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 Mr 34,000 form that lacks the Mr 3,000 N-terminal anti-inflammatory peptide. In nonsmokers, both the Mr 37,000 native annexin I and the Mr 34,000 proteolytically cleaved form are present, with the Mr 37,000 form being most abundant. The NH2-terminal Mr 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 that backs the proteolytic cleavage of annexin I 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 Mr 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). Ferroni 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 NH2-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 (Mr 37,000) form of annexin I has been recovered from BAL 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 Mr 37,000 form of annexin I can be proteolytically cleaved to the Mr 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 predominantly be in the M₃ 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 M₃ 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 i.v. line inserted for delivery of i.v. medication, followed by inhalation of aerosolized albuterol as an airway bronchodilator prior to bronchoscopy. Subjects were then p.o. sprayed with lidocaine for local anesthesia. i.v. remedication consisted of atropine and diazepam. In all cases, bronchoscopy was performed transorally with initial examination of 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 antiserum (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 by us to 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 (Millipore 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) radiographical 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 ImmunoBiologics, Lisle, IL) for a 90-min incubation, fol-
lowed by PBS-Tween washes and addition of 200 µl of peroxidase 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 Smokers BALF Collected from Healthy Smokers and Nonsmokers Were Analyzed for Protein Content.** A significant difference 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 Smokers.** 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 immunoblots. The data shown in Fig. 2 indicate a significant increase in levels of annexin I in smokers’ BALF (2376 ± 456 cpmp) compared with BALF from nonsmokers (670 ± 106). No significant differences were observed in BALF from nonsmokers 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 induction 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 M₉ 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 immunoreactive protein band (Fig. 3). Although annexin I was present in a M₉ 37,000 form from exclusively or predominantly in nonsmokers’ BALF (Fig. 3B), we found a M₉ 34,000 immunoreactive form of annexin I exclusively in most of the smokers and predominantly in two of the smokers’ BALF (Fig. 3A). The M₉ 34,000 form retains immunoreactivity to the polyclonal antisera we used in these experiments. We did not detect immunoreactivity of a M₉ 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 individuals 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 bronchoscopy 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 nonsmoker’s 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 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 i.v. 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 non-smokers 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 fluid. This is further evidence that the increased production of annexin I in smokers’ BALF is caused by cigarette smoke. The speci-
Thus, the Mr examined the percentage of neutrophil content in BAL cells. The difference in annexin I levels between sham-irradiated and irradiated rats 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 Mr, 34,000 form compared with the predominance of the Mr, 37,000 native form in BALF from nonsmokers. This proteolytic degradation is significant because the Mr, 3,000 NH2-terminal fragment has been shown to be anti-inflammatory (19, 27, 33). Thus, the Mr, 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 34,000 protein that lacks the NH2-terminal Mr, 3,000 fragment. This NH2-terminal fragment is necessary for the anti-inflammatory action of annexin I. Thus, the Mr, 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.

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 Mr, 34,000 protein that lacks the NH2-terminal Mr, 3,000 fragment. This Nh2-terminal fragment is necessary for the anti-inflammatory action of annexin I. Thus, the Mr, 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.

**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 HEPS (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 phenylmethyisulfonyl 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.

(22) and by the increased production of cortisol as a result of injury or infection from the hypothalamic-pituitary-adrenal axis (22, 31).

**REFERENCES**


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