Human alveolar macrophages synthesize factor VII in vitro. Possible role in interstitial lung disease.

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Research Article

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Human Alveolar Macrophages Synthesize Factor VII In Vitro

Possible Role in Interstitial Lung Disease

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Abstract

Both fibrin and tissue macrophages are prominent in the histopathology of chronic inflammatory pulmonary disease. We therefore examined the procoagulant activity of freshly lavaged human alveolar macrophages in vitro. Intact macrophages (5×10^5 cells) from 13 healthy volunteers promoted clotting of whole plasma in a mean of 65 s. Macrophage procoagulant activity was at least partially independent of exogenous Factor VII as judged by a mean clotting time of 99 s in Factor VII-deficient plasma and by neutralization of procoagulant activity by an antibody to Factor VII. Immunoprecipitation of extracts of macrophages metabolically labeled with [^{35}S]methionine by Factor VII antibody and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed a labeled protein consistent in size with the known molecular weight of blood Factor VII, 48,000. The addition of 50 μg of unlabeled, purified Factor VII blocked recovery of the 48,000-mol wt protein. In addition, supernatants of cultured macrophages from six normal volunteers had Factor X-activating activity that was suppressed an average of 71% after culture in the presence of 50 μM coumadin or entirely by the Factor VII antibody indicating that Factor VII synthesized by the cell was biologically active. Endotoxin in vitro induced increases in cellular tissue factor but had no consistent effect on macrophage Factor VII activity. We also examined the tissue factor and Factor VII activities of freshly lavaged alveolar cells from nine subjects with clinical and/or histologic evidence of sarcoidosis. Four of the nine subjects expressed increased tissue factor and seven of nine had increased Factor VII activity over the normal range ($P < 0.01$). We estimate the mean Factor VII associated with the cells of sarcoid patients to be 4.7 ng/10^6 cells (range 0.4–20) as compared to a mean of 0.74 ng/10^6 cells (range 0.2–2) for that of normal subjects. Along with previous data showing synthesis of plasminogen activator, these findings indicate that human alveolar macrophages normally synthesize and express measurable amounts of the initial enzymes of proteolytic reactions regulating both fibrin deposition and fibrin resorption. Abnormalities in Factor VII activity in a small group of patients with sarcoidosis raise the possibility that modulation of fibrin turnover by macrophages may contribute to the pathology of this and perhaps other interstitial lung diseases.

Introduction

The participation of coagulation in the inflammatory response was demonstrated by Colvin, Dvorak, and colleagues who established tissue fibrin deposition as a prominent component of delayed cutaneous hypersensitivity reactions in humans (1, 2). Attenuation of the hypercellular characteristics of acute complex glomerulonephritis by antimacrophage antiserum (3) and by defibrinogenation (4) indicated a direct causal role for both the macrophage and fibrin in immunologically mediated tissue injury. Monocytes are induced to express procoagulant activity by T helper lymphocytes directly through cellular interaction after stimulation with endotoxin or immune complexes (5, 6) and indirectly by elaboration of lymphokines in response to mitogens, antigens or allogeneic stimulation (7–10). Studies on experimental animals indicate that macrophages at sites of inflammation express procoagulant activity, respond to similar signals as do monocytes, and show a correlation between their activation state and the amount of procoagulant activity (11).

Initiation of coagulation by human monocytes/macrophages occurs with the expression of tissue factor, the lipophilic protein cofactor required for the extrinsic coagulation pathway (12, 13). Factor VII binds to this cofactor in the presence of calcium to form a proteolytically active complex with the capacity to activate Factors IX and X (14–17). Previous reports suggested that thioglycollate-elicited mouse peritoneal macrophages express a Factor VII-like activity, but these reports did not unequivocally identify this protease (18–20). Recently, Tsao et al. (21) provided biochemical and immunochemical evidence that endotoxin induced human peripheral blood monocytes expressed Factor VII on their surface. However, it remains to be established if monocytes or macrophages can synthesize this proteolytically active zymogen, thereby providing the two essential components for the potential assembly of the extrinsic pathway activation complex on the surface of these cells at extravascular sites.

Fibrin is conspicuous in a number of inflammatory processes involving the lung. Histologic studies show fibrin both in the interstitium and along the alveolar surfaces during the cellular phase of idiopathic pulmonary fibrosis (22). Fibrin is prominent along the alveolar ducts during the exudative phase of the adult respiratory distress syndrome (23). Because pulmonary macrophages are also prominent in the histopathology of these disorders, we examined the procoagulant activity of human alveolar macrophages in vitro. In this report we

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describe the synthesis and expression of Factor VII and tissue factor by these cells. Further, we investigate alveolar macrophage procoagulant activity in a group of patients with an inflammatory lung disease in which macrophages are known to be prominent, sarcoidosis (24).

