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Production of pro-inflammatory polypeptides by airway mucous glands and its potential significance

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Abstract

Burn patients often develop respiratory distress and ARDS several days after injury. An ovine model allows experimental study of this problem. In sheep the injury is characterized by intense acute inflammation in the trachea and bronchi from 3 to 48 h after injury, with accumulation of neutrophils, fibrin and other plasma proteins, and mucus in airway lumens. We have carried out immunostaining for multiple cytokines in this model, including interleukin-8 (IL-8), Interleukin-1 beta (IL-1β), interleukin-1 alpha (IL-1α), tumor necrosis factor-alpha (TNF-α), and vascular endothelial growth factor (VEGF). All of these show intense immunostaining in airway mucous glands. IL-1β and VEGF show substantial constitutive staining in the serous cells of mucous glands, while IL-8, IL-1α, and TNF-α show substantially increased expression after injury. This pattern of expression of cytokines in mucous glands, and the apparent release of cytokines into the lumen after injury, are considered potentially highly significant in the progression of injury in this model. In addition, a proinflammatory function of mucous glands might prove to be important in chronic lung diseases such as chronic bronchitis and asthma.

Keywords: Cytokines; Lung; Airway; Bronchi; Mucous glands; Immunohistochemistry; Pathology; Bronchial circulation

1. Introduction

Some burn patients with inhalation injury develop diffuse lung injury and die primarily as a result of this serious complication. Extensive work in an ovine experimental model designed to reproduce this problem has led to better understanding of the pathophysiology of this complex problem [1,2]. We have been particularly interested in the intense acute inflammatory reaction that is seen in the trachea and bronchi during the first 24 h of this reaction to injury, before there is severe compromise of lung function. We also have been interested in the role of secretion of mucus in airway obstruction in this experimental model [3]. Recently, we have applied immunohistochemical procedures for several cytokines in the sheep, to determine the cellular sources of these important polypeptides. Although several cell types have been found to show positive immunostaining for pro-inflammatory cytokines, we have been impressed that the mucous glands of the bronchi often show substantial expression of cytokines. Neutrophils have also been observed in the tissue around glands and observed within gland epithelium and gland lumens, leading us to question the nature of the stimuli for these patterns of neutrophil migration. This presentation is intended to present evidence for the storage and release of cytokines by gland epithelial cells, and to comment on the possible significance of these observations.

2. Methods

2.1. Experimental animal model

Adult female sheep weighing 25–35 kg (Merino hybrids) were acclimated to the laboratory, were given tracheostomies under ketamine anesthesia (10 mg/kg) and surgical anaesthesia was maintained by inhalation of 1.5% halothane. Cooled cotton smoke, 48 breaths, was administered as previously described [3,4]. In addition, a third-degree burn over 40% of body surface was administered by application of a Bunsen burner to sheared skin, half before

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and half after the inhalation injury. Burn injury was included as recommended by our clinical colleagues in order to make the model more relevant to the condition of hospitalized patients with smoke inhalation injury [5]. Ringer's lactate solution was administered intravenously, 4 ml/kg during the first 24 h after the injury [6]. An analgesic (buprenorphine, 0.01 mg/kg) was given intravenously. Upon recovery from anaesthesia, the animals were maintained on a ventilator, and as a rule they interacted normally and fed spontaneously. They were closely monitored for any signs of distress, and were rapidly killed if they showed evidence of discomfort. 48 h after injury, the animals were given ketamine anaesthesia (10 mg/kg) and sacrificed by IV injection of saturated KCl. The lungs were removed, and a midline cross-section of the right lower lobe was preserved for histological study by injection of 10% phosphate-buffered formalin into multiple sites using a 22-guage needle, and by overnight fixation at room temperature.

