L chain of factor Va, which in turn is bound to phospholipid surface cofactor. The K_d of factor Xa from factor Va bound to surface cofactor (on platelets or lipid vesicles) is $\sim 10^{-10}$ M (5–7). A 100-fold increased K_d is found when factor Xa–Va complexes are formed without surface cofactor (7). Therefore, factor Va associated with surface cofactor, in effect, functions as a high-affinity receptor for factor Xa that promotes increased enzymatic conversion of prothrombin to thrombin. Corresponding with functional binding as determined by enzyme kinetics, direct binding of radiolabeled Xa to platelets has been shown (6) to be specific and saturable.

During blood coagulation, platelets provide membrane surface area expressing prothrombinase cofactor activity. Cofactor activity is also on blood monocytes, lymphocytes, and neutrophils, but apparently not on red cells (5). The platelet and leukocyte membrane surfaces in the vascular space are continuously exposed to factor V. In the absence of an external source of factor V, such as plasma, the prothrombinase complex does not assemble on circulating cells, with the exception of platelets (5, 6), as shown in vitro using the isolated cells and prothrombin activator components.

Endothelial cells and aortic explants have been shown to promote prothrombin activation in vitro, in the presence of Xa and Ca^{2+} (8, 9). In addition, it has been reported (8–10) that cultured endothelial cells synthesize factor V in vitro. Therefore, endothelial cells, like platelets, can provide functional intravascular binding sites for factor Xa in the absence of added factor V.

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By contrast with the considerable details known about coagulation in the vascular space, molecular reactions and membrane surfaces directly involved in extravascular activation of prothrombin to thrombin in the absence of plasma constituents (i.e., platelets and factor V) are unknown. Mechanisms of extravascular coagulation are likely to be of particular importance in the lung. The entire cardiac output circulates through capillaries of the fragile alveolar wall, and pulmonary inflammatory and edematous disorders are associated with fibrin deposition as alveolar hyaline membranes (11). We have recently shown (12) that after short-term culture alveolar macrophages generate factor V activity in a process dependent on cellular maturation and protein synthesis. In the present work, we investigate the kinetics of prothrombin activator complex assembly on the surfaces of these cells. We find that incubated alveolar macrophages express factor Xa receptors that promote assembly of functional prothrombin activator complexes and high rates of thrombin formation, even at extremely low concentrations of factor Xa ($\sim 10^{-10}$ M, or ~0.1% of the factor X concentration in blood). These studies support the thesis that, in the extravascular alveolar compartment of the lung, macrophage factor Xa receptors participate in prothrombinase complex assembly in the absence of plasma factor V during rapid coagulation responses invoked by injury.

Materials and Methods

Cell Isolation and Culture. Alveolar macrophages were isolated from New Zealand White female rabbits determined to be pathogen free (Hazelton, Denver, PA). The cells were obtained by bronchoalveolar lavage and washed as previously described (12). Briefly, animals were killed by intravenous injection of 3 ml of pentobarbital (1 g/ml). The lungs were excised and lavaged with 80 ml of 0.15 M sterile saline. Cells in lavage fluids were pelleted by centrifugation and suspended at 5×10^6/ml in 0.15 M NaCl or in serum-free medium (MEM, Flow Laboratories, Inc., McLean, VA) with 100 U/ml of penicillin, 100 μg/ml streptomycin, and 0.5% lactoalbumin hydrolysate heated to 56°C for 30 min before use (Becton Dickinson & Co., Oxnard, CA). In some experiments the ionophore monensin (13) and the protein inhibitor puromycin (14) were added to cultures at 1 μM and 10 μg/ml, respectively. After overnight culture, alveolar macrophages were detached using a rubber policeman and suspended in the culture medium. Viability was >95% in preparations of freshly isolated cells and 85 ± 5% after culture, as determined by trypan blue exclusion. Cytocentrifuged macrophage suspensions were examined directly using phase microscopy and with transmitted light microscopy after Wright's staining. As previously reported (12, 15), alveolar macrophages isolated as described are highly purified. The preparations were found to be free of contamination with platelets, either in suspension or adhering to the cells, and ~1% neutrophils and lymphocytes were present.