Methods

Bronchoalveolar lavage and cell preparation. 13 healthy community volunteers underwent fiberoptic bronchoscopy as previously described (25). Differential counting of lavaged cells from these subjects showed mean percentages of macrophages, lymphocytes, and neutrophils to be 96, 3, and 1%, respectively. Nine patients with suspected or confirmed diagnosis of pulmonary sarcoidosis also underwent bronchoscopy after informed consent. In eight individuals, the diagnosis was established on histologic evidence of noncaseating granulomata and compatible clinical and radiographic findings. In one subject, the diagnosis was based on clinical criteria alone (26). The sarcoid group consisted of six women and three men, all nonsmokers of ages 22–62 yr. No patient had been treated with steroids within 6 mo of the time of study. Differential cell counting in the patient group revealed increased percentages of lymphocytes, 15–48%, consistent with the diagnosis of sarcoidosis (24). The pooled cellular pellets from volunteers or patients were washed in tissue culture medium and counted by hemocytometer. The cells were immediately studied using modified Eagle's medium (MEM)[1] as the basic tissue culture medium.

Reagents. Citrated, pooled normal human plasma was obtained from the clinical hematology lab, and aliquots were stored at −70°C until use. Factor VII- and Factor X-deficient plasmas were purchased from George King Pharmaceuticals (Kansas City, MO) and stored at −70°C until use. A Westphal preparation of Salmonella enterotoxin obtained from Sigma Chemical Co. (St. Louis, MO) was used as a source of endotoxin without further purification. SDS was bought from Gallard-Schleisinger Chemical Manufacturing Corp. (Carle Place, NY). Blood Factor VII was purified according to Broze and Majerus (27) as modified by Fair (28). Factor X was purified according to Fair and colleagues (29). Thromboplastin (Simplastin) and brain cephalin were obtained from General Diagnostics (Morris Plains, NJ) and stored in dry form at 4°C. Ficoll-Hypaque and Percoll were obtained from Pharmacia Fine Chemicals (Piscataway, NJ). All other reagents were of highest grade commercially available. The media and sera used in these experiments were free of endotoxin contamination as judged by a limulus amoebocyte assay that detects 1 ng/ml of endotoxin (30).

Procoagulant assays. Intact macrophages were assayed for procoagulant activity by a modified one-stage prothrombin time (5). Five $\times 10^5$ lavaged cells in 100 μl of MEM were added to 100 μl of citrated whole plasma. 100 μl of 25 mM $CaCl_2$ was then added and the time of fibrin clot formation determined in a fibrometer (BBL Microbiology Systems, Cockeysville, MD). Tissue culture medium alone did not result in fibrin formation in <200 s. A mixture of rabbit brain and lung thromboplastin (Simplastin), 1 mg/ml in MEM, consistently clotted the plasma in 34–38 s. This concentration of thromboplastin was assigned a value of 1,000 U. There was a linear relationship between thromboplastin, 100–10,000 U and a clotting time in a log–log plot. Data are expressed as mean clotting time (seconds) of duplicate determinations and as units of thromboplastic activity based on the rabbit thromboplastin standard.

In some experiments, alveolar macrophages were incubated in suspension for 18 h in 12 × 75-mm plastic culture tubes (Falcon Labware, Oxnard, CA) at 37°C in the absence or presence of 1 μg/ml of endotoxin. For all cell cultures, the cells were incubated in 5% (vol/vol) human serum that had been heat inactivated at 56°C for 1 h. After the incubation, cells were washed three times in MEM and suspended in MEM at 5×10^5 cells/100 μl for assay of procoagulant activity. Data are expressed as units of thromboplastic activity and as clotting times (seconds).

Factor VII assays. We first assayed cellular Factor VII activity in a one-stage prothrombin assay using plasma genetically deficient in Factor VII. The Factor VII-deficient plasma used was entirely from a single donor, GK702. One of us (Dr. Fair) has determined the Factor VII antigenic level in this plasma to be ~12% of the normal Factor VII concentrations and the procoagulant activity to be about 2% of normal plasma (28). For some experiments, normal plasma was depleted of Factor VII protein by absorption with Sepharose-linked Factor VII antibody. The concentration of Factor VII in this plasma was <1% of that of normal plasma.

Intact cell procoagulant activity in the Factor VII-deficient plasma was also tested for its susceptibility to neutralization by a previously described polyclonal Factor VII antibody (28). For these experiments, 5×10^5 macrophages were suspended in 75 μl of MEM and mixed with either 25 μl of normal rabbit IgG (1 mg/ml) or rabbit IgG that contained Factor VII antibodies (1 mg/ml) for 30 min at 37°C. The cells were then washed once and resuspended in 100 μl of MEM for procoagulant assays in Factor VII-deficient plasma as described above.

