Annexin I Degradation in Bronchoalveolar Lavage Fluids from Healthy Smokers: A Possible Mechanism of Inflammation

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ABSTRACT
Annexin I is a glucocorticoid-inducible, phospholipase A2-inhibitory protein and is proposed to have an anti-inflammatory role. Although annexin I is a cytosolic protein, it is found extracellularly in secreted fluids such as semen. We have examined the expression of annexin I in bronchoalveolar lavage fluids (BALF) from smokers and nonsmokers to investigate the role of annexin I in the airway. We find that annexin I is secreted in BALF. This secretion is not due to cell death or damage, because a cytosolic protein, 3-phosphoglycerate kinase, is not seen in BALF. We observed that BALF from smokers \( (n = 10) \) had high protein concentrations as compared with BALF from nonsmokers \( (n = 11) \). Annexin I levels were higher in BALF from smokers compared with nonsmokers. However, in smokers, annexin I was exclusively found in the \( M_r 34,000 \) form that lacks the \( M_r 3,000 \) N-terminal anti-inflammatory peptide. In nonsmokers, both the \( M_r 37,000 \) native annexin I and the \( M_r 34,000 \) proteolytically cleaved form are present, with the \( M_r 37,000 \) form being most abundant. The \( NH_2 \)-terminal \( M_r 3,000 \) peptide of annexin I exhibits anti-inflammatory actions (G. Cirino et al., Br. J. Pharmacol., 108: 573–574, 1993). Previous studies have implicated neutrophil elastase as the protease cleaving annexin I to the \( M_r 34,000 \) protein. We observed increased elastase levels in BALF from smokers. However, we find no correlation between bronchial sample percent of neutrophils in BALF and the relative amount of the \( M_r 34,000 \) band generated. Our data clearly demonstrate that annexin I is degraded in BALF from smokers, and we propose that proteolytic cleavage of annexin I in BALF from smokers may be a mechanism by which polymorphonuclear neutrophils infiltrate sites of inflammation; thus, inactivation of annexin I in smokers’ lungs may lead to chronic and uncontrolled inflammation.

INTRODUCTION
Increased retention of neutrophils in the pulmonary microvasculature of cigarette smokers is proposed to contribute to the chronic airway and alveolar inflammation believed to be important in the pathogenesis of chronic obstructive pulmonary disease (1–5). Annexin I, also referred to as lipocortin I, is a \( M_r 37,000 \) protein and member of a family of proteins that share sequence homology and bind membrane phospholipids in a calcium-dependent manner (6–9). Annexin I has been suggested to possess anti-inflammatory properties \( in vitro \) and may mediate some of the anti-inflammatory actions of glucocorticoids \( in vivo \) (10–16). Perretti and Flower (17) have shown in a murine air pouch model of inflammation that recombinant human annexin I (lipocortin I) inhibits neutrophil migration induced by interleukin 1. Drost et al. (18) demonstrated that recombinant human annexin I increases neutrophil deformability without concomitant changes in F-actin or neutrophil priming. Recently, the \( NH_2 \)-terminal portions of both mouse and human annexin I have been shown to interfere with activated neutrophil rolling and adhesion, probably by a non-CD11b mechanism such as inhibition of selectins (19). These studies suggest an extracellular (outer surface membrane) site of action for annexin I on neutrophil kinetics.

The native \( (M_r 37,000) \) form of annexin I has been recovered from BAL\(^4\) fluid of humans (20, 21). The source(s) of this protein in human BAL fluid is unknown, although \( in vitro \), annexin I protein has been detected in a variety of cells normally recovered from BAL fluid such as neutrophils, alveolar macrophages, and alveolar epithelial cells. Increased expression of intracellular annexin I by Western blot analysis has recently been shown in bovine bronchial epithelial cells grown in the presence of dexamethasone (22). Smith et al. (21) have demonstrated that the native or \( M_r 37,000 \) annexin I can be proteolytically cleaved to the \( M_r 34,000 \) protein \( in vitro \) by the action of human neutrophil elastase.

If annexin I is an anti-inflammatory protein that is capable of altering neutrophil chemotaxis at sites of inflammation, then it is reasonable to suggest that its extracellular expression may be up-regulated in response to inflammatory stimuli. In the present study, we proposed that increased expression of annexin I protein would be present in the bronchial lavage fluid recovered from otherwise healthy cigarette smokers compared with normal nonsmoking adults. We suggested that the annexin I

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4 The abbreviations used are: BAL, bronchoalveolar lavage; BALF, BAL fluid.
present in the lavage fluid of smokers would not only be quantitatively different from that detected in nonsmokers but would also predominately be in the M, 34,000 form of the protein, if indeed greater proteolytic cleavage of native annexin I occurred in the lower respiratory tract of current cigarette smokers. The relative amount of M, 34,000 protein may then correlate with the extent of bronchial inflammation and/or elastase burden present in bronchial lavage samples. Such findings could be consistent with a functionally less active form of annexin I in the airways of smokers and may provide another mechanism for the chronic polymorphonuclear inflammation observed in the airways of some such individuals.