Surfactant Isolation. Lung surfactant material was obtained from bronchoalveolar lavage, using established methods (16). Cell-free lung lavage fluid was centrifuged at 4°C at 8,000 *g* for 30 min. The pellet obtained was suspended in saline, layered over 0.7 M sucrose, and centrifuged again. Surfactant was collected from the interface and suspended in medium before carrying out kinetic studies of prothrombinase assembly.

Measurement of Prothrombin to Thrombin Conversion. Rates of enzymatic conversion of prothrombin to thrombin were determined in incubation mixtures containing the substrate, prothrombin, at 1.5 μM and factor Xa at 3.5–0.0137 nM together with nonenzymatic components of the prothrombinase complex, including $CaCl_2$ (5 mM), and lipid membranes provided by macrophages at 10^6 cells/ml. Plasma factor V/Va, when added, was at 8–0.8 μg/ml. In some experiments, as described in results, the cells were substituted for surfactant or subcellular fragments. The macrophages and surfactant were at similar

lipid concentration in the final mixture (~40 nmol lipid phosphorus/150 μl), which was composed of 46.3% phosphatidylcholine, 39.1% phosphatidylethanolamine, 4.2% phosphatidylinositol, 2.2% phosphatidylserine, and 5.5% sphingomyelin and lysophosphatidylcholine for alveolar macrophages and 74.0% phosphatidylcholine, 8.0% phosphatidylethanolamine, 10.5% phosphatidylglycerol, 7.4% phosphatidylinositol for lipid surfactant. In some experiments with fresh alveolar macrophages, additional lipid (rabbit brain cephalin, 1.3 μM lipid phosphorus; Sigma Chemical Co., St. Louis, MO) was added to the mixtures. Reagents were in 0.15 M NaCl, except cells, membranes and surfactant, which were in buffered medium. The pH of the reaction mixtures was 7.1–7.3. After initiating enzymatic reactions by addition of the substrate, 10–15-μl samples were successively taken for thrombin assay at intervals of 0.5–5 min for periods of 15–120 min in order to follow the reactions to completion or near completion. The samples were immediately diluted 10–80-fold in cold saline to stop the reaction. To measure thrombin, 90 μl of diluted sample was added to 90 μl of fibrinogen (5 mg/ml) and one-stage clotting tests were performed using a Fibrometer (Becton, Dickinson & Co., Cockeysville, MD). In this assay, log-log plots of measured clotting times versus concentrations of thrombin were linear from 15–250 s, corresponding to thrombin concentrations of 3–100 nM. Standard curves were prepared using active-site titrated thrombin. Without the added prothrombin, factor Xa, or lipid membrane cofactor, no thrombin activity was detected in assay mixtures.

Calculation of the K_d of Factor Xa from Macrophage and Surfactant Membranes. Polynomial equations were derived to relate nanomolar thrombin measured in the reaction mixtures with the respective incubation times using a multiple regression program (Regress II, Human Systems Dynamics, Northridge, CA) on an Apple II microcomputer (Cupertino, CA). Values of initial thrombin formation rates were calculated from the linear coefficients of the derived equations (17). Thrombin formation rates were proportional to low factor Xa concentrations, but approached asymptomatically to a maximum when plotted as a function of increasing factor Xa concentrations. Double reciprocal plots of the reaction rates versus Xa concentrations were linear, allowing calculation of the Xa concentrations resulting in half-maximal rates (7). Because only the fraction of factor Xa that is bound has measurable prothrombinase activity, the concentration of Xa giving half maximal activity was taken as the K_d of Xa binding to its receptor in the prothrombinase complex. Kinetic approaches of this type for determination of factor Xa–Va interaction parameters have been extensively validated in studies using either artificial lipid vesicles or platelets (6, 7).

Coagulation Factors. Coagulation factors X and II were purified to electrophoretic homogeneity from human plasma in collaboration with Dr. George Doellgast, Department of Biochemistry, Bowman Gray School of Medicine. Factors X and II were activated by RVV (Russel viper venom, Sigma Chemical Co.) and TSV (Typan snake venom, Sigma Chemical Co.), respectively, as previously reported (18, 19). Factor V was purified from rabbit plasma by an adaptation of the method of Dahlbäck (20) for purification of factor V from human plasma, including fractional precipitation with polyethylene glycol, barium sulfate absorption, and chromatography on DEAE-shephacel followed by Sephacryl (Pharmacia Fine Chemicals, Piscataway, NJ). The final product had a specific activity ~1,000-fold higher than starting plasma and corrected the clotting defect of human plasma genetically monodeficient in factor V but not plasma monodeficient in either factor II or X. This purified factor V analyzed by SDS-polyacrylamide electrophoresis had three bands of ~300,000, 200,000, and 100,000 M_r. The factor V was activated 2.25-fold by catalytic amounts of thrombin as measured in a one-stage clotting test for factor V activity using human plasma monodeficient in factor V as substrate (20). Although rabbit factor V has not been purified previously, considering the analogy of our results with available information about bovine and human factor V (21, 22), it appears that our purified factor behaves as factor V/Va (mixture of nonactivated and activated factor V).