Cell-dependent procoagulant activity in the Factor VII assays was further analyzed to access the relative contributions of tissue factor alone and Factor VII associated with cellular tissue factor. To do this, we first established the quantitative relationship between dilutions of normal human plasma (as a source of Factor VII) and clotting time (seconds) in Factor VII-deficient plasma for various concentrations of rabbit brain thromboplastin (0.1, 0.3, 0.6, 0.9, 2.2, 6, and 10 mg/ml) in a log–log plot. This consisted of a family of straight lines progressively closer as the concentration of thromboplastin increased. The concentration of Factor VII in the plasma standard as determined by radioimmunoassay was 337 ng/ml (28). The observed clotting times of the cells in Factor VII-deficient plasma were then plotted on the appropriate straight line for the thromboplastin equivalent determined for the same cells in whole plasma. We then expressed the Factor VII activity of alveolar macrophages as nanogram equivalents to the plasma standard per 10^6 cells. In this way, cell-associated Factor VII activity could be separated from the effect of cellular tissue factor binding to any residual Factor VII in the genetically deficient plasma.

Factor X-activating assay. The ability of lysates or cell-free culture supernates of alveolar macrophages to directly activate purified Factor X was assessed by fluorometry. Assays were performed by modifications of the procedure described by Shands (19). Alveolar macrophages (3.5×10^6) were made adherent to 35-mm petri dishes, incubated 2 h at 37°C in 10% fetal bovine serum, washed, and then incubated overnight in 2 ml of serum-free MEM with or without 50 μM coumadin. Preliminary experiments indicated that coumadin had an inconsistent effect on lysate activity unless nascent Factor X-activating activity was at least partially removed. Therefore, before overnight culture in MEM, all cells were incubated in 10 μg/ml of trypsin for 30 min at 37°C followed by neutralization in soybean trypsin inhibitor, 400 μg/ml, for 10 min at 37°C. Neither the trypsin nor the coumadin (up to 100 μM) were toxic to macrophages as judged by morphology and trypan blue exclusion. After overnight culture, the cell-free supernates were made 0.05% in Triton X-100, dialyzed against water, and concentrated 10-fold by lyophilization. The cells were then scraped from the wells and subjected to freeze-thawing and then to four 1-s pulse sonications. The supernatants reconstituted in MEM were made 0.5 mg/ml in brain thromboplastin and assayed for Factor X-activating activity in a reaction mixture consisting of 100 μl of supernatants, 90 μl of 10 mM Tris-HCl pH 7.8, 100 μl of brain cephalin, 10 μl of 0.25 M $CaCl_2$, 10 μg of purified Factor X, and a final concentration of 0.1 mM benzoxycarbonyl - isoleucine - glutamine - glycine - arginine - aminomethyl-coumarinamide (Peninsula Laboratories, San Carlos, CA). The mixture was incubated for 45 min at 37°C, the reaction stopped with 10 μl of 1 mM *p*-nitrophenylguanidinobenzoate, the solution clarified by centrifugation, and fluorescence recorded in a Perkin-Elmer 650-11S spectrofluorometer (Perkin-Elmer Corp., Norwalk, CT). Fluorescence measured in the same reaction mixture containing brain throm-

1. *Abbreviation used in this paper:* MEM, modified Eagle's medium.

boplastin, 0.5 mg/ml, in MEM with 0.5% Triton instead of the reconstituted supernatants, was subtracted from the experimental values. All reactions were done in duplicate. Preliminary data showed the fluorescence to be proportional to purified Factor Xa from 0.5–2 μg in the 45-min assay. Also, the supernatants did not appreciably hydrolyze the Factor Xa substrate in the absence of either added Factor X or brain thromboplastin. Data is expressed as mean picomoles of substrate hydrolyzed in duplicate determinations by standardization of fluorescence with known concentrations of purified product (aminomethylcoumarin). Cell lysates were assayed identically to supernatants except that thromboplastin was omitted and the reaction was stopped after 15 min at 37°C.

Metabolic labeling, immunoprecipitation, and autoradiography. Labeling, immunoprecipitation, and autoradiography were carried out as previously described (25). Briefly, 10^7 alveolar macrophages/25-cm^2 flask were labeled in MEM containing 0.5% fetal bovine serum, 10% stock methionine, and [^{35}S]methionine, 100 μCi/ml, for 18 h at 37°C. The cell monolayers were then washed, scraped, and homogenized in 3 ml of Tris buffer (pH 7.4) containing 5 mM EDTA. Membrane and cytosol fractions of the cell were prepared by ultracentrifugation at 100,000 *g* for 90 min. Immunoprecipitation was done after clarification with Staph A to minimize nonspecific precipitation. The reaction medium consisted of 100 μl of a cellular membrane fraction or the cytosol fraction solubilized in homogenizing buffer containing 0.1% SDS and 0.5% Triton X-100. All samples contained 100 U/ml Trasylol and 0.1 mM phenylmethylsulfonyl fluoride. After 2 h of incubation at room temperature with 15 μl of immune IgG (Factor VII antibody) or control rabbit IgG, the complexes were isolated by Staph A binding, washed, and eluted by boiling in sample buffer for SDS-gel electrophoresis (31). After electrophoresis under reduced conditions in 10% acrylamide slab gels, the gels were dried and autoradiographed for 10–14 d at −70°C.