To test this hypothesis, we obtained the bronchial portions of BALF by bronchoscopy from 11 normal nonsmoking adults and 10 otherwise normal smokers and determined inflammatory cell counts, total quantitative elastase, and expression of annexin I by Western blot analysis. To determine whether bronchial inflammation not induced by cigarette smoke results in a different pattern of annexin I expression than that which is present in smokers, we compared annexin I protein by Western blot in bronchial lavage fluid recovered from five nonsmokers who underwent a single bronchoscopy with BAL and six other nonsmokers who underwent their second bronchoscopy with BAL within 24 h of their initial bronchoscopy. Samples were obtained in this manner, because repeated bronchoscopy within this time interval has been reported to induce neutrophilic inflammation in the lower respiratory tract.

MATERIALS AND METHODS

Collection of BALF. Eleven normal nonsmoking volunteers and 10 otherwise normal cigarette smokers participated in a clinical protocol approved by the institutional review board at the University of Nebraska Medical Center. After informed consent, these subjects all underwent flexible fiberoptic bronchoscopy and BAL, following the methods outlined previously by Reynolds and Newball (23). An Olympus or equivalent bronchoscope (Olympus Corporation of America, New Hyde Park, NY) was used. Briefly, all subjects had an iv. line inserted by Reynolds and Newball (23). An Olympus or equivalent bronchoscope (Olympus Corporation of America, New Hyde Park, NY) was used. Briefly, all subjects had an iv. line inserted. iv. premedication consisted of atropine and diazepam. In all cases, bronchoscopy was performed transorally with anesthesia. iv. premedication consisted of atropine and diazepam. In all cases, bronchoscopy was performed transorally with anesthesia. Initially, the tracheobronchial tree down to subsegmental bronchi. The bronchoscope was then wedged into a segmental or subsegmental bronchus in three separate lobes for the BAL procedure. BAL was performed by infusing five 20-ml aliquots of sterile normal saline at each of the three sites (total, 300 ml) and suctioning return fluid through the suction channel of the bronchoscope. The fluid return from the first aliquot at each site were collected and processed separately from the subsequent four aliquots of infused saline, because this method results in a first return that is enriched for "bronchial" contents (24). Because this initial return was enriched for bronchial material, it was termed the "bronchial" portion of the BAL fluid. In this protocol, up to two additional sites were lavaged with single 20-ml aliquots for additional bronchial BAL samples. These samples were pooled with the other bronchial samples for subsequent analysis. Cells were separated from lavage fluid by centrifugation (300 × g for 5 min), with supernatants stored and frozen at −80°C until used further. Total cell counts were performed on cellular components, and evaluation of cell differentials was performed by a modified Wright-Giemsa stain after cytocentrifuge preparation.

Some of the nonsmoking subjects received a second bronchoscopy 24 h later. Those subjects receiving the second bronchoscopy are indicated by the number 2 after the patient number in all of the figures in this publication. (For example, subject 2847.1 represents the first bronchoscopy of subject 2847, and 2847.2 indicates the second bronchoscopy of the same subject.)

Antibodies and Recombinant Annexin I. Rabbit anti-human annexin I antisera (α 646) was a gift from Dr. Blake Pepinsky (Biogen, Boston, MA). Anti-human 3-phosphoglycerate kinase antiserum was produced in New Zealand White rabbits as described elsewhere (25). Purified recombinant human annexin I was kindly provided to us by Dr. Wonhwa Cho (University of Illinois at Chicago, Chicago, IL).

Protein Determination. Protein concentration in each sample was determined using the bicinchoninic acid assay (Pierce, Rockford, IL).

