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Procoagulant (thromboplastin) activity in human bronchoalveolar lavage fluids is derived from alveolar macrophages

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ABSTRACT: Fibrin deposition in the alveolar space and the lung interstitium is a prominent feature of many types of inflammatory pulmonary diseases. Cells of the monocyte/macrophage line are the primary cells supplying procoagulant activity in inflammatory lesions. In the present study we found that both lung alveolar macrophages (LAM) and bronchoalveolar lavage fluids (BALF) from humans contained procoagulant activities. The procoagulant in BALF was associated with membrane vesicles which sedimented at 100,000 g for 1 h. By electron microscopy the BALF ultrasediment was seen to consist almost exclusively of membrane material and this was confirmed by monitoring the content of different marker enzymes for specific subcellular structures. Using macrophage membrane markers, at least part of the BALF-ultrasediment was shown to be derived from LAM. On the basis of phospholipase C sensitivity, antibody neutralization and the site of action of the procoagulant in the sequential activation of coagulation factors, both the LAM-associated and the BALF-associated procoagulant activity was identified as thromboplastin (tissue factor) or thromboplastin-factor VII complexes. This suggests that alveolar macrophages and the LAM-derived thromboplastin-containing microvesicles may contribute to intraalveolar and interstitial fibrin deposition *in vivo* and probably also have consequences for the development of pulmonary fibrosis.

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Fibrin is a common finding at inflammatory sites. The formation of persistent fibrin in tissues is due to a disturbance in the balance between the coagulation and the fibrinolytic system. In the lungs, fibrin deposition in the alveolar space and in the interstitial tissue is a conspicuous feature of many types of inflammatory reactions [1–3]. In particular, alveolar fibrin deposition has been observed in association with lung injury in experimental animals and patients with the adult respiratory distress syndrome (ARDS) [4–7]. Fibrin probably serves as a nidus for fibrotic processes and fibrin deposition in the lung has been shown to link with the development of subsequent fibrosis in animals [6–8] and has been strongly suggested in humans with interstitial lung disease [1, 2]. Several recent studies have clearly shown disturbances of pathways of clotting and fibrinolysis in interstitial diseases of the lung [7, 9–13].

Mononuclear phagocytes of various types have repeatedly been shown to display procoagulant activities of which thromboplastin (tissue factor) is the best characterized and most widely studied [14]. Macrophages are a prominent cell type of most acute and chronic pulmonary inflammations. Human alveolar macrophages recovered by lavage have been shown to express the two components for the initial enzyme complex of the extrinsic pathway of coagulation, tissue factor and factor VII [9, 10]. Procoagulant activity of tissue factor-type has also been described in alveolar macrophages from rabbits [15, 16] and sheep [17]. On the other hand alveolar macrophages secrete plasminogen activators of the urokinase types [10, 17] and a fibrinolytic inhibitor [18] and thus have a dualistic role in the balance between coagulation and fibrinolysis.

Earlier studies in mice have shown that procoagulant fibrinolytic and antifibrinolytic products of macrophages are coordinately regulated to influence the localization and mobility of these cells in an inflammatory locus [19].

In this report we describe the expression of thromboplastin and factor VII activity by human alveolar macrophages and further the occurrence of non-cell associated procoagulant material in bronchoalveolar lavage fluid. Evidence is presented that this procoagulant is thromboplastin-like and is bound to plasma membrane vesicles probably derived from alveolar macrophages.

Materials and methods

Bronchoalveolar lavage and cell preparation

After informed consent healthy volunteers underwent bronchoscopy with bronchoalveolar lavage, which was performed with a flexible fibreoptic bronchoscope using a segment of the right middle lobe. A total of 100 ml 0.9% saline in five 20 ml aliquots was installed and recovered by suction. Bronchoalveolar lavage fluid was centrifuged at 500 **g** for 10 min to remove cellular material. Differential counting of lavaged cells was performed using a modified Giemsa staining of cytocentrifuged preparations (Diff-Quick, Harleco, Philadelphia, PA, USA). Lavage cells were washed three times in 0.9% saline and finally resuspended in barbital- buffered saline, pH 7.35. Procoagulant activity was tested after freezing and thawing and manual homogenization (10 strokes in a Potter-Elvehjem homogenizer).

