Production of plasminogen activator by alveolar macrophages in normal subjects and patients with interstitial lung disease

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ABSTRACT Increased production of the serum protease plasminogen activator is associated with tissue damage. The in vitro production of plasminogen activator by alveolar macrophages obtained by bronchoalveolar lavage was studied in 22 normal subjects and 28 patients with interstitial lung disease to determine whether plasminogen activator is produced by normal alveolar macrophages and whether this is increased in patients with interstitial lung disease. Plasminogen activator activity, measured with an iodine-125 labelled fibrin release assay, was found to be dependent on time, effector cell numbers, and plasminogen concentration. Plasminogen activator production by alveolar macrophages from 14 normal non-smokers and eight normal smokers was similar and the mean value was 0.78 (SEM 0.16) urokinase (UK) units $\times 10^{-8}$ /cell/hour. Alveolar macrophages from the seven patients with cryptogenic fibrosing alveolitis and six patients with histiocytosis-X produced more plasminogen activator (1.89 (0.25) and 4.54 (1.3) $\times 10^{-8}$ UK units/cell/hour respectively) than macrophages from normal subjects ($p < 0.05$), whereas those from 15 patients with sarcoidosis did not (1.09 (0.2) $\times 10^{-8}$ UK units/cell/hour). Exposure of normal alveolar macrophages to immune complexes enhanced plasminogen activator production to 2.07 (0.27) $\times 10^{-8}$ UK units/cell/hour whereas exposure to products of activated T cells and to purified γ interferon reduced plasminogen activator production (to 0.38 (0.11) and 0.62 (0.11) $\times 10^{-8}$ UK units/cell/hour respectively). These studies show that plasminogen activator is produced by normal human alveolar macrophages and that its production is increased in patients with cryptogenic fibrosing alveolitis and histiocytosis-X.

Introduction

Plasminogen activator is a serine protease that converts plasminogen to the active enzyme plasmin, thus participating in the initiation and enhancement of several important biological processes, including fibrinolysis, inflammation, and connective tissue degradation.¹⁻³

The role of plasminogen activator in inflammation is related to its capacity to activate complement components⁴⁻⁶ and also to activate Hageman factor to generate bradykinin.⁷ During inflammatory processes plasminogen activator is released by several cell types, particularly mononuclear phagocytes, and the amount of plasminogen activator produced corresponds to the intensity of inflammation.⁸ The role of plasminogen activator in the degradation of connective tissue

components relates to its capacity to initiate cleavage of the structural proteins laminin and fibronectin⁹⁻¹¹ and also to activate latent collagenases.¹² It thus plays a part in the remodelling of normal tissues² and in the processes of ovulation, trophoblast implantation, and cancer cell metastasis.^{19,13} The production of plasminogen activator in many of these processes is regulated by external factors. Its release by animal mononuclear phagocytes is considerably enhanced after activation by lymphokines and endotoxin,¹³⁻¹⁵ and production by ovarian granulosa cells is controlled by the gonadotrophic hormones.¹⁶

This study aimed to assess the production of plasminogen activator by normal human alveolar macrophages. As excess plasminogen activator activity is associated with inflammation and tissue damage¹⁷⁻²⁰ and as most interstitial lung diseases are characterised in their active stages by inflammation and alveolar injury,²¹ the production of plasminogen activator in these diseases was also evaluated.

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Methods

STUDY POPULATION

We studied 22 normal volunteers, 14 non-smokers and eight smokers (all with normal chest radiographs and normal pulmonary function and not receiving any treatment), and 28 patients with chronic interstitial lung disease (seven with cryptogenic fibrosing alveolitis, six with pulmonary histiocytosis-X, and 15 with pulmonary sarcoidosis). The diagnosis was confirmed by histological examination of lung biopsy material—open biopsy for patients with cryptogenic fibrosing alveolitis and histiocytosis-X, transbronchial biopsy for those with sarcoidosis—on the basis of established criteria.^{21,22} The protocol received the ethical committee's approval and informed consent was obtained from all subjects before the study.

