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Production of lipocortin-like proteins by cultured human tracheal submucosal gland cells

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Evidence is obtained for the presence of lipocortin-like proteins in human tracheal gland cells in culture. Using polyclonal antibodies to lipocortin I, indirect immunofluorescence studies demonstrate that lipocortin I is mainly confined to the tracheal gland cell surface. From cell membranes, four Ca^{2+}-dependent proteins (35, 40, 45 and 67 kDa) were identified as lipocortin related proteins by using immunoblotting and fluorography following [^{35}S]methionine metabolic labeling experiments. A strong immunoreactivity for the 35 kDa protein was observed. In addition, lipocortin-like proteins with apparent M_r33, 35, 37 and 67 kDa, respectively, were released in the apical culture medium by tracheal gland cells cultured on microporous membrane of a double chamber culture system.

Lipocortin; Human tracheal gland; Cell culture

1. INTRODUCTION

Lipocortins are a family of calcium and phospholopid binding proteins described initially as glucocorticoid-induced and secreted proteins inhibiting phospholipase A_2 in vitro and in vivo [1-15], and thus preventing the release of inflammatory mediators, prostaglandin E_2 and leukotrienes. Human respiratory diseases including acute and chronic bronchitis, asthma and cystic fibrosis are associated with excessive airway mucus production, mainly originating from submucosal gland secretory cells [6,7]. Mechanisms possibly responsible for chronic mucus hypersecretion involve the increase in total number of mucus-producing cells [6] and/or the secretory effects of a number of locally released mediators including arachidonic acid metabolites and prostaglandins [8,9] as well as mediators released from activated inflammatory cells [10]. Organ cultures (explants) of human [8] and cat [11] trachea, as well as human tracheal epithelium-cell cultures [12-15] generate numerous metabolites of arachidonic acid, predominantly prostaglandin E_2 and 15-hydroxyeicosatetraenoic acid (15-HETE) which have powerful stimulatory effects on respiratory glycoconjugate secretion [8,9]. Glucocorticoids have been shown to inhibit secretion of mucus in asthmatic airways [16,17] and in in vitro experiments using human and cat airway explants [18,19]. Lundgren et al. [19] speculated that the inhibitory action of dexamethasone on mucus glycoprotein secretion is mainly due to the inhibition of arachidonic acid product formation through the induction of lipocortin synthesis. Nevertheless, using whole airway explant cultures, it is impossible to know the cell types or tissues (surface ciliated cells, goblet cells, submucosal gland cells, migratory cells, vessels) responding to glucocorticoid treatment. Recently, Shimura et al. [20] demonstrated a direct inhibitory action of glucocorticoid on both basal glycoconjugate secretion and stimulated secretion from cultured cat airway submucosal gland cells. According to the authors, these inhibitions are due to a reduction in the release of glycoconjugates without significant alteration in synthesis. As there is evidence that a lipocortin-like protein (calpactin) binds to cytoskeleton (actin) and promotes contact between the secretory vesicles and the plasma membrane during the exocytosis process [21,22], we have investigated the possibility that human airway gland secretory cells in culture produce lipocortin-like proteins.

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2. MATERIALS AND METHODS

2.1. *Cell culture on permeable filters*

Isolation and culture of airway submucosal gland cells were realized from human tracheas obtained 6-12 h post-mortem by using a method of enzymatic dissociation previously described by Tournier et al. [23]. Exponentially growing tracheal submucosal cells, cultured on type IV collagen-coated T_{25} flasks in RPMI 1640 medium (Intermed, France) supplemented with 2% Ultroser G (a serum substitute from IBF, France), were harvested by treatment with 0.25% trypsin, 0.5 mM EDTA in Ca^{2+}-free phosphate buffered saline. The cell suspensions were rinsed 3 times with fresh culture medium and seeded on (24.5 mm diameter, 0.4 μm pore size) type I and III collagen-coated membranes (Transwell-COL membranem, Costar, Cambridge, MA) at a density of 0.5×10^6 cells per cm^2. Filter-grown tracheal gland cells were cultured in the presence of 2% Ultroser G added to the apical (1.5 ml) and basal (2.5 ml) compartments of the culture chamber and maintained at 37°C under 5% CO_2 in air. Ten days after