Assay of marker enzymes

The following enzymes were assayed: Lactate dehydrogenase (EC 1.1.1.27), and alkaline phosphatase (EC 3.1.3.1.) according to the Scandinavian Committee on Enzymes [20]; phosphodiesterase I (EC 3.1.4.1.) as described by TOUSTER *et al.* [21]; succinic dehydrogenase (EC 1.3.99.1) according to PENNINGTON [22]; esterase (EC. 3.1.1.2.) as described by BEAUFAY *et al.* [23]; β-acetylglucosaminidase (EC. 3.2.1.30), catalase (EC. 1.11.1.6) and acid phosphatase (EC. 3.1.3.2) according to BARRET [24], BAUDHUIN *et al.* [25] and KACHMAR [26], respectively.

Electron microscopy

Samples of cell-free bronchoalveolar lavage fluid were pelleted at 100,000 **g** for 1 h in a Beckman ultracentrifuge, model L8-70, rotor SW 60Ti, washed twice in 0.9% saline, fixed in 2% glutaraldehyde at 6°C for 2 h, and washed in 0.2 N cacodylate buffer (pH 7.3). The preparations were postfixed in 1% OsO_4 for 2 h, washed in cacodylate buffer, dehydrated in graded ethanol (30, 50, 70%, absolute) and propylene oxide (70%) and finally embedded in LX epoxy resin. Ultrathin sections were cut from the specimen, stained with uranyl acetate and examined in a Philips 3015 electron microscope.

Antibodies

A monoclonal antibody (1D5, IgG_1) against a plasma membrane antigen of human monocytes [27] was a gift from G. Gaudernach, Institute of Transplantation Immunology, Rikshospitalet, Oslo, Norway. Purified 1D5 was radioiodinated (^{125}I) by the Iodogen method [28] to a specific activity of 8.5 $mCi \cdot mg^{-1}$ protein. Bronchoalveolar lavage fluid containing cells were incubated for 2 h with the labelled 1D5 antibody. Washed and homogenized cells and washed, ultrapelleted material from lavage fluid were assayed for radioactivity using a Packard Model 578 gamma scintillation spectrometer.

The polyclonal neutralizing antiserum to thromboplastin (apoprotein III) was a gift from H. Prydz, Research Institute for Internal Medicine, Rikshopitalet, Oslo, Norway and the Ig fraction was isolated as described by BJØRKLID and STORM [29].

Protein and procoagulant activity determination

Protein was determined by a modified Lowry procedure as described by MARKWELL *et al.* [30]. Bovine serum albumin was used as a standard.

Procoagulant activity was determined in a one-stage clotting system [31] consisting of 0.1 ml cell or ultrapellet homogenate in barbital-buffered saline, (pH 7.35); 0.1 ml citrated human plasma (from single donor) (or human plasmas deficient in coagulation factor VII, VIII, XII, X, V, or prothrombin) and 0.1 ml $CaCl_2$ (30 mM). Clotting times were determined in duplicates by the manual tilt method and the mean of two measurements was used to calculate thromboplastin units. Standard curves for thromboplastin activity were established by using a standard human brain thromboplastin preparation [32] arbitrarily taken to contain 100 units per ml. Specific thromboplastin activity was expressed as units of thromboplastin per mg protein.

Reagents

Human plasmas deficient in clotting factor VII, XII, V and VIII were a gift from H. Stormorken, Research Institute for Internal Medicine, Rikshopitalet, Oslo, Norway, Factor X and II-deficient plasmas were obtained from Merz & Dade AG (Düdingen, Switzerland).

Phospholipase C was purified to homogeneity from *Bacillus cereus* culture supernatants [33].

