Lung type II cell and macrophage annexin I release: differential effects of two glucocorticoids

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Annexin I is a glucocorticoid-inducible member of the annexin family of 13 structurally related proteins (26). It has anti-inflammatory, immunosuppressive, and anti-proliferative effects in a number of in vivo and in vitro models and appears to mediate some of the anti-inflammatory actions of exogenous glucocorticoids (reviewed in Ref. 1).

Specific binding sites for annexin I have been localized on the plasma membranes of a number of cell types, including peripheral blood leukocytes (16, 17). Neutralizing antibodies to annexin I (which do not readily penetrate living cells) inhibit its anti-inflammatory activity (e.g., Ref. 14), suggesting that it requires release, probably followed by receptor occupancy and activation, for biological activity. In A549 cells, an epithelial carcinoma cell line, annexin I binding inhibits its activation of cytosolic phospholipase A2 activity, which, in turn, suppresses phospholipase A2-dependent inflammatory processes such as eicosanoid production (9, 10). Annexin I is particularly abundant in the lung, suggesting that it has an important physiological function in this organ (41). Furthermore, induction of annexin I may contribute to the anti-inflammatory actions of glucocorticoids in the respiratory tract (13, 29). In addition to its putative anti-inflammatory action, annexin I (also called phospholipid binding protein) may play a role in the regulation of pulmonary surfactant synthesis and secretion by alveolar epithelial type II (ATII) cells (11, 35, 36). However, the ATII cell is only one of a number of cell types present in the respiratory tract that contains annexin I. Others include a variety of epithelial and tracheal gland cells (19, 21), neutrophils (16), and alveolar macrophages (AMS) (2). Annexin I is also present extracellularly in the epithelial lining fluid of the rabbit lung (25) and secretions from all levels of the human respiratory tract (32). At least two of the cell types present in the peripheral lung, ATII cells and AMS, can secrete the protein and thus could contribute to the extracellular annexin I pool (2, 3, 31), although the relative contribution of annexin I released from ATII cells and AMS is unclear. Neither is it known whether the relative contributions from these cells change with disease status or glucocorticoid administration. Investigations utilizing bronchoalveolar lavage (BAL) fluid (BALF) have shown that concentrations of annexin I within BAL cells (predominantly AMS) increase after therapeutic doses of oral glucocorticoid (13); whether the AM is responsible for the parallel rise in extracellular annexin I in BALF is unknown (4, 31). However, the increase in cellular and extracellular annexin I further supports the view that this mediator may contribute to the anti-inflammatory effects of oral glucocorticoids in the human lung. Whether inhaled glucocorticoids such as budesonide (which are used extensively for the treatment of asthma) induce a similar response has not been adequately investigated.

Therefore, a primary aim of the present study was to establish the effect of an inhaled anti-inflammatory glucocorticoid, budesonide, on the release of annexin I in the respiratory tract. Thus we measured annexin I in BALF from asthmatic patients after a short course of inhaled budesonide or a placebo. We hypothesized that both AMS and ATII cells secrete annexin I into the epithelial lining fluid and that annexin I secretion by...
these cells is upregulated by budesonide. To test this, we quantified the release, ex vivo, of annexin I from ATII cells and AMs in the presence and absence of budesonide. In addition, we compared the effect of budesonide on annexin I release with that of dexamethasone, a synthetic glucocorticoid that Smith et al. (29) showed stimulates release of annexin I into pulmonary epithelial lining fluid in vivo.

METHODS

In Vivo Study

Patients. The study was carried out on 11 subjects with mild asthma [age 28.5 ± 1.4 (SE) yr; 6 men] receiving treatment only with the inhaled β₂-adrenergic agonist aerosol albuterol for relief of intermittent wheeze. All patients demonstrated a >15% improvement in forced expiratory volume in 1 s (FEV₁) after 200 µg of albuterol and an airway hyperresponsiveness to methacholine, with a provocative concentration of <4 mg/ml producing a 20% fall in FEV₁. All patients were atopic as defined by two or more positive skin prick tests to common allergens. None of the subjects studied had received any oral or inhaled glucocorticoid for the preceding 12 mo. All subjects were nonsmokers or exsmokers of ≥5 pack·yr, and all had an FEV₁ > 80% predicted.