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reaching confluency, filter-grown cells were rinsed 3 times with Ultroser G-free RPMI 1640 medium and cultured for 3 days in Ultroser G-free medium containing insulin (1 μg/ml), transferrin (1 μg/ml), epidermal growth factor (10 ng/ml), hydrocortisone (0.5 μg/ml) and retinoic acid (10 ng/ml). The hormone-supplemented medium was placed in each compartment and changed daily. All culture media contained 2 mM L-glutamine, streptomycin (100 μg/ml) and penicillin (100 U/ml). We confirmed that confluent cells grown in Transwell-COL membranes exhibited characteristics of epithelial and secretory cell, including dome formation and positive immunocytochemical staining for cytokeratin. Lysozyme, a secretory protein marker specific to the human tracheal serous gland cell [24], was detected in the culture media (data not shown).

2.2. *Tightness of filter-grown cell monolayers*

To demonstrate the polarized secretion of proteins in apical and basal compartments, we verified that the confluents cells grown in Transwell-COL filters remained impermeable to secretory proteins during the course of the experiments. The tightness of confluent cells was assessed by means of the technique described by Chambard et al. [25]. Ultroser G-free RPMI 1640 medium was added to the apical side while 2% Ultroser G-supplemented medium was added to the basal side. The culture chambers showing $<1\%$ of the basal concentration in the apical side after 24 h at 37°C were selected for this study.

2.3. *Metabolic labeling*

Culture chambers with confluent cells grown in the Transwell-COL membrane were maintained in the hormone-supplemented medium (for 3 days) and rinsed 3 times with methionine-free RPMI 1640 medium. After 1 h, the medium was then replaced with methionine-free RPMI 1640 medium containing 30 μCi/ml [^{35}S]methionine (1000 Ci/mmol, Amersham International) added to the basal compartment for 16 h, washed once quickly with fresh RPMI 1640 medium containing an excess of unlabeled methionine and then, chased for 5 h at 37°C in fresh RPMI 1640 medium in both compartments. Newly synthesized proteins, secreted in apical and basal medium, were collected separately, proteinase inhibitors (0.25 mM PMSF, 1000 U/ml aprotinin) were added and cell debris was removed by centrifugation. The Ca^{2+} and phospholipid-binding proteins were selectively extracted with EGTA from cell membranes, as previously described [26]. All samples were dialyzed exhaustively (Spectrapor tubing, MW cut off 6000 to 8000, Spectrum Medical Industries, L.A.) against deionized water for 8 days (2 changes a day), lyophilized and then stored at −80°C until used for biochemical analysis. Total protein content was measured by the BioRad Protein Microassay Procedure [27]. Radioactivity was evaluated by mixing samples with Hionic-Fluor (Packard) and counting by liquid scintillation spectrometry.

2.4. *Immunofluorescence*

Procedures for indirect immunofluorescence of cultured human tracheal submucosal gland cells were as previously described [28]. We applied the biotin-streptavidin fluorescein technique using rabbit polyclonal antiserum to lipocortin I (dilution 1/25), fluorescein streptavidin and biotinylated goat anti-rabbit IgG (dilution 1/25, Amersham). Immunostaining was performed on fixed (3% paraformaldehyde) and either non-permeabilized or permeabilized (0.1% Triton X-100) cells grown on type I collagen-coated glass coverslips.

2.5. *Electrophoresis and immunoblotting*

SDS-polyacrylamide gel electrophoresis was performed in 10–15% gradient Phast gels according to the manufacturer's instructions (Pharmacia, France). After electrophoress, the dried gels were exposed to X Omat AR films (Kodak, France). For immunoblotting, proteins were transferred to nitrocellulose and probed with rabbit antiserum to lipocortin I (dilution 1/1000). Immunoreactive bands were visualized as described [29] using the biotinylated anti-rabbit IgG and streptavidin-biotinylated horseradish peroxidase complexes (dilution 1/1000, Amersham).