Subcellular Fractionation. Subcellular membrane fractions from uncultured alveolar macrophages were obtained as previously described (23, 24). Cells were lysed in 1 mM $NaHCO_3$ and the cellular fragments were pelleted at 2,000 *g*. The pellet material was overlayered with sucrose solutions at 50, 45, 40, and 35% and fractions in interfaces were

collected after centrifugation at 90,000 *g* for 2 h. The cell lysates, the supernatant from the low-speed centrifugation and each of the fractions obtained by the 90,000 *g* centrifugation step were analysed for lipid phosphorus content, protein concentration, alkaline phosphodiesterase I activity and membrane cofactor activity in the prothrombinase reaction. For the latter reaction, addition of purified factor V was necessary in that uncultured cell lysates have negligible factor Xa receptor activity.

Antibodies. Goat antibodies to rabbit factor V were raised by innoculation of purified factor V/Va antigen in complete Freund's adjuvant and subsequent challenge with the antigen in saline. Immunoglobulin G containing anti-factor V was purified from the hyperimmune serum by ammonium sulfate precipitation and DEAE chromatography (25). The antibody obtained inhibited factor V activity in human and rabbit plasma but did not affect activities of factors VII, X, XII, II, VIII, or XI. Tests for individual coagulation factors were performed using genetically monodeficient plasmas as previously described (15, 26). In functional prothombinase tests using fresh (uncultured) alveolar macrophages as the lipid surface, the activity in 1 μg of purified factor V/Va was 64% inhibited by 40 μg of purified anti-factor V IgG, as compared with the activity measured in the absence of antibody. Similarly raised and isolated goat anti-human factor VII IgG and a commercial purified goat nonimmune IgG were used as controls.

Miscellaneous. Protein concentrations were measured by the Lowry method using bovine serum albumin as the standard. Lipid phosphorus concentrations were measured by the method of Rouser et al. (27) after extracting lipids from samples by the method of Bligh and Dyer (28). Phospholipid composition of alveolar macrophages and lipid surfactant was determined using HPLC (29) by the personnel of the Lipid Core Laboratory of the Biochemistry Department, Bowman Gray School of Medicine. Factor Xa and IIa (thrombin) were active-site titrated using *p*-nitrophenyl-*p'*-guanidinobenzoate as previously described (18, 30). Experiments were repeated at least three times except subcellular fractionation and phospholipid composition analyses, which were performed twice. Data are presented as means ± standard error.

Results

Prothrombin Activation by the Prothrombinase Complex Assembling on Alveolar Macrophages and Surfactant. To determine whether prothrombinase complex assembly can occur on membrane surfaces available in the alveolus, rates of prothrombin to thrombin conversion in the presence of either isolated alveolar macrophages or surfactant were determined. Rates were measured by quantitating concentrations of the product, thrombin, at 0.5–2-min intervals after adding the substrate, prothrombin (1.5 μM), to mixtures containing the enzyme, Xa (3.5 nM), Ca^{2+} (5 mM), and either freshly isolated macrophages, cultured macrophages, or surfactant. The findings obtained demonstrate that after culture, alveolar macrophages support assembly of prothrombinase components and thrombin generation from prothrombin at high initial rates in the absence of exogenous factor V/Va, i.e., the cultured cells produce factor Xa–binding sites that function like the V/Va receptor molecules described for platelets (6). Mixtures containing freshly isolated alveolar macrophages or surfactant did not reach similar high rates unless factor V/Va was added.