Protocol. The study design was a double-blind, randomized crossover trial in which the subjects took budesonide (600 µg twice daily via a Turbhaler) or a placebo for 1 mo. The placebo Turbhaler appeared identical to the test device but contained only lactose powder. The washout time between each treatment period was 4 wk. A bronchial provocation test was performed on day 26 of each treatment period with a nebulized buffered isotonic methacholine solution (6). After an initial nebulized saline challenge, doubling doses of methacholine were administered via a dosimeter. The initial methacholine concentration was 0.06 mg/ml. Spirometric values were recorded 2 min after administration until a 20% fall in FEV₁ was achieved; the provocative concentration value was obtained from a concentration-response curve. BALF was collected on day 28. In brief, the tip of the bronchoscope was wedged at the level of the second or third generation of airways and 4 × 60-ml portions of warmed, sterile 0.15 M NaCl were sequentially instilled through the biopsy channel and immediately aspirated into the same sterile trap. The pooled aspirate was centrifuged at 300 g at 4°C for 15 min to remove the BAL cells. The cell-free supernatants were stored in aliquots at −20°C or below until analyzed. The study was approved by the Royal Brompton Hospital Ethics Committee (London, UK), and all patients gave their informed consent.

In Vitro Study

Protocol. The in vitro study was performed on AMs and ATII cells isolated from male Wistar rats 200 g in body weight, and bred in-house. The cells from two to six animals were pooled for each experiment; a total of nine experiments was performed.

Isolation of AMs. After death by an overdose of pentobarbital sodium, the lungs were perfused free of blood, dissected, and lavaged with normal saline as previously described (29). A sample of lavage fluid was taken for a total cell count while the remainder was centrifuged for 10 min at 300 g to pellet the lavage cells. The cells, which were identified as >95% AMs, were resuspended in low-protein hybridoma medium containing 2 mM glutamine, 100 µg penicillin/ml, and 60 µg streptomycin/ml. They were plated in 12-well plates at a density of 1.5 × 10⁶ cells/well or in 6-well plates at 3.5 × 10⁶ cells/well and incubated for 3 h in 95% O₂-5% CO₂ at 37°C.

Isolation of ATII cells. ATII cells were isolated from the lavaged lungs by the method of Bingle et al. (5). ATII cells from each experiment were cultured for 96 h, by which time they were confluent and stained positive for alkaline phosphatase. Transmission electron microscopy showed that the cells possessed microvilli on their apical surfaces and contained lamellar bodies, thus confirming their identity as ATII cells (Fig. 1).

Exposure of AMs and ATII cells to glucocorticoid. AMs were allowed to adhere for 3 h as described in Isolation of AMs; then the medium was changed, and adherent cells were exposed to either budesonide or dexamethasone (10⁻⁹ to 10⁻³ M) for 24 h. ATII cells were cultured for 96 h; then, after a change of medium, the confluent cells were cultured for a further 24 h in the presence of exogenous glucocorticoid.

In each repetition of the experiment, the effect of every treatment was determined on triplicate wells. Stock solutions of budesonide were dissolved in ethanol; the final concentration of vehicle in culture was 0.001%. Preliminary experiments showed that ethanol at the concentrations used in this study had no effect on annexin I levels or release in either AMs or ATII cells (data not shown). Stock solutions of dexamethasone sodium phosphate were dissolved in water. Throughout the study, the term “basal” refers to the appropriate vehicle control.

Preparation of samples for analysis of annexin I. The medium was removed, and the adherent cells (AM and ATII) were washed in Ca²⁺-free medium to remove any cell surface annexin I. The washings were pooled with the conditioned medium, then centrifuged at 300 g for 15 min to pellet any nonadherent cells. The supernatant was concentrated fivefold through a Centricon unit (molecular-mass cutoff, 3 kDa) and stored at −20°C until analysis.

Adherent and nonadherent cell pellets were lysed in 2% Nonidet P-40 in phosphate-buffered saline containing 10 mM EDTA and 1 mM phenylmethylsulfonyl fluoride. The lysates from adherent and nonadherent cells were pooled and centrifuged, and the supernatants containing the cellular annexin I were concentrated and stored as described above.