Statistical analyses

The results are presented as mean ±SEM

Results

Procoagulant activity of human alveolar macrophages

Differential counting of lavaged lung cells from healthy donors showed mean percentages of macrophages, lymphocytes and neutrophils to be 96, 3 and 1%, respectively. Freshly isolated alveolar macrophages from these individuals reduced markedly the recalcification time of normal human plasma (72±12 s *vs* 240±21 s). Alveolar macrophages from smokers and nonsmokers reduced the recalcification time to the same degree. The procoagulant was characterized using plasma deficient in single coagulation factors (table 1). Cellular procoagulant activities were not demonstrated in plasmas deficient in

factors X, V and II (common pathway). Total cellular procoagulant activity was also tested in factor VIII and factor XII deficient plasmas and reduced the recalcification time to the same degree as in normal plasma. This indicates that the procoagulant was not dependent on factors of the intrinsic pathway for its expression. The procoagulant had a partial, although not complete, requirement for factor VII, indicating that the procoagulant partly exists in a form which can activate factor X directly. Treatment of alveolar macrophage lysates with a goat anti-human apoprotein III antiserum and phospholipase C, respectively, completely extinguished the procoagulant activity (table 1).

Table 1. – Characterization of procoagulant activities in LAM homogenates and BALF sediment

	Coagulation time (s)	
Plasma	LAM homogenate	BALF ultrasediment
Normal human	60.9	39.7
F II deficient	>180	>180
F V deficient	>180	>180
F X deficient	>180	>180
F VII deficient	71.8	68.9
F VIII deficient	62.0	37.2
F XII deficient	62.0	37.8
Thromboplastin activity in normal plasma ($U \cdot mg^{-1}$)	0.80	26.2
After incubation with apoprotein III antiserum*	0.04	0.16
After incubation with PLC**	<0.01	<0.01

Upper panel: functional characteristics of procoagulant activity assayed with normal and coagulation factor deficiency plasmas; lower panel*: thromboplastin activity neutralization was accomplished by incubating samples for 1 h at 37°C with an equal volume of antiapoprotein III antiserum in 1:25 dilution; **: phospholipase C sensitivity; samples were incubated for 10 min at 37°C with PLC 5 $\mu g \cdot ml^{-1}$ final concentration. Each value is the mean of two experiments.

These data suggest that most of the LAM-associated procoagulant activity acts *via* the extrinsic pathway (factor VII dependent) and is immunochemically related to or identical to thromboplastin (tissue factor). Assuming this, conversion of the recalcification times for alveolar macrophages in normal plasma to thromboplastin units using a standard human brain thromboplastin preparation gave specific thromboplastin activities of 0.75±0.09 and 0.82±0.14 units per mg cell protein for smokers and non-smokers, respectively.

Procoagulant activity of bronchoalveolar lavage fluid

Bronchoalveolar lavage fluid from which alveolar macrophages have been removed by centrifugation contains a procoagulant activity which is sedimentable and totally recovered at 100,000 g for 1 h in the ultracentrifuge, but fails to sediment at 5000 g and 15000 g (data not shown). As a mean 61% of the procoagulant activity recovered after bronchoalveolar lavage resided with the alveolar macrophages and 39% with the high velocity sedimentable material. Characterization of the procoagulant by means of deficiency plasmas, gave exactly the same profile as shown for alveolar macrophages (table 1) which means that most of the activity is accounted for by thromboplastin or thromboplastin/factor VII complexes. The same observation also seems to be valid when BAL fluid from patients with high levels of procoagulant activities is examined (data not shown). The specific thromboplastin activity in BAL ultrasediment was increased about 20-fold when compared with homogenized lung alveolar macrophage material (table 2).

Table 2. – Relative specific activity of subcellular markers in homogenized human alveolar macrophages and ultrasedimentable material from cell-free bronchoalveolar lavage fluid. The values given are the specific activity of each enzyme/marker in BALF ultrasediment related to the specific activity of the same enzymes in homogenized LAM

Enzyme/marker	Subcellular fraction	Relative specific activity
Phosphodiesterase I	Plasma membranes	29.50±8.53
Alkaline phosphatase	Plasma membranes	38.13±10.52
Thromboplastin	Plasma membranes	19.87±6.99
1D5 antigen	Plasma membranes	39.75±8.27
Catalase	Peroxisomes	0.00
Esterase	Microsomes	0.00
β-acetylglucos-aminidase	Lysosomes	0.03±0.02
Acid phosphatase	Lysosomes	0.06±0.02
Succinic dehydrogenase	Mitochondria	0.08±0.03
Lactate dehydrogenase	Cytosol	0.27±0.05

Values are the means (±SEM) of duplicate analyses from 2–4 experiments.