PREPARATION OF EFFECTOR CELLS

Lung mononuclear cells were obtained by bronchoalveolar lavage.²³ The cells were washed in RPMI supplemented with glutamine 50 mM, penicillin 100 units/ml, and streptomycin 100 µg/ml medium (Gibco, Grand Island, NY). Cell viability (as judged by trypan blue dye exclusion) was always over 95%. In some experiments lung mononuclear cells from patients with sarcoidosis were depleted of adherent cells by passage through nylon wool columns (30 min, 37°C) before being assayed for plasminogen activator activity. These lymphocyte populations were over 97% pure (on the basis of morphology) and over 97% viable.

PREPARATION OF FIBRIN COATED PLATES

Fibrinogen (supplied by J Finlayson, Bureau of Biologics, Food and Drug Administration, Bethesda, Maryland) was labelled with iodine-125 (¹²⁵I) by a lactoperoxidase method (New England Nuclear, Boston, Massachusetts) and separated from unbound ¹²⁵I by sephadex G25 gel filtration at 22°C (Pharmacia, Piscataway, New Jersey). The ¹²⁵I labelled fibrinogen was added to 24 well tissue culture plates (Falcon 3047, Becton Dickinson, Oxnard, California) at 10⁵ cpm per well with 30 µg unlabelled fibrinogen in a final volume of 250 µl. The plates were then dried for 72 hours at 50°C and stored at 22°C until they were used.

ASSAY OF PLASMINOGEN ACTIVATOR ACTIVITY

Medium containing 10% fetal calf serum (Gibco) as a source of thrombin was added to each well for two hours at 37°C, to convert the fibrinogen to fibrin.²⁴ Alveolar macrophages were added to the wells coated with ¹²⁵I—from 10⁵ to 10⁶ in each well in a final volume of 500 µl of medium in the presence of plasminogen. In the initial experiments the source of plasminogen was 5% acid treated (30 min, 22°C, pH 2.0) fetal calf

serum, and in subsequent experiments it was purified plasminogen (Sigma, St Louis, Missouri). Parallel experiments showed similar levels of measured plasminogen activator activity for given macrophage numbers for the two sources of plasminogen (0.25 (SEM 0.11) v 0.18 (0.09) UK units × 10⁻⁸/cell/h (p > 0.2; n = 4)). Purified plasminogen concentrations from 5 to 50 µg/ml produced similar plasminogen activator activity in these experiments but, as more than 50 µg/ml produced increased ¹²⁵I release (probably owing to autocatalytic generation of plasmin¹), 10 µg/ml was used in all experiments. Plasminogen activator activity in each well was assessed at various times from one to 72 hours, at 37°C, from the concentrations of fibrin breakdown products in 25 µl aliquots of supernatant in a gamma counter. Total releasable counts were obtained by adding 500 µl of 0.25% trypsin (Gibco) to wells. Background release was determined by adding medium plus plasminogen to wells in the absence of effector cells; it was always below 0.5% of the total releasable counts per hour. Neither source of plasminogen altered background release (p > 0.3 compared with medium alone), confirming that the plasminogen preparations were completely free of plasmin and plasminogen activator.

SPECIFICITY OF ¹²⁵I RELEASE

To confirm that the activity observed was due to plasminogen activator and not to other fibrin degrading proteases such as elastase, we performed two additional experiments. Firstly, macrophages were studied in the absence of plasminogen and, secondly, a specific inhibitor of plasminogen activator, ac-gly-gly-arg chloromethyl ketone (supplied by R Goldfarb, Pfizer, Groton, Connecticut), was added to wells containing alveolar macrophages plus plasminogen. Maximum inhibition occurred at 100 µg/ml and was used in all experiments. This inhibitor had no effect on background release of ¹²⁵I (p > 0.3).

To determine whether preformed or intracellular plasminogen activator was present, 10⁶ alveolar macrophages were disrupted by sonication before assay and their plasminogen activator activity was compared with that of an equal number of intact alveolar macrophages over 90 minutes.