Analytic Techniques

Quantification of annexin I. Annexin I levels in BALFs, cultured cells, and conditioned medium were quantified with a previously described enzyme-linked immunosorbent assay (30). Human recombinant annexin I (0.625–40 ng/ml) was used as a standard.

Total protein assay. A bichoninic acid assay was used for the estimation of total protein in the cells. The assay was carried out on cell lysates according to the manufacturer’s instructions (Pierce and Warriner, Chester, UK), and bovine serum albumin (0.02–2 mg/ml) made up in 0.15 M NaCl, Hanks’ buffered salt solution, or low-protein hybridoma medium, as appropriate, was used as a standard.

Albumin assay. Albumin levels in BALF were quantified by rocket immunoelectrophoresis with specific antisera (DAKO, High Wycombe, UK) and standardized against standard human serum containing albumin of a known concentration (Behring, Hounslo, UK).

Statistics. The Wilcoxon signed rank test for paired data was used to evaluate the effects of glucocorticoid on annexin I in vivo and in vitro. The level of significance was P < 0.05. Data are means ± SE unless otherwise specified.
RESULTS

In Vivo Study

The BALF recovery was not significantly different after the placebo [49.5 ± 1.3% (SE), median 50%, range 42–58%] and budesonide (53.7 ± 2.8%, median 54%, range 31–62%; P = 0.167; n = 11 subjects). Neither the total protein nor albumin concentrations in the BALF were affected by budesonide treatment (protein after placebo: 166 ± 22 µg/ml, median 155 µg/ml, range 87–295 µg/ml; protein after budesonide: 171 ± 21 µg/ml, median 159 µg/ml, range 92–328 µg/ml; P = 0.657; n = 11 subjects; albumin after placebo: 55 ± 6 µg/ml, median 45 µg/ml, range 28–94 µg/ml; albumin after budesonide, 54 ± 6 µg/ml, median 45 µg/ml, range 28–94 µg/ml; P = 0.721; n = 11 subjects). Similarly, extracellular annexin I in BALF was not significantly different after budesonide treatment compared with after the placebo whether the data are expressed as nanograms of annexin I per milliliter of BALF (after placebo: 5.4 ± 0.7 ng/ml, median 5.7 ng/ml, range 1.4–9.0 ng/ml; after budesonide: 5.8 ± 1.0 ng/ml, median 7.1 ng/ml, range 1.6–9.7 ng/ml; P = 0.476; n = 11 subjects), nanograms of annexin I per milligram of protein (after placebo: 35 ± 5 ng/mg, median 31 ng/mg, range 19–70 ng/mg; after budesonide: 34 ± 6 ng/mg, median 27 ng/mg, range 10–72 ng/mg; P = 0.959; n = 11 subjects) or nanograms of annexin I per milligram of albumin (after placebo: 107 ± 16 ng/mg, median 90 ng/mg, range 44–203 ng/mg; after budesonide: 110 ± 20 ng/mg, median 89 ng/mg, range 31–226 ng/mg; P = 0.722; n = 11 subjects; Fig. 2).

In Vitro Study

Morphology and detachment of adhered AMs and ATII cells. Glucocorticoid treatment had no effect on the morphology of AMs or ATII cells at the light-microscopic level in the doses used in this experiment. Neither did it alter cell detachment, which was 1–2% of the cells for all conditions. After treatment with glucocorticoid, all adherent ATII cells and AMs were viable as assessed by exclusion of trypan blue (data not shown).

Total protein. Basal levels of total protein did not differ between AMs and ATII cells (AMs: 0.31 ± 0.10 mg protein/10^6 cells, n = 6 experiments; ATII cells: 0.28 ± 0.06 mg protein/10^6 cells, n = 5 experiments; P > 0.05). Dexamethasone had no significant effect on the total protein concentration of either cell type (Table 1). Treatment of AMs, but not of ATII cells, with 10^{-3} M budesonide caused a substantial and significant reduction in total protein. ATII cells were more responsive than AMs to lower concentrations of budesonide; small but significant falls in intracellular total protein were observed at 10^{-5} and 10^{-4} M budesonide (Table 1).