In reaction mixtures containing macrophages cultured for 20 h ($0.66 \pm 0.056 \times 10^6$ cells/ml), it was found that thrombin content increased linearly starting at an incubation time of 0 s (extrapolated from ~30 s when the first measurements were made). The initial reaction rate was 159 ± 16 nM thrombin/min, and conversion of prothrombin to thrombin was ~90% complete within 15 min (Fig. 1). In mixtures containing freshly isolated (uncultured) macrophages (10^6 cells/ml), prothrombin conversion followed a prolonged lag (≅10 min) and the

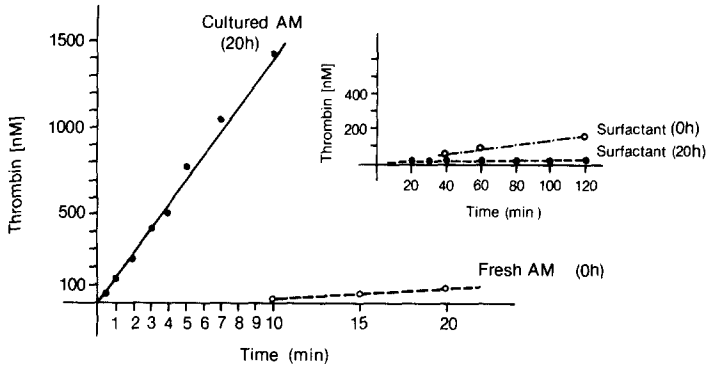

FIGURE 1. Prothrombinase complex assembly on alveolar macrophages and surfactant. Cultured or freshly isolated alveolar macrophages were incubated at 37°C in MEM, pH 7.3, with factor Xa (3.5 nM), Ca^{2+} (5 mM), and prothrombin (1.5 μM) in a 150-μl mixture. Thrombin was assayed in samples taken at the times shown (*abcsissa*). Lipid phosphorus concentrations with cultured or fresh cells were 53.1 ± 4.5 and 39.43 ± 7.0 nmol (per 150 μl of mixture), respectively. Results of similar experiments with surfactant (40 nm lipid phosphorous) rather than cells are shown in the insert (note differences of scale). The surfactant was studied fresh and after incubation at 37°C for 20 h under conditions comparable to those used to alveolar macrophage culture.

measured reaction rate after this period was very low (5 ± 3 nM thrombin/min, Fig. 1). In studies with surfactant rather than macrophages, thrombin formation was also found to follow lags ~10 min, and subsequent initial reaction rates (1.2 ± 0.2 nM/min) were likewise very low (Fig. 1, *inset*). However, purified factor V/Va (8 μg/ml) added to reaction mixtures containing fresh cells or surfactant promoted efficient prothrombinase assembly, so that initial lags were not detected and reaction rates were high (477 ± 115 and 166.9 ± 19 nM thrombin/min, respectively). Therefore, freshly isolated macrophages and lipid surfactant do not have receptors for direct binding of Xa, but their membranes provide the phospholipid cofactor indispensable for prothrombinase assembly. Addition of rabbit brain cephalin (1.3 μM lipid phosphorus) to mixtures containing fresh alveolar macrophages without added V/Va did not result in significantly increased levels of prothombinase activity (8.5 ± 1.24 and 5.6 ± 0.61 nM of thrombin/min with and without added cephalin, respectively). Incubation of surfactant for 20 h at 37°C under the same conditions used for culturing alveolar macrophages had no effect on its lipid cofactor function nor on the requirement for addition of factor V/Va to promote prothrombinase assembly (Fig. 1, *inset*).

Inhibition of Functional Factor Xa Assembly on Cultured Alveolar Macrophages by Antibodies Raised against Plasma Factor V. Goat antibody raised against factor V isolated from rabbit plasma was preincubated for 1 h at 37°C, with cultured alveolar macrophages. As controls, goat anti-factor VII and nonimmune goat IgG were used at the same concentrations as anti-factor V IgG (1 mg/ml). After preincubation of antibody with cultured macrophages, factor Xa, prothrombin, and Ca^{2+} were added and rates of prothrombin conversion were measured in the reaction mixtures. Final concentration of the antibody in these mixtures was 230 μg/ml. Anti-factor V antibody was found to greatly diminish rates of thrombin formation. By contrast, the anti-factor VII and IgG controls at the