RELEASE OF PLASMINOGEN ACTIVATOR

To determine whether the observed alveolar macrophage plasminogen activator activity was released or membrane bound, we cultured alveolar macrophages at 10⁶ cells/ml medium in 5 ml polypropylene tubes (Falcon 2063, Becton Dickinson) for four hours at 37°C and then centrifuged them (500 g, 15 min). An aliquot of the supernatant was removed carefully and assayed for plasminogen activator activity as described above.

STANDARDISATION OF ASSAYS

In all assays a standard curve of plasminogen activator activity was constructed with purified human urokinase (UK) (Sigma) diluted in normal saline and with 10 $\mu\text{g/ml}$ purified plasminogen. This standard curve was used to relate the percentage of maximal releasable counts to Sigma UK units, one Sigma UK unit being equivalent to 5–10 000 Plough units. Urokinase was stored at -20°C at 1 U/ml in phosphate buffered saline and did not lose activity during storage for six months.

Each fibrinogen coated plate prepared from the same ^{125}I fibrinogen batch had similar levels of ^{125}I release for a given dose of urokinase over periods of up to two months. Slight differences in the ^{125}I fibrinogen coated plates occur with each new batch, because of variability in ^{125}I labelling, "cold" fibrinogen concentrations, and the respective proportions of these components in each batch. Alveolar macrophages from at least three normal individuals were therefore studied with each of the three batches. Mean (SEM)% cpm released by these normal macrophages from each of the three plate batches were 31.3 (4.1), 26.3 (4.4), and 50.7 (4.6). To validate comparisons with macrophages from individuals with interstitial lung disease, we related all data to the initial plate batch, using this formula: urokinase activity = (% released by test macrophages – mean % released by normal macrophages with that batch) $\times$ 31.3. Assays were performed in triplicate.

EFFECT OF MACROPHAGE STIMULI ON PLASMINOGEN ACTIVATOR PRODUCTION

The capacity of immune complexes and lymphokines to stimulate normal alveolar macrophages to produce increased plasminogen activator activity was determined as follows. Ox red blood cell (ORBC)–anti-ORBC antibody complexes were formed.²⁵ A T cell lymphokine preparation was prepared by incubating normal peripheral blood mononuclear cells in medium containing 10% fetal calf serum plus 20 $\mu\text{g/ml}$ concanavalin A (Sigma) for 24 hours, purifying the T cells by double sheep red blood cell (SRBC) rosette formation (95% (SEM 2%) pure on the basis of SRBC rosette formation and over 98% viable as judged by trypan blue dye exclusion), and then culturing them in medium without serum at 5×10^6 cells/ml for 24 hours at 37°C before the supernatant was collected. This preparation was found to contain 800 international units of interferon by bioassay²⁶; this was shown to be γ interferon as the interferon activity was abrogated only by a monoclonal antibody to γ interferon (Melo Laboratories, Springfield, Virginia).²⁶ Purified γ interferon was obtained from Interferon Sciences (New Brunswick, New Jersey), aliquoted at 10^5 units/ml (assayed as above) and stored in medium containing

1% bovine serum albumin (Sigma) under liquid nitrogen.

The effect of these preparations on plasminogen activator production by normal alveolar macrophages was determined as follows. The ORBC–anti-ORBC antibody immune complexes (1% by volume), lymphokine preparation (20% by volume), purified interferon (200 units/ml), or medium alone was added to wells containing 2.5×10^5 normal alveolar macrophages, and plasminogen activator activity was measured as described above. None of the stimuli altered macrophage adherence, background counts, or release of ^{125}I by purified urokinase plus plasminogen ($p > 0.3$, all comparisons); this confirmed that the observed alterations in plasminogen activator production were due to a direct effect on macrophages rather than to effects on the plasminogen activator assay.

DATA

All data are presented as mean values with standard errors in parentheses.