Gel Electrophoresis and Immunoblotting. Proteins were resolved on 4–15% polyacrylamide gels under denaturing conditions and electrotransferred onto Immobilon-P (Milipore Corp., Bedford, MA) transfer membranes as described previously (25). The blots were soaked for 30 min in blocking buffer (150 mM NaCl, 15 mM Tris-HCl, pH 7.5), containing 1% BSA, and then incubated for 3 h at room temperature with the primary antibody diluted appropriately in the blocking buffer. The membranes were washed three times with TBS (150 mM NaCl, 15 mM Tris-HCl, pH 7.5) containing 0.05% NP40 and once with TBS. Subsequently, the blots were incubated with 0.25 μCi/ml of 125I-labeled Staphylococcus aureus protein A (NEN Research Products, Boston, MA) in TBS containing 5% BSA for 45 min at room temperature. After three washes with 2× TBS-0.05% NP40 and one wash with 4× TBS, the blots were exposed to a Kodak X-Omat AR film with a Cronex-plus intensifying screen at −80°C. For quantitation of the immunoreactive band, the blot was scanned on a Betascope 603 (Betagen, CT) radioanalytical imager. We have found a correlation of 0.996 for the scanned data from Betascope, and the radioactivity was determined by counting the membrane pieces in a gamma counter.

Estimation of Total Quantitative Elastase. An indirect ELISA was used to evaluate elastase burden in the bronchial lavage fluid. Each well of the micro ELISA plates was coated with 2 μg/ml of purified human neutrophil elastase (Elastin Products Co., Pacific, MO). Serial dilutions of purified human elastase (0.5 μg/ml) was also used as a standard. Undiluted bronchial lavage fluid (167 μl) was added to each well, along with rabbit anti-human elastase (Sigma Chemical Co., St. Louis, MO) for a 30-min incubation. Unbound material was directly transferred to flat-well plates coated with purified human elastase for an additional 30-min incubation, followed by PBS-Tween washes. To these plates were added goat anti-rabbit IgG conjugated with horseradish peroxidase (ICN ImmunoBiologicals, Lisle, IL) for a 90-min incubation, fol-
lowed by PBS-Tween washes and addition of 200 μl of peroxi-
dase substrate until a visible color change was evident. The reaction was stopped with 8 M sulfuric acid with the
chromophore produced read at 492 nm.

Statistical Analysis of Data. All data are expressed as
means ± SE. Statistical analysis was performed using one-way
ANOVA, followed by Duncan’s test (P < 0.05).

RESULTS

Elevated Protein Concentration in BALF from Smok-
ers BALF Collected from Healthy Smokers and Nonsmokers
Were Analyzed for Protein Content. A significant differ-
ence was observed between the protein content of the two
groups. Although individual variations were observed within
each group, BALF from smokers, as a group, had a 10- to
25-fold higher protein content (Fig. 1). This increase in protein
content of the BALF is not due to increased epithelial cell
permeability, cell death, or damage in smokers, as evidenced
by lack of detection of the cytosolic proteins phosphoglycerate
kinase and annexin II in these fluids by immunoblot analyses
(data not shown). Thus, the increase in protein concentration is
due to increased secretion of proteins from bronchial epithelial
cells. We did not observe significant differences between the
first and second bronchoscopy on protein concentrations in
BALF from nonsmokers.

Increased Secretion of Annexin I in BALF from Smok-
ers. We examined the secretion of annexin I in lavage fluids
by immunoblot analysis. Annexin I was readily detectable in
BALF from both nonsmokers and smokers. The level of annexin
I in BALF was determined by radioanalytical scanning of im-
munoblots. The data shown in Fig. 2 indicate a significant
increase in levels of annexin I in smokers’ BALF (2376 ± 456
cpm) compared with BALF from nonsmokers (670 ± 106). No
significant differences were observed in BALF from nonsmok-
ers undergoing first versus second bronchoscopy. We have
previously demonstrated the presence of annexin I in secretory/
columnar cells in bovine bronchial epithelia (22) and the in-
duction of annexin I secretion by dexamethasone. However, none
of the smokers and nonsmokers in our study group received any
steroid treatment. Thus, the increase in annexin I secretion may
be mediated by exposure to cigarette smoke.

Annexin I in BALF from Smokers Is Predominantly in
the M, 34,000 Form. When we examined the immunoblots of
annexin I in BALF from smokers (n = 9) and nonsmokers (n =
9), we found a striking change in the mobility of the immuno-
reactive protein band (Fig. 3). Although annexin I was present in
a Mr 37,000 form from exclusively or predominantly in non-
smokers’ BALF (Fig. 3B), we found a Mr 34,000 immunoreac-
tive form of annexin I exclusively in most of the smokers and
predominantly in two of the smokers’ BALF (Fig. 3A). The Mr
34,000 form retains immunoreactivity to the polyclonal antisera
we used in these experiments. We did not detect immuno-
reactivity of a Mr 3,000 or other smaller proteolytic products.