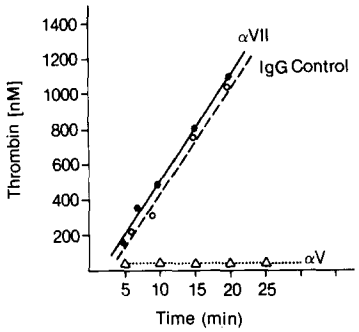

FIGURE 2. Factor V antibody inhibition of prothrombinase assembly on cultured alveolar macrophages. Cultured macrophages were preincubated with either anti-factor V IgG, anti-factor VII IgG, or nonimmune IgG (1 mg/ml) for 30 min at 37°C. After this period, factor Xa (1.7 nM), prothrombin (1.5 μM), and Ca^{2+} (5 mM) were added. The incubation was continued and concentrations of thrombin in the mixtures (*ordinate*) was measured as for Fig. 1. Cells were at $0.63 \pm 0.02 \times 10^6$ cells/ml and final IgG concentration was 250 μg/ml.

same protein concentration had no effect (Fig. 2). This specific inhibition of thrombin formation by antibody to plasma factor V/Va indicates that similar antigenic reactivity is exhibited by the latter protein and the functional binding sites for factor Xa produced by cultured alveolar macrophages.

Factor Xa Binds to Functional Assembly Sites on Cultured Alveolar Macrophages with High Affinity. To characterize the interaction between factor Xa and its surface receptors on cultured alveolar macrophages, rates of thrombin formation were measured at nine different concentrations of factor Xa (from 0.0137 to 3.5 nM) in mixtures containing fixed concentrations of the cultured cells, prothrombin and Ca^{2+}. Rates of prothrombin to thrombin conversion in these mixtures increased with the Xa concentration. After a maximum rate was reached, further increases in the Xa concentration did not affect the rate of reaction significantly, indicating saturation of the assembly sites with Xa (Fig. 3). Reciprocal plots of measured initial rates of thrombin formation versus factor Xa concentrations showed a linear relationship (Fig. 3), and the K_d calculated from the abscissa intercept was $2.11 \pm 0.94 \times 10^{-10}$ M.

The K_d of factor Xa binding to freshly isolated alveolar macrophages and of Xa binding to surfactant were also measured. Neither the uncultured cells nor surfactant amplified enzymatic effects of factor Xa significantly unless factor V/Va was added (see above). Therefore, fixed amounts of isolated plasma factor V/Va were included together with varying amounts of factor Xa in the factor Xa titration experiments. Rates of thrombin formation also reached a maximum, indicating membrane factor V/Va complex saturation with factor Xa. The factor Xa interaction with uncultured macrophages and surfactant complexed with added factor V/Va were observed to be of high affinity (Table I). These values resemble the K_d determined for cultured alveolar macrophages without added factor V/Va, indicating that binding sites produced by the cells in culture are molecules that function like plasma factor V/Va in the prothrombinase complex.

Monensin and Puromycin Inhibit Production of Factor Xa Receptors by Cultured Alveolar Macrophages. To examine further the effect of culture on production of the high-affinity factor Xa–binding sites, either monensin or puromycin were

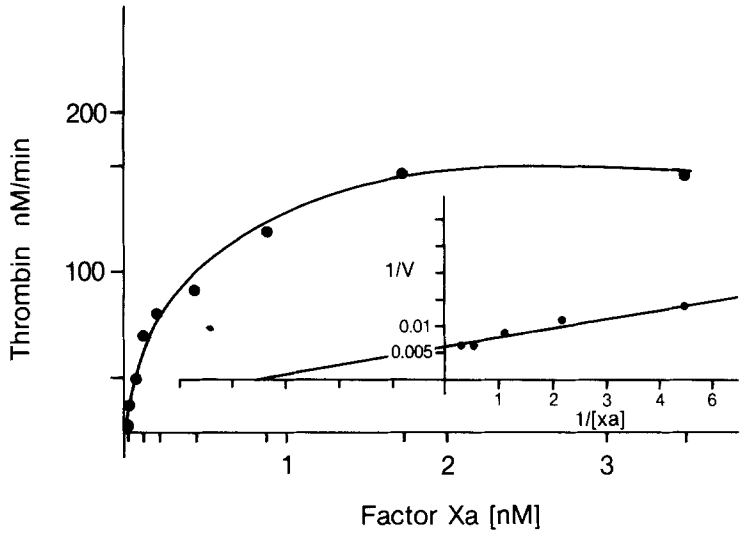

FIGURE 3. Functional binding of factor Xa to V receptors on alveolar macrophages. Initial rates of prothrombin catalysis (*ordinate*) were measured in mixtures containing fixed amounts of cultured alveolar macrophages (0.94×10^6/ml), Ca^{2+} (5 mM), prothrombin (1.5 μM), and concentrations of factor Xa from 3.5–0.00137 nM (*abscissa*). Initial reaction rates were calculated (see Materials and Methods) from thrombin concentrations measured in samples taken at 0.5–1-min intervals. The inserted reciprocal plot shows 1/V (initial rates) versus 1/[Xa] (factor Xa concentrations). One of three similar experiments is shown.