Results

Procoagulant activity of human alveolar macrophages. Intact macrophages from each of 13 healthy volunteers promoted fibrin formation in a one-stage prothrombin assay using pooled human plasma as a source of coagulation factors (Fig. 1). The mean clotting time and one standard deviation, for the group was 65±14 s. This clotting time is equivalent to 480 U thromboplastic activity (1 mg/ml = 1,000 U). 6 of the 13 volunteers smoked at least 1 pack/d; the remaining subjects were nonsmokers. There was no difference in procoagulant activity between these subgroups.

We first investigated the chemical nature of this procoagulant activity. Preliminary studies with five individuals demonstrated that all procoagulant activity required exogenous Factor X (not shown). Previous reports indicated that stimulated murine peritoneal (18–20) and rabbit alveolar (32) macrophages activate the coagulation cascade via the extrinsic pathway and the studies in mice suggested a partial involvement of Factor VII-like activity. We thus tested the ability of human macrophages to clot plasma genetically deficient in Factor VII. We observed a mean clotting time of 99±22 s (Fig. 1). In paired samples of cells assayed in both plasmas, the clotting times were consistently longer in the Factor VII-deficient plasma than whole plasma (not shown). The total procoagulant activity is not explained simply as tissue factor activity as incubation of Factor VII-deficient plasma with 480 U of thromboplastin gave a clotting time of only 150 s. These findings suggest that the human cells express a procoagulant in addition to thromboplastin.

To test the possibility that freshly lavaged macrophages express Factor VII, we determined whether a neutralizing antibody prepared against purified Factor VII would block the procoagulant activity. Control experiments with nonimmune IgG showed no prolongation of macrophage associated procoagulant activity assayed in Factor VII-deficient plasma (Fig. 2). In contrast, preincubation of cells with Factor VII antibody significantly decreased the cellular procoagulant activity in seven subjects tested from 107±22 s to 182±21 s. The Factor VII activity in the presence of Factor VII antibody may actually be lower as the assay was terminated after 200 s. These data cannot be explained by interaction of the Factor VII antibody with residual Factor VII in the genetically deficient plasma. First, we observed similar findings with plasma immunochemically depleted of residual Factor VII by absorption with Sepharose-linked Factor VII antibody. This plasma had virtually nondetectable levels of Factor VII antigen and activity. And second, the Factor VII antibody did not prolong the clotting times of the deficient plasma initiated by rabbit thromboplastin, when added at the start of the 200-s assay.

By comparing the clotting times observed with dilutions of normal human plasma as a source of Factor VII (337 ng/ml) to that of the cells in identical assays, we have estimated the Factor VII equivalents associated with normal human alveolar macrophages. The mean ng/10^6 cells for the 13 normal volunteers was 0.74 with a range of 0.2–2 ng/10^6 cells. When the procoagulant activities in Fig. 2 are expressed as nanograms of Factor VII/10^6 cells, preincubation of alveolar macrophages with the Factor VII antibody abrogated over 90% of the Factor VII equivalents in six of the eight subjects tested and >80% in the remaining two subjects.

Synthesis of Factor VII by human alveolar macrophages in vitro. The experiments reported above indicated that the procoagulant activity of normal, intact alveolar macrophages

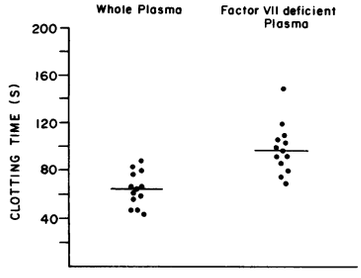

Figure 1. Procoagulant activity of human alveolar macrophages. Intact cells, 5 × 10^5, were added to fibrometer wells containing 100 μl of whole plasma, and a clotting time recorded after addition of 100 μl of 25 mM $CaCl_2$. Data represents the mean of duplicate determinations from each of 13 volunteers. The mean clotting times in seconds±1 SD for the whole and Factor VII-deficient plasmas were 65±14 and 99±21 s, respectively.