Results

PLASMINOGEN ACTIVATOR ACTIVITY OF NORMAL ALVEOLAR MACROPHAGES

Normal alveolar macrophages released little ^{125}I in the absence of plasminogen (fig 1). When plasminogen

Fig 1 Plasminogen activator (PA) activity of normal alveolar macrophages, showing plasminogen dependence and inhibition by Ac-Gly-Gly-Arg-chloromethylketone, a specific plasminogen activator inhibitor. PA activity = urokinase units $\times 10^{-8}$ /cell/hour.

Fig 2 (a) Kinetics and (b) cell number dependence of plasminogen activator (PA) activity of normal human alveolar macrophages. PA activity = urokinase units $\times 10^{-8}$ /cell/hour.

was present significant fibrinolysis occurred ($p < 0.001$ compared with no plasminogen). When ac-gly-arg chloromethyl ketone was added almost complete inhibition of ^{125}I release was observed (fig 1, $p < 0.001$ compared with no inhibitor). Thus virtually all the fibrinolytic activity observed was attributable to plasminogen activator.

Normal human alveolar macrophages produced plasminogen activator at a constant rate over 24

hours, after which the rate of ^{125}I fibrin breakdown declined (fig 2a). Sonicated alveolar macrophages produced less than 20% of the plasminogen activator activity of an equal number of cultured alveolar macrophages, suggesting that plasminogen activator was largely synthesised *de novo* during the culture period. The addition of a fresh source of plasminogen activator activity (fresh alveolar macrophages or urokinase) to these wells after 24 hours produced further ^{125}I fibrin breakdown (data not shown), implying that this decline in plasminogen activator production with time was largely due to decreased production by effector cells rather than to substrate depletion.

The degradation of ^{125}I fibrin by normal alveolar macrophages was linear with respect to cell number up to 5×10^5 macrophages/well (fig 2b). When more macrophages were added to each well the rate of ^{125}I release declined.

Plasminogen activator was partially released into the supernatant and not totally membrane bound as appreciable plasminogen activator activity was detected in the supernatant after four hours of culture in suspension (9.8% (1.3%) of the plasminogen activator activity of an equivalent number of adherent cells) and maximal ^{125}I release was observed with submaximal cell numbers in each well in the presence of ample substrate (fig 2a).

No significant difference was noted in plasminogen activator production by alveolar macrophages

Fig 3 Comparison of alveolar macrophage plasminogen activator (PA) activity from normal non-smokers (NSm) and normal smokers (Sm). PA activity = urokinase units $\times 10^{-8}$ /cell/hour.

Fig 4 Plasminogen activator (PA) activity of alveolar macrophages from patients with cryptogenic fibrosing alveolitis (CFA), histiocytosis-X (HX), and sarcoidosis (S). N indicates the mean and standard error of normal values.

obtained from normal smokers and from normal non-smokers (fig 3: 0.98 (0.19) v 0.70 (0.13) UK units $\times 10^{-8}$ /cell/h; $p > 0.1$).

PLASMINOGEN ACTIVATOR IN PATIENTS WITH INTERSTITIAL LUNG DISEASE

Alveolar macrophages from most patients with cryptogenic fibrosing alveolitis and histiocytosis-X had increased plasminogen activator activity (fig 3: 1.89 (0.25) and 4.74 (2.6); $p < 0.05$) for both groups compared with normal subjects. In patients with pulmonary sarcoidosis, however, alveolar macrophage plasminogen activator activity was similar to that observed in normal subjects at 1.09 (0.1) UK units $\times 10^{-8}$ /cell/h (fig 3: $p > 0.1$). All plasminogen activator activity in mononuclear cell populations from sarcoid lungs was associated with alveolar macrophages as purified lung lymphocytes from these patients showed negligible plasminogen activator activity ($< 10^{-9}$ units per cell/h).