3. RESULTS AND DISCUSSION

The ability of human tracheal gland cells to produce lipocortin-like proteins was investigated by using in-

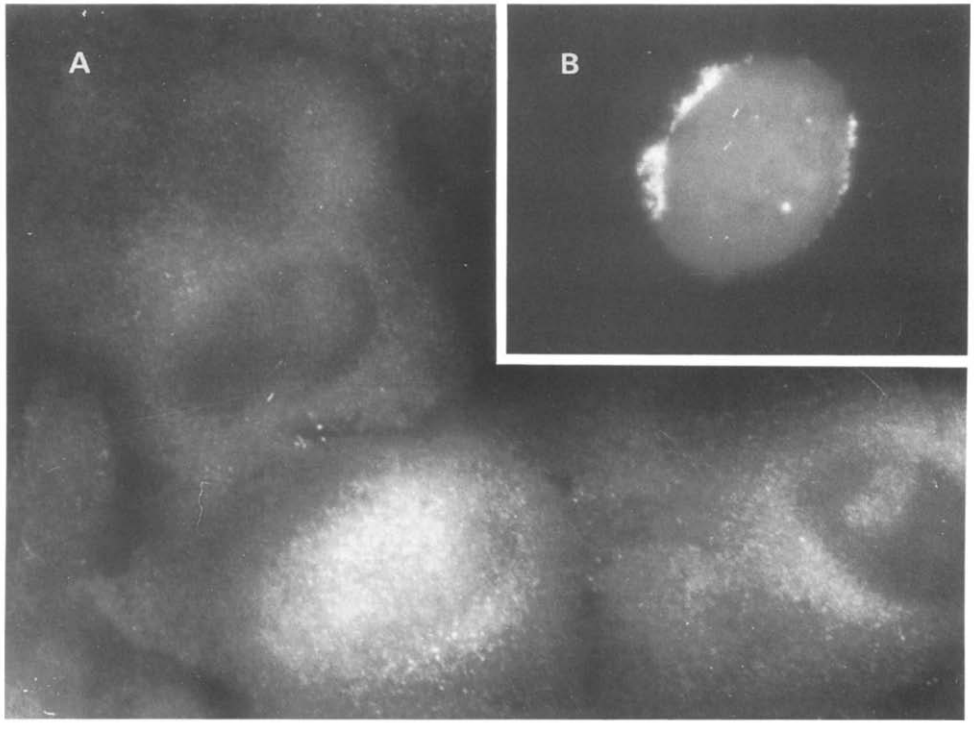

Fig. 1. Surface immunofluorescence localization of lipocortin I-like proteins from cultured human tracheal submucosal gland cells. Cells were fixed and non-permeabilized (A) or permeabilized with 0.1% Triton X-100 (B). Control cells using normal rabbit serum gave no staining.