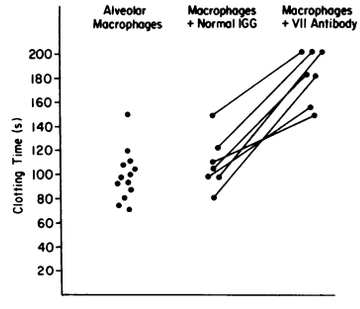

Figure 2. Neutralization of macrophage procoagulant activity by a Factor VII antibody. Single-stage clotting times for alveolar macrophages assayed in Factor VII-deficient plasma (Fig. 1) are regraphed. These are compared to the procoagulant activity of 5 × 10^5 macrophages preincubated with either nonimmune or anti-Factor VII rabbit IgG, then washed, and assayed in the same plasma.

is largely or entirely due to the extrinsic pathway of initiation of the coagulation cascade. These experiments do not, however, determine the source of the Factor VII activity. Alveolar macrophages may be stimulated to express thromboplastin activity and then bind blood-derived Factor VII as reported for human monocytes by Broze (17). We examined the alternative possibility that macrophages may synthesize Factor VII as suggested by Tsao et al. (21). We metabolically labeled macrophages for 18 h with [^{35}S]methionine, prepared cytosol and membrane fractions of the labeled cells, immunoprecipitated the cellular fractions with nonimmune IgG or IgG that contained Factor VII antibody, and analyzed the precipitates by SDS-polyacrylamide gel electrophoresis. Fig. 3 *A* illustrates a typical autoradiograph of the immunoprecipitates of the cytosol fraction of the cell. The major labeled protein precipitated by the Factor VII antibody was 48,000±2,000 mol wt and corresponded closely with the known molecular weight of human Factor VII (27, 28, 33). In addition, there were four to five faint bands between 45,000 and 150,000 mol wt. To further confirm the specificity of this immunoprecipitate, we used unlabeled purified Factor VII to compete with the labeled protein for the antibody (Fig. 3 *B*). Addition of 50 μg of Factor VII to the labeled cytosol fraction before the precipitation step totally blocked recovery of the labeled 48,000-mol wt protein. There was also partial loss of the high molecular band (~150,000 mol wt) but not the intermediate bands that were more prominent in this experiment. These findings demonstrate that human alveolar macrophages synthesize a protein indistinguishable by size or immunochemistry from Factor VII. The relationship between the higher molecular protein and Factor VII is unclear.

In our initial attempts to immunoprecipitate Factor VII, we used a buffer containing 1 mM EDTA. The 48,000-mol wt protein under these conditions is recovered in both the membrane and cellular fractions of the cell (not shown). After increasing the concentration of EDTA to 5 mM, we observed all the 48,000-mol wt protein in the cytosol fraction. These observations are consistent with the known peripheral membrane nature of cell-associated Factor VII (17).

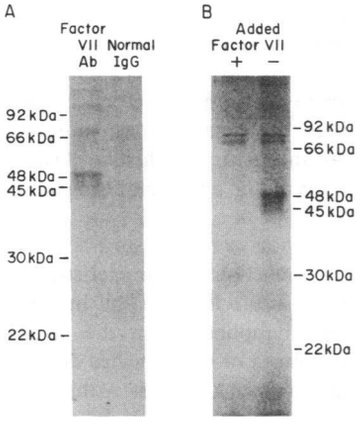

Figure 3. Factor VII synthesis by alveolar macrophages in vitro. (*A*) Autoradiograph of the cytosol of [^{35}S]methionine-labeled alveolar macrophages immunoprecipitated by either control, nonimmune IgG (*right*), or Factor VII antibody (*left*) after electrophoresis in 10% acrylamide slab gels. We observed identical results with cells from each of three smoking subjects. (*B*) Autoradiograph of the cytosol from labeled cells immunoprecipitated with Factor VII antibody in the presence (+) or absence (−) of 50 μg of unlabeled, purified Factor VII. The high molecular weight band (150,000) was faint but visible on the original autoradiograph even in the immunoprecipitation done in the presence of unlabeled Factor VII (+). The molecular weight standards for both experiments were phosphorylase *b* (92,000), bovine serum albumin (66,000), ovalbumin (45,000), carbonic anhydrase (30,000), and soybean trypsin inhibitor (22,000). KDa, molecular weight.