TABLE I

The K_d of Factor Xa Binding to Alveolar Macrophages and Comparisons with Surfactant

	K_d ($\times 10^{-10}$ M)
Fresh alveolar macrophages	6.03 ± 1.7 (3)
Cultured alveolar macrophages	2.11 ± 0.94 (3)
Lipid surfactant	8.21 ± 2.60 (5)

Fresh alveolar macrophages and surfactant were studied after adding factor V/Va to assay mixtures. Cultured alveolar macrophages were studied without factor V/Va addition. The apparent K_d values were calculated from intercepts on the abscissa of double reciprocal plots constructed as indicated in Fig. 3. The K_d values shown are means ± standard error. The numbers of experiments performed are indicated in parentheses.

included in the medium at 1 μM and 10 μg/ml, respectively. Monensin has been found to inhibit protein glycosylation and to interfere with secretory processes in various cell types, including macrophages (13). Puromycin inhibits protein synthesis (14). Both compounds interfered with expression of factor Xa receptors, suggesting that the alveolar macrophage is either involved in complex metabolic processing or in de novo synthesis of the receptor molecule (Fig. 4).

Macrophage Phospholipid Surface Cofactor for Prothrombinase Assembly Is Expressed on Cytoplasmic and Endoplasmic Membranes. Experiments were carried out to determine whether alveolar macrophage membranes other than those on the cytoplasmic surface can provide lipid surface cofactor. Subcellular fractions were isolated by centrifuging macrophage lysates through a sucrose density

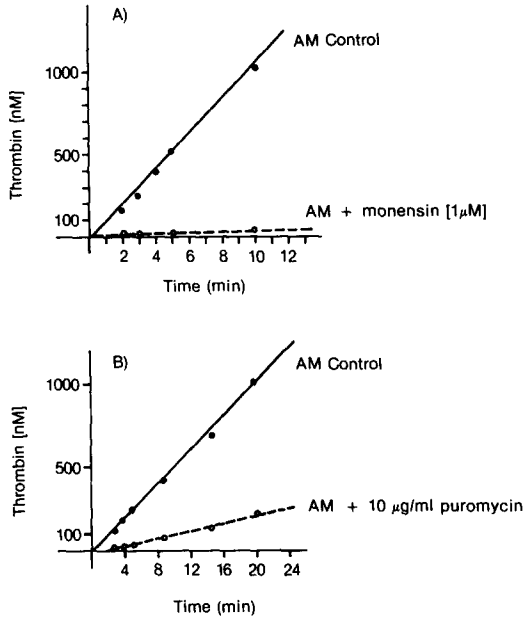

FIGURE 4. Effect of monensin and puromycin on factor Xa receptor production by cultured alveolar macrophages. Rates of prothrombin to thrombin conversion in mixtures containing alveolar macrophages cultured in the presence or absence of 1 μM monensin (*A*) or 10 μg/ml of puromycin (*B*) were measured. Reagent concentrations are the same as for Fig. 1. Sampling times (*abscissa*) and thrombin concentrations (*ordinate*) are shown.

gradient. The ability of each membrane fraction to serve as lipid cofactor in assembly of prothrombinase was determined after adjusting each fraction to the same lipid concentration. It was found that prothrombinase activity was similar in all fractions and when normalized per milligram of cellular protein, the activity correlated highly with the respective lipid phosphorus content but not with alkaline phosphodiesterase I activity in the fractions (Table II). The latter enzyme is in alveolar macrophage cytoplasmic membranes, but not in other macrophage membranes (23). Correlation of prothrombinase activity with lipid phosphorus (i.e., phospholipid content), rather than with phosphodiesterase (i.e., cytoplasmic membrane content) indicates that membranes in all subcellular fractions studied provide equally efficient phospholipid cofactor activity.