Quantification of annexin I. ALVEOLAR MACROPHAGES. Annexin I was present in all preparations of AMs examined (Fig. 3). The amount of extracellular annexin I was variable but averaged 28% of the total (cellular plus extracellular) annexin I content (Fig. 3).
Treatment with dexamethasone resulted in a dose-dependent increase in total annexin I levels, which was maximal and significant at $10^{-2}$ M (Fig. 4A). It was noted that at $10^{-5}$ M dexamethasone, intracellular annexin I was significantly increased, whereas at $10^{-3}$ M, increased extracellular protein was responsible for the overall increase in total annexin I (Fig. 4A).

In contrast, budesonide reduced total AM annexin I at the highest concentration tested and significantly suppressed its release into the medium at all concentrations of drug (Fig. 4B). Increased intracellular annexin I concentrations were observed at some concentrations of budesonide (Fig. 4B).

ATII cells. In the absence of exogenous glucocorticoid, annexin I was always present in ATII cells, although the concentrations varied considerably between preparations (Fig. 3). The amount of annexin I released into the medium also varied but averaged 27% of the total (cellular plus extracellular) value (Fig. 3). In the absence of glucocorticoid, the cellular content of annexin I and the amount of annexin I release did not differ significantly between ATII cells and AMs (Fig. 3).

Dexamethasone caused an increase in total annexin I levels that was significant at $10^{-5}$ M. Annexin I release was significantly greater than baseline release at $10^{-9}$ and $10^{-7}$ M dexamethasone (Fig. 5A).

In contrast, $10^{-9}$ M budesonide caused a significant fall in total annexin I. It had no effect on intracellular annexin I concentrations of ATII cells at any dose tested but was associated with a reduction in annexin I release at the lowest doses tested (Fig. 5B).

DISCUSSION

In this study, we have quantified annexin I content and release from two of the most abundant cell types in the peripheral lung, AMs and ATII cells. We found that under basal conditions, the two cell types contain and release equivalent amounts of annexin I. Thus, potentially, both cell types could contribute to the...
extracellular annexin I pool found in respiratory tract secretions (25, 32). Similarly, both cell types responded to dexamethasone with concentration-related increases in annexin I content and release, the ATII cell being particularly responsive. This agrees with previous studies that have shown that human subjects (4, 13, 31) and rodents (29) receiving oral glucocorticoid (prednisolone or dexamethasone) show an increase in cellular and extracellular levels of annexin I in the respiratory lumen. However, neither cell released more annexin I after treatment with budesonide. This observation complements the parallel in vivo study on allergic asthmatic patients, in which inhaled budesonide was found to have no effect on the extracellular annexin I content in BALF (Fig. 1), although it relieved asthmatic symptoms (23) in these patients. Our results provide new information that extends previous investigations in this field.

Published immunocytochemical studies indicate that, in the respiratory tract, annexin I is most abundant in bronchial gland cells (20), epithelial cells (15, 20, 25, 39), and phagocytes (15, 25), including AMs, and that...
these are the most likely cellular sources of the annexin I in airway and pulmonary secretions. Such studies of the lung are valuable indicators of the likely sources of annexin I in vivo but are purely qualitative. Although annexin I content and release have been assessed in AMs and ATII cells in vitro (2, 3, 24, 35), only one cell type was examined at a time in each of these earlier investigations. To our knowledge, this is the first comparative study of annexin I production by AMs and ATII cells. Thus we have been able to assess the relative contributions of these cell types to the total and extracellular annexin I pools within the lung. Under basal conditions, intracellular annexin I levels in AMs and ATII cells were similar, as was annexin I release (Fig. 2). However, because there are four to five times as many ATII cells as AMs in the peripheral lung of the normal rat (8, 18), it is likely that under basal conditions, ATII cells contribute more annexin I to the total lung pool than AMs. Such a conclusion is compatible with the data of Tsao et al. (35), who showed that rabbit ATII cells were enriched with phospholipid binding protein (another name for rabbit annexin I) and thus a plausible source of that protein in respiratory tract secretions. In the present study, the concentration of annexin I in both AMs and ATII cells was 10–15 ng/mg protein, similar to that in human AMs in vitro (31) but much lower than that in rabbit ATII cells (9 µg/mg protein) (35). This may reflect species differences in either the rates of production, metabolism, or clearance of annexin I or different antisem specificities.