Assay of human alveolar macrophage Factor VII-activator activity. Although the data show that human alveolar macrophages synthesize Factor VII antigen, the question arises whether this protein is biologically active. Reported evidence indicates that murine peritoneal macrophages contain and secrete a coumadin-sensitive Factor X activator (19). The cell-associated Factor X-activator activity increased in response to endotoxin in vitro. These observations indicate rodent macrophages may contain the γ-carboxylase necessary to activate Factor VII. We tested whether human alveolar macrophages released active Factor VII that was coumadin sensitive. If so, this would imply the cells can synthesize biologically active Factor VII inasmuch as coumadin has no direct inhibitory effect in the procoagulant assay. In these experiments we assayed Factor VII activity by a one-stage Factor X-activator assay using a fluorescent oligopeptide specific for Factor Xa. Data in Table I show the results of experiments with cell-free supernatants from cultured macrophages of six separate normal volunteers. In each case, Factor X-activating activity was observed in the concentrated cell-conditioned media. However, as shown by the representative result with subject 6, expression of activity required the addition of brain thromboplastin to the supernatants. This finding correlated with our observation that no procoagulant activity was measurable in the nascent supernatants (not shown). That the cultured cells' release of Factor X-activating activity represented secretion of Factor VII was indicated by the fact that in two cases tested 25 μl of the Factor VII antibody completely blocked Factor X-activating activity (not shown). In every case, the addition of 50 μM coumadin to the start of the cultures at least partially blocked the subsequent recovery of Factor X-activating activity (Table I). The mean percent inhibition by coumadin was 71% (range 23–100%). In additional experiments, we tested the lysates of cultured cells for the Factor X activator. We observed coumadin to have a small and inconsistent effect on human alveolar macrophage Factor X-activator activity after 18–20 h culture in vitro. However, by removing much of the nascent activity with a 30-min exposure to trypsin (10 μg/ml) a consistent coumadin effect could be observed on the Factor X-activator activity of these cultured cells. Trypsin removed an average of 55% of the initial Factor X-activator activity (range 30–86%). Subsequent culture of the cells in 50 μM coumadin suppressed Factor X-activator activity by an average of ~25% compared with that of cells cultured without coumadin. In contrast, coumadin had no measurable effect on cellular tissue factor activity when tested in a standard one-stage prothrombin assay identically to that described in Fig. 1. These data indicate that at least some—and possibly all—of the Factor VII antigen synthesized by human alveolar macrophages is biologically active.

Effect of endotoxin on human alveolar macrophage Factor VII activity in vitro. A number of investigators have shown that freshly isolated human monocytes from peripheral blood have little or no procoagulant activity. These cells, in collaboration with lymphocytes, generate substantial procoagulant activity upon stimulation with endotoxin in vitro (5, 34). We examined whether alveolar macrophages would also respond to endotoxin in vitro. Lavaged cells from seven volunteers were cultured overnight in MEM containing 5% heat-inactivated human serum or in this medium supplemented with 1 μg/ml

Table I. Effect of Coumadin on Factor X-activating Activity Released by Cultured Human Alveolar Macrophages

Nonsmoking normal volunteer	Factor Xa substrate hydrolyzed		
	No coumadin	50 μM coumadin	% Inhibition by coumadin
	pmol	*pmol*	*%*
1	56	28	50
2	38	14	63
3	86	9	90
4	43	33	23
5	63	0	100
6	37	0	100
Supernatant (6) without thromboplastin	2	ND	—
Supernatant (6) without added Factor X	1	ND	—

Cell-free supernatant after overnight culture of macrophages (3.5×10^6/2 ml) in MEM±50 μM coumadin were made 0.05% in Triton X-100, dialyzed, and concentrated 10-fold by lyophilization. Reaction mixtures consisted of 100 μl of reconstituted supernatant in MEM to which 0.5 mg/ml brain thromboplastin was added, 10 μg of Factor X, 90 μl of Tris buffer, pH 7.8, 100 μl of cephalin, 10 μl of 0.25 M $CaCl_2$, and 0.1 mM Factor Xa substrate. After 45 min at 37°C, the reactions were stopped and fluorescence recorded. Data are expressed as mean picomoles substrate hydrolyzed in duplicate determinations where the coefficient of variation was routinely <5%. The mean percent inhibition by coumadin was 71%. Fluorescence observed in reaction mixtures containing 0.5 mg/ml thromboplastin in MEM with 0.5% Triton X-100 but no cell-conditioned media were subtracted from the reported values in each case. ND, not determined. The number in parentheses indicates the volunteer used in the experiment.

Table II. Effect of Endotoxin on Human Alveolar Macrophage Procoagulant Activity

Endotoxin	Tissue factor activity		Factor VII activity	
	Clotting time	Thromboplastin units	Clotting time	Equivalents per 10^6 cells
	s		*s*	*ng*
None	85±12	87±35	127±27	0.54
1 μg/ml	55±3	290±44	111±27	0.56
	$t < 0.05$	$t < 0.05$	$t > 0.05$	$t > 0.05$

Lavaged alveolar macrophages were incubated overnight in 5% heat-inactivated human serum±1 μg/ml endotoxin. The cells (5×10^5) were then washed three times and assayed intact in whole or Factor VII-deficient plasma. Cells from seven separate volunteers were tested in Factor VII-deficient plasma and cells from four subjects were tested in both plasmas. Mean clotting times±1 SD for the two assays are shown in the table. Data are also expressed as units of thromboplastin and (in four subjects) as nanogram equivalents of blood Factor VII for the tissue factor and Factor VII assays, respectively. The increases in procoagulant activity in whole plasma but not Factor VII deficient plasma were statistically different ($t < 0.05$ vs. $t > 0.05$, respectively) in a two-tailed, paired t test (44).