The fibrinolytic activity of the alveolar macrophages from each group of patients like those from normal subjects, was shown to be due to plasminogen activator since it was plasminogen dependent and inhibited by the specific plasminogen activator inhibitor ac-gly-gly-arg chloromethyl ketone ($p < 0.001$ for both cryptogenic fibrosing alveolitis and histiocytosis-X compared with macrophages plus plasminogen without inhibitor).

EFFECT OF EXTERNAL STIMULI ON PLASMINOGEN ACTIVATOR PRODUCTION

Immune complexes stimulated the production of plasminogen activator by normal alveolar macrophages to 2.07 (0.27) UK units $\times 10^{-8}$ /cell/h (fig 4: $p < 0.01$ compared with macrophages alone). Serum free activated T cell supernatant containing γ interferon decreased the production of plasminogen activator by normal alveolar macrophages—to 0.38 (0.11) UK units $\times 10^{-8}$ /cell/h (fig 5: $p < 0.001$ compared with macrophages alone)—as did purified γ interferon—to 0.62 (0.11) UK units $\times 10^{-8}$ /cell/h (fig 5: $p < 0.001$ compared with macrophages alone).

The effects of these stimuli were manifest within two hours of being applied to alveolar macrophage cultures.

Discussion

Plasminogen activator has a variety of physiological and pathological activities, depending on the cell origin, the location of its production, and the presence or absence of factors that regulate its production.

PRODUCTION OF PLASMINOGEN ACTIVATOR BY NORMAL ALVEOLAR MACROPHAGES

The immediate and sustained plasminogen activator production contrasts with the production of neutrophil chemotactic factor under similar conditions,²⁵ and with murine plasminogen activator production,

Fig 5 Effects of stimulation of normal alveolar macrophages by immune complexes (IC), activated T cell supernatants (TCS), and γ interferon (IFN γ). Plasminogen activator (PA) activity = urokinase units $\times 10^{-8}$ /cell/hour.

implying that normal human alveolar macrophages are "primed" to produce plasminogen activator *in vivo*. This suggests that the production of plasminogen activator has a role *in vivo* in the normal human lung, a suggestion that is supported by the observation that plasminogen activator activity is present in extracted normal lung tissue²⁷ and in the epithelial lining fluid of the normal lower respiratory tract (paper in preparation). The role of plasminogen activator in the normal lung may be similar to its role in other normal tissues—that is, tissue remodelling and prevention of clot formation² by clearance of fibrinogen.²⁸

Although appreciable amounts were released, the activity of plasminogen activator in normal alveolar macrophages was largely bound to the cell membrane, as noted in human blood monocytes.²⁹ Membrane bound plasminogen activator is largely protected from inhibition by antiproteases³⁰ and is thus able to act freely on the substrates with which the macrophage makes contact.

EFFECTS OF SMOKING ON PLASMINOGEN ACTIVATOR PRODUCTION

Although cigarette smoking induces human alveolar macrophages to produce increased amounts of some mediators—for example, oxygen radicals³¹—this study shows no such effect on plasminogen activator production. Although total plasminogen activator activity in the lower respiratory tract of smokers is likely to be increased because of their increased number of alveolar macrophages,³² whether plasminogen activator has a role in the destruction of alveolar structures from cigarette smoking remains uncertain.

PLASMINOGEN ACTIVATOR RELEASE IN INTERSTITIAL LUNG DISEASES

Plasminogen activator release was increased in most patients with cryptogenic fibrosing alveolitis and histiocytosis-X. Loss of type I epithelial cells and basement membrane damage are seen in cryptogenic fibrosing alveolitis³³ and histiocytosis-X.²² In view of the capacity of plasminogen activator to degrade laminin and fibronectin, important structural and cell attachment components of basement membrane,¹⁰ and to activate latent collagenases,¹² possibly increased plasminogen activator activity in these diseases induces damage to basement membrane, which may then prevent type II epithelial cells from reconstituting the epithelial surface, thus leading to repair by fibrosis.³⁴ Plasminogen activator also directly degrades other lung connective tissue proteins³⁵ and this may also contribute to the tissue damage in these disorders.