Use of synthetic glucocorticoids highlighted differences in annexin I release between the two cell types that were undetectable under basal conditions. With respect to annexin I release, the ATII cell was more responsive than the AM to dexamethasone, having a lower threshold and a greater response. This suggests that, in vivo, at concentrations of dexamethasone that might be achieved clinically (≤10^{-7} M) (28), ATII cells, but not AMs, would respond with increased annexin I release. In contrast, in experimental studies, when higher doses of dexamethasone may be used, AMs may secrete (or leak) more annexin I into the epithelial lining fluid than ATII cells.

In contrast to dexamethasone, budesonide appeared to subdue annexin I release from both cell types. The reason for this is unknown. Because budesonide is not normally metabolized in the lung (7), it seems unlikely that it is being degraded by lung-derived cells in vitro. Both drugs are glucocorticoid-receptor (GR) agonists, with budesonide binding having an eightfold higher affinity than that for dexamethasone (12). Thus the data suggest that either budesonide is not activating GRs in AMs and ATII cells or that the action of dexamethasone on annexin I in AMs and ATII cells is GR independent. Studies with a GR antagonist, RU-38486, indicate that, in control animals, GR-independent pathways account for 50% of the annexin I in peripheral blood leukocytes (27). Administration of lipopolysaccharide to adrenalectomized rats (in which GR cannot be activated) increased annexin I in lung homogenates (33), confirming that expression of the protein can be modulated by GR-independent pathways.

Because, to our knowledge, this is the first study of this aspect of budesonide activity, we have nothing with which to compare our data. However, our in vitro study is compatible with our observations in vivo (Fig. 2). The lack of annexin I release in vivo may reflect the inability of inhaled budesonide to reach the peripheral lung, but our in vitro studies show that, even when treated directly with this drug, ATII cells and AMs do not secrete increased amounts of annexin I. Because all the patients in the in vivo study showed an improvement in bronchial hypersensitivity after the budesonide treatment period (23), the data support the hypothesis that the mechanism of action of this drug is independent of peripheral annexin I release. The airway epithelium may be a more important target for inhaled budesonide when this drug is used for its antiasthmatic properties, consequently suggesting that the action of budesonide on annexin I in airway epithelial cells and secretions merits investigation.

The effect of lung pathology on annexin I distribution and content is unknown, but changes in cell profile may alter the annexin I content of the lung. Smith et al. (31) and van Hal et al. (37) showed before that the extracellular annexin I concentrations in BALF from nonsmoking healthy and asthmatic volunteers were similar (~100 ng/mg albumin). In contrast, BALF from asthmatic smokers contained twice as much annexin I (~220 ng/mg albumin; 37). However, when standardized to AM number, extracellular annexin I was the same in smokers and nonsmokers, suggesting that the increase in the smokers' BALF was due to release from the increased number of AMs (37), supporting our hypothesis that annexin I concentration in BALF depends, in part, on cell profile.

ATII cell proliferation often occurs as part of a normal repair mechanism after inflammatory insult. For example, intratracheal instillation of rats with an inflammmagen, carrageenan, resulted in increased AM and ATII cell numbers (29) and a doubling in extracellular annexin I, suggesting a relationship between increased cell number and increased annexin I. Concurant treatment with oral dexamethasone (1 mg/kg) resulted in an eightfold increase in extracellular annexin I, although AM and ATII cell numbers were normal (29). The present study suggests that at the maximum dexamethasone plasma concentration probably achieved in that experiment (~10^{-6} M; 38), increased synthesis by both cell types contributed to the annexin I pool.

It has been suggested that the glucocorticoids upregulate annexin I gene expression via the "classic" mechanism of increased gene transcription (42); the appropriate response elements have been identified on both the rat and human annexin I genes (22). Posttranscriptional modifications (40) and intracellular redistribution of annexin I (34) may also be important glucocorticoid effects. Our data suggest that the action of glucocorticoids may also depend on the target cell and the specific drug employed.
In summary, we have shown that the ATII cell is likely to be a major cellular source of annexin I in secretions from the healthy peripheral lung under basal conditions and after clinical doses of dexametha-
sone. This study shows that the more numerous ATII cells are more responsive than AMs to dexamethasone with regard to annexin I release. We have also observed that only dexamethasone, not budesonide, can increase annexin I release by ATII cells and AMs in vitro. The effects of these glucocorticoids in vitro is consistent with their actions on annexin I release in the lung in vivo (Fig. 2) (29).

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