of endotoxin. After 18 h, the cells were washed three times and assayed for procoagulant activity in Factor VII-deficient plasma. In four cases the cells were also assayed in whole plasma. Data in Table II show results expressed as mean±1 SD of the clotting times as well as in units of thromboplastin or Factor VII activity. In each of the four subjects' cells tested in whole plasma, endotoxin increased procoagulant activity. The thromboplastic activity increased from a mean of 85 U to a mean of 290 U in response to endotoxin. However, when alveolar macrophages were assayed in Factor VII-deficient plasma, no significant difference could be observed between the endotoxin-treated and untreated cultures in Factor VII activity (Table II). Further, no trend in the Factor VII activity was evident as two of seven individuals showed an increase in their clotting times after incubation with endotoxin (not shown).

Factor VII activity of lavaged cells from patients with sarcoidosis. Previously reported data indicate that the alveoli of patients with sarcoidosis contain increased numbers of both macrophages and lymphocytes (24). We measured the procoagulant activity of freshly lavaged cells from nine such subjects in whole and Factor VII-deficient plasmas. Assays were performed without further purification of macrophages, because preliminary experiments indicated that gradient centrifugation (Percoll) resulted in some loss of activity and a cellular adherence step precluded comparison with the control group. Moreover, all evidence to date indicates that lymphocytes, the only cell contaminating these preparations, do not express procoagulant activity (7, 35, 36). Table III depicts the procoagulant activities of nine patients with sarcoidosis. Data are expressed both as tissue factor (thromboplastin equivalents) and nanogram equivalents of blood Factor VII associated with the intact cells. Four of nine subjects with sarcoidosis had increased tissue factor above the normal range and seven of nine subjects had increased Factor VII activity. The mean Factor VII equivalent per 10^6 cells for the sarcoid subjects was 4.7 ng as compared to 0.74 ng for the control group. By the nature of antigen determination being dependent on activity, we can only estimate the amount of Factor VII present. We do not know the activation state of Factor VII on these cells, i.e., one chain versus the more active two-chain enzyme. As a group, the increases in both tissue factor and Factor VII were statistically significant. In three of these nine patients, we measured procoagulant activity in Factor X-deficient plasma. In each case, there was no activity in the absence of exogenous Factor X.

We considered whether there was a relationship between observed procoagulant activity and clinical characteristics of the patient group. As the data in Table III show, all but two of the individuals had 20–50% lymphocytes in the lavage preparations. However, the percentage of lymphocytes did not correlate with the procoagulant activities at least within this small population.

Discussion

Previous observations in humans indicate that upon appropriate stimulation monocytes initiate coagulation via the extrinsic pathway by expression of tissue factor (7, 11, 17, 36). More recently, Tsao et al. (21) indicated that human monocytes can

Table III. Procoagulant Activities of Alveolar Macrophages from Sarcoid Patients

Patient	Age/sex	Percentage lymphocytes in lavage fluid	Procoagulant activities	
			Tissue factor	Factor VII equivalent
	yr		*U/10⁶ cells*	*ng/10⁶ cells*
1	22/M	47	330	3.2
2	58/F	31	>10,000	>50
3	24/M	33	2,000	2.4
4	50/F	25	8,000	2.7
5	62/M	20	500	0.4
6	27/F	10	800	3.4
7	50/F	48	270	18.2
8	27/F	11	600	7.4
9	53/F	25	5,500	0.9
		Mean	2,250	4.7
		SD	2,910	5.8
		Normal mean	480	0.74
		Normal range	140–1,000	0.2–2
			$P < 0.05$	$P < 0.01$

Alveolar macrophages (5×10^5) from sarcoid patients were assayed intact in whole plasma for tissue factor and in Factor-VII deficient plasma for Factor VII activity. Tissue factor is expressed in unit equivalents to rabbit brain thromboplastin (1,000 U = 1 mg/ml). Nanogram equivalents to blood Factor VII/10^6 cells were determined by plotting the observed clotting time in Factor VII-deficient plasma on a log-log transformation of dilutions of normal human plasma vs clotting time (seconds). Both the tissue factor and Factor VII activities of the sarcoid group were statistically higher than control by a Wilcox nonparametric rank-sum analysis (44). Data from subject 2 were excluded from the calculations.