Elastase Levels and Neutrophil Counts in BALF from
Smokers and Nonsmokers. Total quantitative elastase levels
were determined by ELISA techniques. The data shown in Fig.
4 indicate significantly higher levels of elastase in BALF from
smokers. Although differences were observed between individ-
uals in each group, the elastase levels were 2- to 11-fold higher
in smokers’ BALF. We did not measure the elastolytic activity
in BALF.

The total cell and percentage of neutrophil counts were
determined in smokers’ and non-smokers’ BALF. The data
shown in Table 1 indicate an increase in total cell count in
BALF from smokers. Among the nonsmokers, there was not a
significant increase in total cell number between the first bron-
choscopv and the second bronchoscopy. However, there was a
significant increase in the percentage of neutrophil content,
indicating the presence of significant inflammation in these
subjects as a result of the first bronchoscopy.

Lack of in Vitro Degradation of Annexin I by BALF.
We examined whether the proteolytic activity persists in the
smokers’ BALF and whether this proteolytic activity can be
measured in vitro using purified annexin I. Purified recombinant
human annexin I was incubated with BALF from a smoker and
a nonsmoker in the presence or absence of protease inhibitors.
The data shown in Fig. 5 indicate lack of any proteolytic activity
with annexin I as a substrate in either the smoker’s or nonsmok-
er’s BALF.
Annexin I in BALF

DIscussion

The annexins are a family of structurally related proteins that bind membrane phospholipids in a calcium-dependent manner. The annexin family of proteins have been implicated in multiple biological functions. Of these, the role of annexin I as an anti-inflammatory protein has become evident from investigations in recent years. Annexin I was originally thought to be a cytosolic protein and to exert its anti-inflammatory effects through inhibition of phospholipase A2. However, annexin I is known to be found at the cell surface and as a secreted protein. Putative cell surface binding sites for annexin I are present on both neutrophils and monocytes, with rapid surface binding of annexin I observed within 5 min after its intravenous injection in mice (26). Murine leukocytes removed from air-pouch and peritonitis models of inflammation have a markedly reduced ability to bind annexin I, and in patients with rheumatoid arthritis, a reduction in specific, saturable binding sites for annexin I has been reported (26, 27). Goulding and Guyre (28) reported that annexin I inhibited IgG binding to its Fc receptor on neutrophils and monocytes but not lymphocytes, suggesting it may modulate immune function. These observations have led to the suggestion by Goulding and Guyre (29) that annexin I may be a regulator of systemic inflammatory events.

We investigated the presence of annexin I BAL fluids from healthy smokers and nonsmokers. We found significantly higher secretion of proteins in BALF from smokers. Annexin I was detected with immunoblot analysis in BALF from both nonsmokers and smokers. We have also used RIA for detecting annexin I in BALF (data not shown). Previously, annexin I has been detected in human BAL fluid, with annexin I BAL concentrations increased both in normals and in patients with idiopathic pulmonary fibrosis after systemic administration of corticosteroids (20). We observed that annexin I concentration in BALF was higher in smokers than nonsmokers. Our data are consistent with the observation of van Hal et al. (30), who found increased annexin I in BALF from normal and asthmatic patients who smoked compared with their nonsmoking counterparts. However, they did not investigate the molecular form of annexin I present in the BALF from smokers. Annexin I production and secretion is increased as a response to corticosteroids (22, 31). However, our study population did not receive corticosteroids before or during these studies. Thus, increased annexin I is produced by airway epithelial cells as a response to cigarette smoke. This may be a natural defense mechanism to protect against cigarette smoke-induced inflammation. We did not find any significant difference in annexin I secretion between nonsmokers undergoing the first versus the second bronchoscopy. However, our data in Table 1 clearly indicate that subjects undergoing second bronchoscopy had inflammation as evidenced by the increased neutrophil content of their BAL.

Conclusions

This is further evidence that the increased production of annexin I in smokers’ BAL is caused by cigarette smoke. The speci-

Discretionary

Table 1 Total cell number and percentage of neutrophils in BALF from smokers and nonsmokers

<table>
<thead>
<tr>
<th>Group</th>
<th>Bronch</th>
<th>n</th>
<th>Total cells (X 10^6)</th>
<th>% neutrophils</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonsmokers</td>
<td>1</td>
<td>5</td>
<td>5.8 ± 3.5</td>
<td>3.2 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>6</td>
<td>4.8 ± 1.8</td>
<td>39 ± 2.9</td>
</tr>
<tr>
<td>Smokers</td>
<td>1</td>
<td>10</td>
<td>8.84 ± 3.7</td>
<td>7.25 ± 2.05</td>
</tr>
</tbody>
</table>

*From each individual, three bronchial washes were collected as indicated in “Materials and Methods.” These three bronchial washes were pooled, and the data represent the pool of the three bronchial washes.
Thus, the examined the percentage of neutrophil content in BAL cells. Thus, neutrophil elastase may not be the only radiated rabbit lungs. 