Discussion

Conversion of prothrombin to thrombin is accelerated to physiologic levels by calcium-dependent association between factor Xa (a serine protease), factor Va, and membrane phospholipid (1). During coagulation within the circulation, assembly of factor Xa–Va likely occurs on platelet membranes (31). Factor V in platelets and soluble factor V in plasma are present at concentrations sufficient to saturate factor V/Va–binding sites on platelet membranes. It is not known which of the two factor V sources is involved in prothrombinase assembly in vivo.

Blood leukocytes also have phospholipid surface cofactor that functions in

TABLE II

Correlations between Lipid Surface Cofactor Activity, Total Phospholipid, and Membrane Marker Protein in Alveolar Macrophage Lysates and Membrane Fractions

Fraction	Alkaline phospho-diesterase*	Prothrom-binase activity‡	Lipid phos-phorus§
	nmol	*μM*	*nmol*
Alveolar macrophage lysate (unfractionated)	70.0	4.5	369
2,000 *g* supernatant	26.5	8.5	227
0/30% sucrose	0.0	34.9	4,188
30/40% sucrose	4,295.0	10.8	598
40/45% sucrose	6,705.0	9.4	616
45/55% sucrose	1,610.0	9.3	564
Pellet	569.0	2.2	222

Subcellular fractions from uncultured alveolar macrophages were obtained as described in Materials and Methods. Prothrombinase activity in each fraction was correlated with lipid phosphorus and alkaline phosphodiesterase activity. The respective correlation coefficients are 0.98 and −0.145.

* Alkaline phosphodiesterase activity, a cytoplasmic membrane marker, was measured using thymidine 5-monophosphate-*p*-nitrophenyl as substrate and is expressed as nmol of *p*-nitrophenol generated per minute per milligram protein.

‡ Prothrombinase activity was measured to estimate the ability of each fraction to provide lipid surface cofactor activity. Mixtures (150 μl) contained 20 nmol lipid phosphorus from each fraction, 0.8 μg of factor Va, and the rest of the prothrombinase components as in Fig. 1. Values shown express μM of thrombin generated per milligram of cell protein per minute.

§ Lipid phosphorous is expressed as nanomoles per milligram protein and reflects the total phospholipid content of each fraction.

prothrombin to thrombin conversion (5). Studies using radioimmunoassay have shown that leukocytes additionally contain factor V antigen. The factor V antigen found within leukocytes did not promote prothrombinase assembly on these cells, as indicated by requirements for addition of factor Va to demonstrate enzyme activity (5).

Although there is a substantial amount of information describing possible mechanisms of thrombin formation and coagulation on platelets within blood vessels, there is little known about clotting factor interactions on cells or lipid surfaces outside the vascular bed at sites removed from plasma and platelets. In the present study, we examined a possible mechanism of extravascular prothrombinase assembly in airspaces of the lung. The alveolar airspace is found to contain fibrin deposits in a variety of inflammatory and edematous disease states, including bacterial pneumonia, congestive heart failure, and disorders associated with the adult respiratory distress syndrome (11, 32–34). However, molecular reactions activating coagulation proteases and inducing extravascular fibrin deposition on the alveolar wall are largely unknown.

In edematous states, plasma ultrafiltrates can leak into the alveolar space

without extravasation of platelets (11, 32–34). Molecular radii and plasma concentrations are properties influencing protein concentrations reached in extravascular filtrates (35). Transudation of factor V, which is a large, asymmetric, trace plasma protein (M_r ~300,000) across minimally damaged alveolar capillary membranes and epithelial cell barriers would in all likelihood be restricted as compared with passage of smaller plasma clotting proteins such as factor VII and X (M_r ~50,000) or large, more abundant clotting factors such as fibrinogen. Therefore, after minimal alveolar injury, concentrations of factor V in the airspace may be rate limiting for coagulation reactions. Requirements for rapid extravascular clotting on the surface of the pulmonary alveolus are evident. In the absence of adequate hemostasis, even minimal damage of the alveolar epithelial and endothelial barriers could result in flooding of the alveolus due to transudation of plasma and interstitial fluid.