Further support for a role for plasminogen activator in the inflammation and tissue destruction in patients with interstitial lung disease comes from several animal disease models. Plasminogen activator

correlates closely with the degree of inflammation and damage in cutaneous leprosy in mice,¹⁷ chronic subcutaneous inflammation in sheep,¹⁸ and chronic hypersensitivity pneumonitis in rabbits.¹³ In addition, increased plasminogen activator production corresponds to the degree of inflammation and tissue damage in patients with chronic inflammatory bowel disease,¹⁹ rheumatoid arthritis,²⁰ and psoriasis.³⁶ Interestingly, sarcoidosis, a disease that is generally less aggressive and destructive,²¹ is not associated with the production of abnormal amounts of plasminogen activator by alveolar macrophages. As the alveolar macrophages in sarcoidosis are activated,²⁶ clearly the nature of this activation differs from that seen in cryptogenic fibrosing alveolitis and histiocytosis-X, suggesting either that a different population of cells is present or that the activation signals in the different diseases induce different "types" of alveolar macrophage activation.

EFFECTS OF EXTERNAL STIMULI ON PLASMINOGEN ACTIVATOR PRODUCTION

The aetiology of cryptogenic fibrosing alveolitis is unknown but immune complexes might play a part by activating macrophages.²¹ In this study immune complexes induced *in vitro* changes in normal alveolar macrophage plasminogen activator production that mimic the changes seen in alveolar macrophages in patients with cryptogenic fibrosing alveolitis *in vivo*. Animal studies using alveolar macrophages have also shown that immune complexes enhance plasminogen activator production.¹⁵ Interestingly, the addition of immune complexes to alveolar macrophages from patients with cryptogenic fibrosing alveolitis did not further increase plasminogen activator production (data not shown). The aetiology of histiocytosis-X is unknown, but immune complexes have been found in this disease also and may have a role in pathogenesis. Possibly, however, the abnormal cell type that accumulates in the lung in this disorder, the "histiocytosis-X cell," which has some similarities to the antigen presenting dendritic cell,³⁷ is able to produce large amounts of plasminogen activator.

The aetiology of pulmonary sarcoidosis is also unknown but the disease is associated with the accumulation of large numbers of activated T cells that release large amounts of the macrophage activating factor γ interferon.²⁶ The finding in this study that products of activated T cells, particularly γ interferon (though not necessarily exclusively), reduce plasminogen activator production is consistent with the observation that its production by alveolar macrophages from patients with sarcoidosis is not increased, even though these macrophages, like those in cryptogenic fibrosing alveolitis and histiocytosis-X, are activated.²⁶ This effect of activated T cell products and

purified γ interferon on plasminogen activator production contrasts with that noted with animal peritoneal¹⁴ and alveolar macrophages¹³ and with human peripheral blood monocytes,²⁹ where activated T cell products enhance plasminogen activator production. This reduction in fibrinolysis induced by γ interferon may be due to reduced plasminogen activator release, "internalisation" of membrane bound plasminogen activator, negative feedback on the producer cell, cell cytotoxicity, or the release of a plasminogen activator inhibitor. None of these possibilities is directly addressed in this study.

This differential production of plasminogen activator in response to different activation stimuli shows that its production is not useful as a "general" marker for macrophage activation, as has been suggested for animal macrophages.³⁸ It also implies a heterogeneity of response of normal human alveolar macrophages to different stimuli. These observations confirm the complexity of human alveolar macrophage responses to different stimuli and implies that study of the production of mediators such as plasminogen activator by alveolar macrophages in different diseases may provide clues about the initiating events in these disorders.

This study shows that normal human alveolar macrophages produce plasminogen activator and that increased amounts of this are released by alveolar macrophages in patients with destructive interstitial lung diseases such as cryptogenic fibrosing alveolitis and histiocytosis-X. Possibly suppressing the release or the effects of plasminogen activator in the lung may be useful in patients with cryptogenic fibrosing alveolitis and histiocytosis-X.

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