The difference in annexin I levels between sham-irradiated and irradiated rabbit lungs. The specificity of cigarette smoke induced annexin I is further supported by studies of Ts'ao et al. (32), who found no appreciable difference in annexin I levels between sham-irradiated and irradiated rabbit lungs.

Analysis of the immunoblots revealed that in smokers' BALF, annexin I was present as a proteolytically degraded M₄ 34,000 form compared with the predominance of the M₄ 37,000 native form in BALF from nonsmokers. This proteolytic degradation is significant because the M₄ 3,000 NH₂-terminal fragment has been shown to be anti-inflammatory (19, 27, 33). Thus, the M₄ 34,000 annexin I form in smokers' BALF cannot function as an anti-inflammatory protein. These findings, then, may be consistent with an augmentation of airway inflammation in smokers due to cleavage of annexin I to a less active form of the protein (21). Neutrophil elastase is one of the candidate proteases that cleaves annexin I to a M₄ 34,000 form. Thus, we examined the percentage of neutrophil content in BAL cells. Our data indicate no correlation between production of the M₄ 34,000 form of annexin I and the percentage of neutrophil content in BAL. Thus, neutrophil elastase may not be the only protease involved in this degradation, and additional proteases produced in response to cigarette smoke may be involved in the production of the M₄ 34,000 annexin I. However, we do not find any proteolytic activity in either the smoker's or nonsmoker's BALF ex vivo when purified annexin I was used as a substrate.

We propose a mechanism for the anti-inflammatory action of annexin I and the degradation of annexin I in cigarette smoke-induced inflammation (Fig. 6). In this mechanism, we propose that cigarette smoke induces the production of annexin I from bronchial epithelial cells and also stimulates the polymorphonuclear neutrophils to produce elastase and other proteases. The increased proteolytic activity results in degradation of the native annexin I (M₄ 37,000) to the cryptic annexin I (M₄ 34,000) that can no longer bind to the polymorphonuclear neutrophils. In addition to cigarette smoke, annexin I secretion is also induced by treatment of bronchial epithelial cells with glucocorticoids and by the increased production of cortisol as a result of injury or infection from the hypothalamic-pituitary-adrenal axis (22, 31).

In summary, we have demonstrated that annexin I is present in BALF, but the predominant form of annexin I in the smokers' BALF is the M₄ 34,000 protein that lacks the NH₂-terminal M₄ 3,000 fragment. This NH₂-terminal fragment is necessary for the anti-inflammatory action of annexin I. Thus, the M₄ 34,000 annexin I in the smokers' BALF is ineffective as an anti-inflammatory protein. The inactivation of annexin I by neutrophil elastase and/or additional proteases stimulated by cigarette smoke may be an important event in chronic inflammation observed in smokers' lungs.

REFERENCES


Fig. 5 Purified annexin I is not degraded by BALF in vitro. Purified recombinant human annexin I (2 μg) was incubated with 5 μl of BALF from a nonsmoker (Lanes 1–4) and a smoker (Lanes 5–8) in a total volume of 30 μl containing 65 mM HEPES (pH 7.5), 6.5% DMSO, and 13 mM NaCl. After incubation for 30 min at 37°C, the reactions were terminated with SDS-PAGE sample buffer. SDS-PAGE, immunoblotting, and autoradiography were performed as described in “Materials and Methods.” Lanes 4 and 8 did not have purified annexin I in the incubation mixture. Lanes 2 and 6 contained 1 mM phenylmethylsulfonyl fluoride, and Lanes 3 and 7 contained 1 mM EDTA in addition to the reaction mixture mentioned above. Lane 9 contained purified annexin I in the absence of any added BALF. The position of molecular weight markers run on the same gel are indicated.

Fig. 6 Proposed mechanism for production of annexin I from bronchial epithelial cells and the effect of cigarette smoke on annexin I secretion and degradation. BEC, bronchial epithelial cells; HPA, hypothalamic-pituitary-adrenal; PMN, polymorphonuclear neutrophils.

Fig. 6 Proposed mechanism for production of annexin I from bronchial epithelial cells and the effect of cigarette smoke on annexin I secretion and degradation. BEC, bronchial epithelial cells; HPA, hypothalamic-pituitary-adrenal; PMN, polymorphonuclear neutrophils.


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