direct immunofluorescence, selective extraction procedure of Ca^{2+} and phospholipid-binding proteins from cell membranes, fluorography following [^{35}S]methionine metabolic labeling and immunoblotting. We first performed immunofluorescence studies on fixed, non-permeabilized and permeabilized cells (Fig. 1). On subconfluent cell cultures, a significant surface staining was observed using the lipocortin I antibody (Fig. 1A). After permeabilization, the cell membrane was clearly stained (Fig. 1B). On the other hand, no detectable fluorescence was obtained intracellularly. This indicates that human submucosal secretory cells contain a significant amount of membrane-associated lipocortin I-like proteins. The Ca^{2+} and phospholipid-binding proteins from cell membranes were then analyzed by fluorography and immunoblotting following SDS-PAGE of EGTA extracts obtained from submucosal gland cells labeled with [^{35}S]methionine. Two major radiolabeled calcium-dependent phospholipid-binding proteins with apparent M_r values of 45 and 67 kDa were detected, respectively (Fig. 2, lane 1). To demonstrate that these Ca^{2+}-dependent proteins belong to the lipocortin family, immunoblotting was performed using the lipocortin I antiserum. As shown in Fig. 2, lane 2, at least 4 polypeptides with apparent M_r values of 35, 40, 45 and 67 kDa were revealed, respectively, with the strongest immunoreactivity for the 35 kDa protein. We also investigated the vectorial delivery of exogenous secretory proteins to either the apical or basal cell surface of human tracheal gland cells cultured on a permeable substrate. The newly synthesized proteins released by filter-grown cell monolayers during a 5 hour-chase period were analyzed by fluorography and immunoblotting following SDS-PAGE. More than 90% of radiolabeled proteins (93%, mean of 8 filters, range 4.5%) released by filter-grown cell were present in the apical medium. The amount of secretory proteins released during the 5 hour-chase period was approximately 45 μg and 5 μg/culture chamber in the apical and basal medium, respectively. Fluorography of proteins released in the apical medium (Fig. 3, panel I, lane A) revealed, at least, 15 radiolabeled polypeptides with M_r values ranging from 14 to 95 kDa. Two major polypeptides of apparent M_r values of 14.4 and 40 kDa were detected, respectively. Based on the molecular mass and immunoblotting characteristics followed by immunoenzymatic assays of the apical medium, the labeled M_r 14.4 kDa protein band has been identified as lysozyme, a secretory protein marker specific to the human tracheal serous gland cell [24] (F. Dupuit et al., in preparation). As yet, the identity of the labeled M_r 40 kDa band is unknown. Using immunoblotting, antilipocortin I antiserum revealed a group of 3 polypeptides with apparent M_r values of 33, 35 and 37 kDa, respectively, and a 68 kDa protein, secreted in the apical medium (Fig. 3, panel II, lane A).

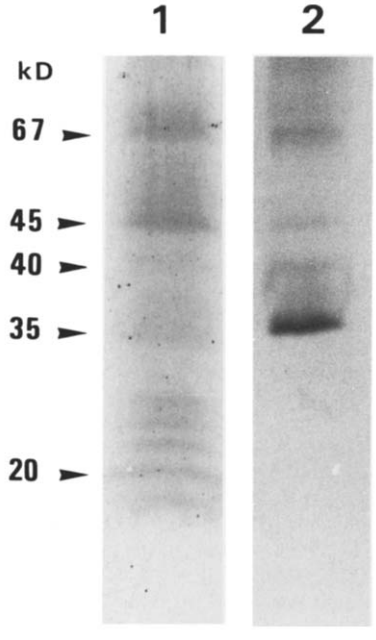

Fig. 2. Identification of Ca^{2+} and phospholipid-binding proteins present in EGTA extracts from cultured human tracheal submucosal gland cells. SDS-PAGE of proteins from cell membranes after fluorography (1) and immunoblotting using antiserum to lipocortin I (2).