Characterization of BALF ultrasediment

Electron microscopy of sections from BALF ultrasediment consisted almost exclusively of vesicles with the appearance of plasma membranes (fig. 1) with only a few scattered mitochondria and remnants of endoplasmic reticulum. Lamellar bodies probably derived from pneumocytes type II were only occasionally seen, as lamellar bodies are transformed into tubular myelin once they are released into the alveoli [34]. The membrane material was at least partly derived from alveolar macrophages. This was shown by incubating BALF material with the radioiodinated monoclonal antibody to a monocyte membrane antigen (1D5) which is also reactive with human alveolar macrophages (Gaudernack, personal communication). Further, determination of phosphodiesterase I and alkaline phosphatase activity as additional plasma membrane markers were performed. The 1D5 antigens and phosphodiesterase I partitioned

between alveolar macrophages and BAL fluid in about the same ratio as did thromboplastin activity (66/34% for 1D5 and 55/45% for phosphodiesterase I, mean of three different experiments). The specific activity of each membrane marker or enzyme was calculated as the enzyme activity in relation to the amount of protein in BALF ultrasediment and LAM homogenate, respectively. The relative specific activity of each enzyme was calculated as the specific enzyme activity in BALF ultrasediment related to the specific activity of the same enzymes in homogenates of LAM. The specific activity of the applied plasma membrane markers in the BALF ultrasediment were 20–40 fold above that in LAM homogenates (table 2).

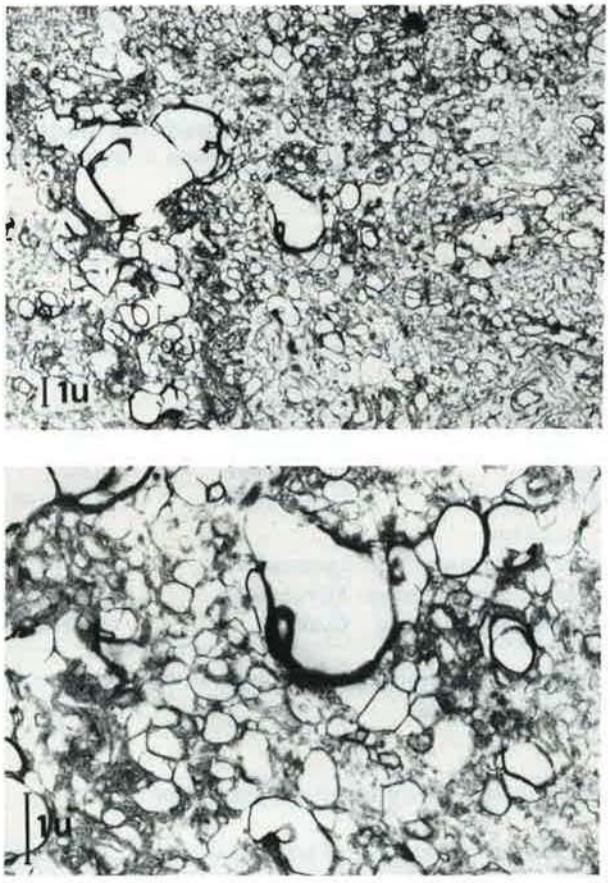

Fig. 1. – Electron micrographs of bronchoalveolar lavage fluid ultrasediment (100,000 g, 1 h) showing mainly vesicles with the appearance of plasma membrane material.

The content of subcellular structures in BALF ultrasediment other than plasma membranes was monitored by measurement of different enzyme markers. The recovery in BALF ultrasediment of succinic dehydrogenase (mitochondria), β-acetylglucosaminidase and acid phosphatase (lysosomes), catalase (peroxisomes), esterase (microsomes) and lactate dehydrogenase (cytosol) was very small (<1%). In contrast to the membrane markers, the specific activity of the other subcellular markers were negligible in BALF ultrapellet compared with homogenized lung alveolar macrophage material (table 2), indicating low contamination with these subcellular structures which was also confirmed by electron microscopy.