also express Factor VII on their surface upon endotoxin stimulation in vitro in the absence of an exogenous source of Factor VII. We found that normal human alveolar macrophages, without in vitro stimulation, initiate the coagulation cascade by a Factor VII-dependent mechanism (Fig. 2). This was characterized by the ability of monospecific Factor VII antibodies to neutralize this activity and the requirement of 5 mM EDTA to completely dissociate the Factor VII activity from cellular membranes. Moreover, our evidence directly shows for the first time that cells of the monocyte/macrophage series have the capacity to synthesize Factor VII in vitro (Fig. 3). These cells also possess the carboxylase enzyme system required for biological activity of this protein as judged by coumadin sensitivity (Table I). The fact that the released Factor VII activity was more dramatically inhibited by coumadin than cell-associated activity (71 vs. 25%) suggests that the bulk of Factor VII synthesized by the cells in vitro may be secreted. These cells have the potential then to synthesize and express the extrinsic complex of blood coagulation on their surfaces for the initiation of hemostasis at extravascular sites of inflammation. We cannot be sure, however, what fraction of the Factor VII-dependent procoagulant activity in either normal volunteers or the patient group is due to Factor VII made by the cells and what fraction is derived from blood.

The only species of synthesized Factor VII observed was the 48,000-mol wt single-chain zymogen and not the two-chain activated derivative of Factor VII, Factor VIIa. Several lines of evidence indicate that this zymogen binds to tissue factor and exhibits coagulant activity equivalent to 1–2% of that of Factor VIIa (37). This coagulant activity appears sufficient to initiate coagulation upon exposure of whole blood to tissue factor (37). Our data support the view that normal human alveolar macrophages can initiate coagulation by this mechanism. It remains possible that undetectable but functionally important Factor VIIa is also present on the cell surface. Moreover, it is likely that the cells can utilize exogenous Factor VII as we consistently observed a shorter clotting time in normal as compared with Factor VII-deficient plasma (Fig. 1).

The role of Factor VII activity in the biology of pulmonary macrophages is unclear. Fibrinogen is undetectable in lavaged alveolar fluid, and fibrin is not normally found in extravascular lung tissue (38). The procoagulant activity of these cells may simply reflect a programmed response to cellular stimulation, inasmuch as alveolar macrophages are exposed continuously to the environment and a variety of inducers. Factor VII could also serve a role in normal lung physiology. Although we have not tested whether thrombin formation occurs in vivo, the generation of thrombin in a fibrinogen-free environment would allow the expression of previously reported functions of thrombin in addition to clot formation. For example, thrombin is chemotactic for monocytes and stimulates lung fibroblasts to elaborate arachidonic acid metabolites (39, 40). These processes offer possible mechanisms for continued influx of young macrophages onto the alveolar surfaces.

Fibrin is a conspicuous part of acute and chronic inflammatory disorders of the lung. A number of pathways, in addition to macrophages, could initiate fibrin deposition after the leakage of plasma constituents into the interstitial and alveolar compartments of the lung. Strong but circumstantial evidence suggests that such tissue fibrin may not only be a marker of injury but also exert an influence on the course of healing. Astrup has reviewed evidence that fibrin deposition and the rate of its resorption regulate the events in scar formation, particularly fibroblast proliferation (41). Fibroblasts utilize fibrin and fibronectin substrata as a matrix for proliferation and collagen deposition (42). Moreover, Spencer and Pratt in separate pathologic studies have noted the correlation between persistent fibrin deposits and subsequent localization of fibrotic processes in the lung (23, 24). Our observations with alveolar macrophages may be germane, because we have previously shown that normal human alveolar macrophages synthesize and express a plasminogen activator (25). Macrophages thus synthesize the rate-limiting enzymes in proteolytic cascades initiating both fibrin deposition (Factor VII-tissue factor) and resorption (plasminogen activator). These cells are in a position then to influence fibrin turnover in the lung. By coordinating fibrin turnover with the elaboration of other factors important to scar formation, e.g., fibronectin and fibroblast growth factor, lung macrophages may well modulate healing.

We report here our initial findings in a group of patients with sarcoidosis, a chronic inflammatory disease that may result in pulmonary fibrosis (43). We observed increased Factor VII activity in seven of nine subjects (Table III). Because the Factor VII activity of monocytes is induced by T-helper lymphocytes (21), the increase in alveolar macrophage associated Factor VII activity in sarcoid patients may be due

to the disproportionate increase in alveolar T helper cells known to occur in these individuals (24). Based on these observations, we retrospectively examined biopsy specimens from five sarcoid patients for evidence of tissue fibrin deposition. We observed fibrin in the central, macrophage-rich areas of sarcoid granulomata by immunoperoxidase stains of lung or lymph node biopsy specimens from three of five subjects. The peripheral areas rich in fibroblasts and collagen were negative (Elizabeth Hammond and Harold Chapman, unpublished observations). These findings support the hypothesis that fibrin deposition may precede and support subsequent scar formation in sarcoidosis. Whether the observed increased macrophage Factor VII activity is related to the observed tissue fibrin and whether the Factor VII activity and/or tissue fibrin is actually linked to the course of the disease requires further study.

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