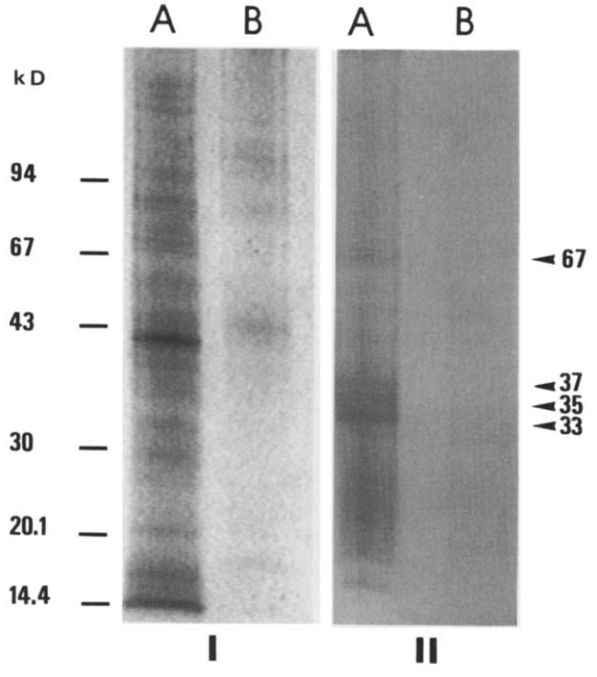

Fig. 3. Polarized secretion of newly synthesized proteins from human tracheal gland cells. The apical (A) and basal (B) culture medium from the 5 hour-chase period were analyzed by SDS-PAGE and fluorography (panel I) or by immunoblotting using antiserum to lipocortin I (panel II). Control immunoblotting using normal rabbit serum gave no immunoreactive staining. The positions of molecular mass markers (phosphorylase b, bovine serumalbumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and α-lactalbumin) are indicated.

This finding suggests that lipocortin I, recently demonstrated in human lung lavage fluid and defined as two lipocortin forms probably produced by alveolar macrophages with apparent M_r values of 34 and 37 kDa, respectively [30], originates at least in part, from tracheal submucosal gland cells. The lipocortin I (35 kDa) and other lipocortin-related proteins (15, 30, 32, 36, 45 and 67–70 kDa) have been also identified in various cells and tissues such as bovine lung [31], cultured rat renomedullary interstitial cells [4], porcine thyroid cells [32], human endothelial cells [33] and human mononuclear cells [34]. Several reports have shown that the 35 kDa and 67–70 kDa lipocortin-like protein are closely related with respect to sequence, antiserum cross-reactivity and binding of Ca^{2+} and phospholipid (see [35] for refs). Lipocortin I is a substrate for both the EGF receptor tyrosyl kinase [36] and the protein kinase C (PKC) [2,37]. Antonicelli et al. [32] recently showed that among four proteins (32, 35, 36 and 67–70 kDa) of the lipocortin family, only lipocortin I was the endogenous substrate for the PKC in thyroid-stimulating hormone (TSH) treated cells.

This is the first evidence that lipocortin-like proteins can be produced and released by human airway submucosal secretory cells. This finding is consistent with a previous study [19] demonstrating that lipocortin is a possible mediator of the suppressive effect of dexamethasone on respiratory glycoconjugate secretion. Airway gland cells are known to be targets for glucocorticosteroids [20]. The 35 kDa lipocortin identified in EGTA extracts from cultured tracheal submucosal gland cells could be a candidate for the glucocorticosteroid action. The 35 and 36 kDa lipocortins also bind phospholipid and actin in a Ca^{2+}-dependent manner, and may have a role in crosslinking the plasma membrane and cytoskeleton during the secretory vesicle release [38]. The extracellular presence of lipocortins has been also reported from cultured cell lines, and acted exogenously by inhibiting the formation of arachidonic acid metabolites [4,34,39,40]. In airways, it is now becoming clear that human tracheal epithelial cells can produce several cyclooxygenase products such as 15-HETE and prostaglandin E_2 [12–15], and may be partly responsible for the inflammatory diseases of the lung such as asthma. The anti-inflammatory actions of glucocorticoids include the inhibition of the release of arachidonic acid derivatives and the increase of lipocortin levels [40] associated with a significant reduction in respiratory glycoconjugate secretion [19]. Lipocortins are presumed to be responsible for the anti-inflammatory effect of the corticosteroids (see [41] for refs). However, recent data have shown, in human cultured endothelial cells [33], that an increased synthesis of lipocortin cannot account for the inhibition of prostaglandin synthesis brought about by dexamethasone. In L969 fibrosarcoma cells [42], the protective effect of dexamethasone appears not to be mediated by lipocortins. It has been proposed that lipocortins are involved in phospholipid-cytoskeleton interaction, exocytosis and membrane traffic [21,22,38].

These data emphasize the importance of human tracheal submucosal gland cell cultures for studies of the regulatory effects of glucocorticoids on respiratory glycoconjugate secretion. Further experiments are required to elucidate the exact role of these lipocortins produced by human tracheal submucosal gland cells.

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