Discussion

It is well documented that human monocytes upon appropriate stimulation initiate coagulation by generating and expressing thromboplastin activity. Other procoagulants such as factor X activator [35, 36] and prothrombinase [37, 38] have been described but are not so extensively studied. Alveolar macrophages are part of the mononuclear phagocyte system and share a common bone marrow progenitor cell with the monocytes [39]. Tissue thromboplastin activities have been demonstrated in human alveolar lining fluid and are expressed by alveolar macrophages [9, 40–42]. Functional heterogeneity with respect to procoagulant activity expression has been demonstrated both in rabbits [15, 43] and in man [42]. In the human system the specific thromboplastin activity of LAM subpopulations varied inversely with their density, and low-density subpopulations of LAM (believed to represent the most differentiated cells) showed a high specific thromboplastin activity [42]. Unlike blood monocytes, LAM seem to have a limited capacity for thromboplastin induction *in vitro* [42] and it has been inferred that the alveolar compartment is functionally saturated with active complexes of thromboplastin/factor VII, and that the procoagulant pathway is regulated by the availability of distal clotting factors necessary for thrombin formation [11, 41].

Due to the hydrophobicity of apoprotein III (the protein component of thromboplastin) thromboplastin is firmly anchored to plasma membranes and has the characteristics of an integral membrane protein which demands detergents for its extraction. Direct evidence that most of the thromboplastin activity in stimulated monocytes is associated with a purified membrane preparation has been presented by HETLAND *et al.* [44].

According to this, thromboplastin is, as a rule, not released from simulated monocytes. However, monocytes stimulated with phorbol ester (TPA) have a specially high surface availability of thromboplastin and TPA is so far the only known stimulant which causes release of considerable amounts of thromboplastin to the medium where it is associated with membrane vesicles which sediment at 100,000 **g** for 1 h and which are possibly shed from the cellular surface [44]. Microvesicular release of membrane components has been observed in a variety of cells and may represent a common biological phenomenon [45]. Procoagulant activity has been demonstrated in membrane vesicles shed from several types of tumour cell lines [46, 47] and alveolar macrophages [15, 42] cultured *in vitro*. TPA is further known as a substance causing terminal differentiation of several cell types including immature cells of the monocyte/macrophage lineage. Terminal differentiation by TPA of human promyelocytic cell line HL-60 as well as the histiocytic cell line U-937 is associated with increased thromboplastin activity [48–50], and shedding of thromboplastin activity from HL-60 cells has been observed [51].

The present experiments demonstrate that both alveolar macrophages and cell-free bronchoalveolar lavage fluids contain procoagulant activities which seem to have the same characteristics as judged by their profiles in clotting factor deficiency plasmas. Most of the procoagulant activity is identified as thromboplastin based upon the absence of its procoagulant activity in factor VII deficient plasma and loss of activity following PLC-incubation and treatment with antibodies to apoprotein III. However, part of the procoagulant seem to act directly on factor X independent of factor VII. This activity probably represents a thromboplastin/factor VII complex because it is sedimentable in the ultracentrifuge and completely sensitive to PLC. Similar complexes have been reported by SHANDS [36] for murine peritoneal macrophages and MAIER and ULEVITCH [35] for rabbit hepatic macrophages.

The specific thromboplastin activity of BALF ultrasedimentable material was regularly increased about 20 times compared with homogenized LAM and the absolute values for specific activity corresponded closely to those recorded for purified plasma membranes from unstimulated monocytes [44]. These results suggest that most of the thromboplastin activity must be regarded as a definite plasma membrane constituent. Phosphodiesterase I is a cytoplasmic membrane marker which is present in human alveolar macrophages [52]. The monoclonal 1D5 antibody binds exclusively to monocytes and macrophages (including LAM) with high avidity and with a very high binding constant [27, G. Gaudernack, unpublished work]. Both these membrane markers as well as alkaline phosphatase showed specific activities which were 30–40 times higher in BALF ultrasediment than in LAM, and thus of the same order of magnitude as recorded for thromboplastin. The copurification of these membrane markers (thromboplastin, 1D5, phosphodiesterase I and alkaline phosphatase), the low contamination with markers of other subcellular structures and the electron microscopy give strong evidence that the majority of sedimentable material in BAL fluid consists of membraneous material which for a large part is derived from alveolar macrophages. Similar results have been obtained by MCGEE and ROTHBERGER in a rabbit system [15]. The procoagulant microvesicles found in BALF may well be derived from the surface of alveolar macrophages by a shedding process. Fractionation of LAM based on density separation suggests that this kind of shedding process only takes place in the low-density fractions, *i.e.* the most differentiated LAM [42, 53]. It is therefore tempting to speculate that the shedding process observed after TPA stimulation and differentiation of monocytes *in vitro* is comparable to the processes which seem to occur naturally by lung macrophage differentiation *in vivo*.