Using a rabbit model in the present study, we show that alveolar macrophages and pulmonary surfactant can directly contribute to thrombin formation, a central event in hemostasis and coagulation reactions. We find that after a short period of culture (several hours), the macrophages interact functionally with factor Xa. This interaction is saturable, of high affinity ($K_d \sim 10^{10}$ M), and does not require added factor V, indicating that the macrophages express receptors for factor Xa. The prothrombinase reaction rates, K_d values and specific antibody inhibition observed demonstrate that these receptors are functionally and antigenically similar to plasma factor V. Expression of the receptors is abrogated by adding puromycin and monensin in culture, which are inhibitors of cellular protein synthesis and processing, respectively (13, 14). The K_d of interaction between factor Xa and alveolar macrophage receptors is similar to K_d values reported for Xa interaction with purified factor Va added to artificial lipid vesicles, platelets, or blood leukocyte membranes (5). It is also similar to the reported K_d of interaction between factor Xa and intrinsic platelet factor V/Va (6).

Additionally, we find that alveolar macrophages are sources of surface cofactor activity. Cytoplasmic membranes, and membranes in subcellular fractions were equally effective. In view of the fast turnover of macrophages in the lung (5 d in mice) (36), the observed lipid surface cofactor on intracellular membranes may contribute significantly to prothrombinase assembly in vivo after macrophage death and disintegration. We also found that surfactant isolated from alveolar lavage fluid has surface cofactor activity, but unlike alveolar macrophages, surfactant did not express factor Xa receptor activity.

High-affinity factor Xa receptors and surface cofactor on alveolar macrophages may promote efficient prothrombin to thrombin conversion within the extravascular airspace of the alveolus at extremely low concentrations of factor Xa, independently of blood platelets and plasma factor V. Further, it is possible that factor VII, complexed with tissue factor on alveolar macrophages (12, 37), catalyzes activation of factor X to factor Xa at cellular surface sites very near to prothrombinase assembly, thereby minimizing exposure of nascent factor Xa to protease inhibitors. The latter inhibitors are abundant in the lung (38). In view of the ability of alveolar macrophages to initiate the clotting sequence with tissue factor and to facilitate thrombin formation owing to surface cofactor activity and

high-affinity factor Xa receptors, we propose the existence of an extravascular coagulation pathway centered about these cells. Considering the apparent K_d found for the interaction between factor Xa and macrophages (10^{-10} M), efficient prothrombinase assembly could occur in the alveolus at concentrations of Xa 500–1,000-fold lower than the concentration of the factor X zymogen in plasma, which is estimated to be $\sim 10^{-7}$ M in human and bovine blood (39). Therefore, macrophages that provide a major immune defense system in the alveolus may also be crucial for thrombin formation and coagulation reactions in the extravascular alveolar space.

Summary

Efficient prothrombin activation occurs after assembly of factors Va, Xa, and phospholipid surface cofactor as a multimolecular complex. These components are provided by platelets and plasma within the vascular space, but molecules and membranes for prothrombin activator assembly in extravascular spaces have not been identified. In the present study, purified alveolar macrophages were found to produce high-affinity factor Xa receptors that mediate formation of enzymatic prothrombinase complexes and rapid prothrombin to thrombin conversion in the absence of exogenous factor V/Va or platelets. Thus, in reaction mixtures with alveolar macrophages cultured for 20 h in serum-free medium, the thrombin formation rate was 152 nM/min/0.66 × 10^6 cells, after adding prothrombin (1.5 μM), Ca^{2+} (5 mM), and factor Xa (3.7 nM). The observed K_d of factor Xa interaction with macrophage receptors is 2.1 ± 0.94 × 10^{-10} M. Kinetic analysis and inhibition studies using isolated factor V and anti–factor V antibody show that macrophage Xa receptors are functionally and antigenically similar to plasma factor V. By contrast, freshly isolated cells lacked receptors promoting prothrombin conversion at high rates. Inhibitors of protein synthesis and glycosylation, puromycin and monensin, respectively, abrogated production of Xa receptors in culture. Additionally, subcellular fractionation and enzyme-marker studies (alkaline phosphodiesterase I) indicate that internal and external membranes of alveolar macrophages have phospholipid surface cofactor activity required for prothrombinase complexes. Pulmonary surfactant is also shown to express this cofactor activity. Alveolar macrophages and surfactant comprise an efficient prothrombin activator system that is independent of plasma factor V. This system may facilitate rapid extravascular alveolar thrombin formation even at very low concentrations of factor Xa during lung defense reactions to inflammation or edema.

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