The present findings suggest that even under physiologic conditions alveolar macrophages are able to shed microvesicles from their surface. The distribution of these microvesicles in the alveolar lining fluid may contribute to the lipid-protein complexes normally defined as surfactant, and which facilitates a decrease in surface tension when the lungs deflate. Apart from the localized procoagulant activity of macrophages *in situ*, the high level of thromboplastin activity in shedded microvesicles may be taken to mean that the alveoli are endowed with a more widespread protective barrier with potential procoagulant activity and with the ability to trigger the coagulation of extravasated clotting factors and the formation of fibrin in inflammatory lesions. Several diseases of the lung have been shown to cause the leakage of plasma proteins, including the clotting factors into the alveolar space [54].

Intraalveolar and interstitial fibrin deposition commonly occurs in the lungs of patients with the adult respiratory distress syndrome (ARDS) [5]. The presence of active procoagulants [40] together with increased alveolocapillary leak of clotting factors [55] may explain the formation of hyaline membranes on bronchoalveolar surfaces of patients with ARDS [11] and in animal models of acute lung injury [6–8, 12]. Similar mechanisms may also be involved, but to a more limited extent, in the pathogenesis of interstitial lung disease like idiopathic pulmonary fibrosis and sarcoidosis [10, 11, 13, 56]. Unsuccessful dissolution of fibrin deposits may be due to imbalance between expression of procoagulant and fibrinolytic activities. Fibrin and fibronectin deposits are likely to provide a chemotactic stimulus for fibroblast recruitment and a provisional matrix for fibroblast adhesion and replication [57]. Fibroblast ingrowth into such fibrin deposits may have important consequences for subsequent scar formation and promote the fibrosis known to complicate interstitial lung diseases.

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L'activité procoagulante (thromboplastine) des liquides de lavages broncho-alvéolaires humains provient des macrophages alvéolaires. T. Lyberg, B. Nakstad, Ø. Hetland, N.P. Boye.
RÉSUMÉ: Les dépôts de fibrine dans l'espace alvéolaire et dans l'interstitium pulmonaires sont une caractéristique dominante de nombreux types de maladies pulmonaires inflammatoires. Les cellules de la lignée monocytaire macrophagique sont les principales cellules assurant l'activité procoagulante dans les lésions inflammatoires. Dans l'étude actuelle, nous avons trouvé que, aussi bien les macrophages alvéolaires pulmonaires (LAM) que les liquides de lavages broncho-alvéolaires (BALF) provenant d'humains, avaient une activité procoagulante. L'activité procoagulante du BALF est associée à la présence de vésicules membranaires qui sédimentent à 100.000 g pendant 1 heure. En microscopie électronique, l'ultrasédiment du BALF consiste quasi exclusivement en matériel de membrane et ceci a été confirmé par l'étude du contenu de différents enzymes marqueurs pour des structures subcellulaires spécifiques. En utilisant des marqueurs de membrane macrophagiques, au moins une partie de l'ultrasédiment du BALF paraît provenir de macrophages alvéolaires pulmonaires. L'activité procoagulante, associée aux macrophages alvéolaires et au liquide de lavage, est dentifiée comme due à la thromboplastine (facteur tissulaire) ou à des complexes thromboplastine - facteur VII, sur la base de leur sensibilité à la phospholipase C, de la neutralisation des anticorps et du site d'action du procoagulant dans l'activité séquentielle des facteurs de coagulation. Ceci suggère que les macrophages alvéolaires et les microvésicules contenant la thromboplastine provenant de ces macrophages, peuvent contribuer aux dépôts intra-alvéolaires et interstitiels de fibrine in vivo, et probablement aussi avoir des conséquences sur le développement de la fibrose pulmonaire.
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