2.2. Immunohistochemical technique

Tissue was processed into paraffin, embedded and sectioned at 4 μm. Samples from 3 to 5 injured sheep and 3 to 5 uninjured sham control sheep were studied each time immunostaining was done, and staining for each cytokine was repeated at least once. Deparaffinized sections were stained with haematoxylin and eosin (H&E) for histological study and orientation. Heat-induced epitope retrieval in citrate buffer was carried out on unstained sections for 15–20 min in a steamer. Sections were rinsed in buffer, rinsed 5 × with 0.6% hydrogen peroxide in methanol to suppress endogenous peroxidase and incubated for 30 min in blocking serum of the species providing the second antibody to block nonspecific staining. Sections were incubated overnight in primary antibody at 4 °C in humidified chambers. The sections were rinsed, incubated in biotinylated second antibody (goat anti-rabbit IgG or rabbit anti-mouse IgG), treated with an avidin-biotin-peroxidase kit (Vector, Burlingame, CA), and visualized using diaminobenzidine, with the aid of an automated immunostainer (Biogenex, San Ramon, CA). In all cases the results were compared to slides incubated with the same concentration of nonimmune serum corresponding to the primary antibody as a negative control. Sections were lightly counterstained with haematoxylin. The primary antibodies used for this study were: polyclonal rabbit antiserum to recombinant ovine interleukin-8 (IL-8) at dilutions of 1:4000–6000 (Chemicon AB1840, Raleigh, NC), murine monoclonal antibody to recombinant ovine IL-8 at dilutions of 1:4000–6000 (Serotec MCA-1660, clone 8M6, Temecula, CA), murine monoclonal antibody to recombinant ovine interleukin-1 beta (IL-1β) at 0.5 μg/ml (Serotec MCA 1658, clone 1D4, Temecula, CA), polyclonal rabbit antiserum to recombinant human interleukin-1 α (IL-1α) diluted 1:800 (Abcam ab7632, Cambridge, MA), murine monoclonal antibody to a synthetic N-terminal peptide of human tumor necrosis factor-α (TNF-α) at 0.125 μg/ml (Chemicon MAB 1096, Raleigh, NC), and rabbit polyclonal antiserum to a recombinant peptide of human vascular endothelial growth factor (VEGF) at a dilution of 1:500 (Santa Cruz sc-152, Santa Cruz, CA).

3. Results

The larger bronchi of sheep have numerous glands in the stroma between the muscular wall of the bronchus and the cartilage, a few between the lining epithelium and the muscular wall, and a few near the external surface of the bronchial cartilage rings. Most of the glands contain both clusters of tall goblet cells filled with pale mucin (mucous cells) and regions lined by columnar cells with eosinophilic cytoplasm (serous cells) in an irregular arrangement. Histological study of large bronchi showed that most gland acini and ducts had small lumens and columnar lining epithelial cells in uninjured "sham" animals that received the same operative preparation, anaesthesia, ventilation with 100% oxygen and fluid resuscitation as the injured animals, although a few gland acini appeared dilated. As time progressed from 4 to 48 h, the glands became progressively dilated and had thinner walls lined by much shorter or flattened cells, and the mucous goblet cells were no longer recognizable because they no longer contained mucin. At 4–48 h after injury, polymorphonuclear neutrophils were seen scattered in the connective tissue around the glands, and often neutrophils were visible within the lining epithelium of gland acini and within acinar lumens and gland ducts.

Immunohistochemical staining for IL-8, IL-1β, IL-1α, TNFα and VEGF all showed positive staining in bronchial gland epithelial cells. For each cytokine, at some time point the immunostaining was at least as intense as that in other cell types in the bronchus, and persisted as the primary antibody was diluted out. The patterns of staining and the changes after smoke inhalation injury were different for each cytokine.

Immunostaining for *IL-8* (Fig. 1a and b) showed distinct selective staining of secretory cells in airway glands in sham control animals that were uninjured but intubated and ventilated. Usually scattered mononuclear cells in the adjacent stroma showed more intense cytoplasmic immunostaining than did the epithelial cells of glands. After combined smoke inhalation and burn injury, the apparent intensity of immunostaining for IL8 increased with time, such that mucous glands stained as intensely as the residual surface lining epithelium or any other tissue by 48 h after injury. In addition, a larger proportion of gland cells appeared to stain with the antibody to ovine IL-8 and, by 48 h after injury, almost all epithelial cells lining the bronchial glands were stained.

Immunostaining for *IL-1β* (Fig. 1c and d) showed positive staining of moderate intensity of the apical half of the cytoplasm of serous cells. In some areas there was light punctuate staining of mucous goblet cells or staining

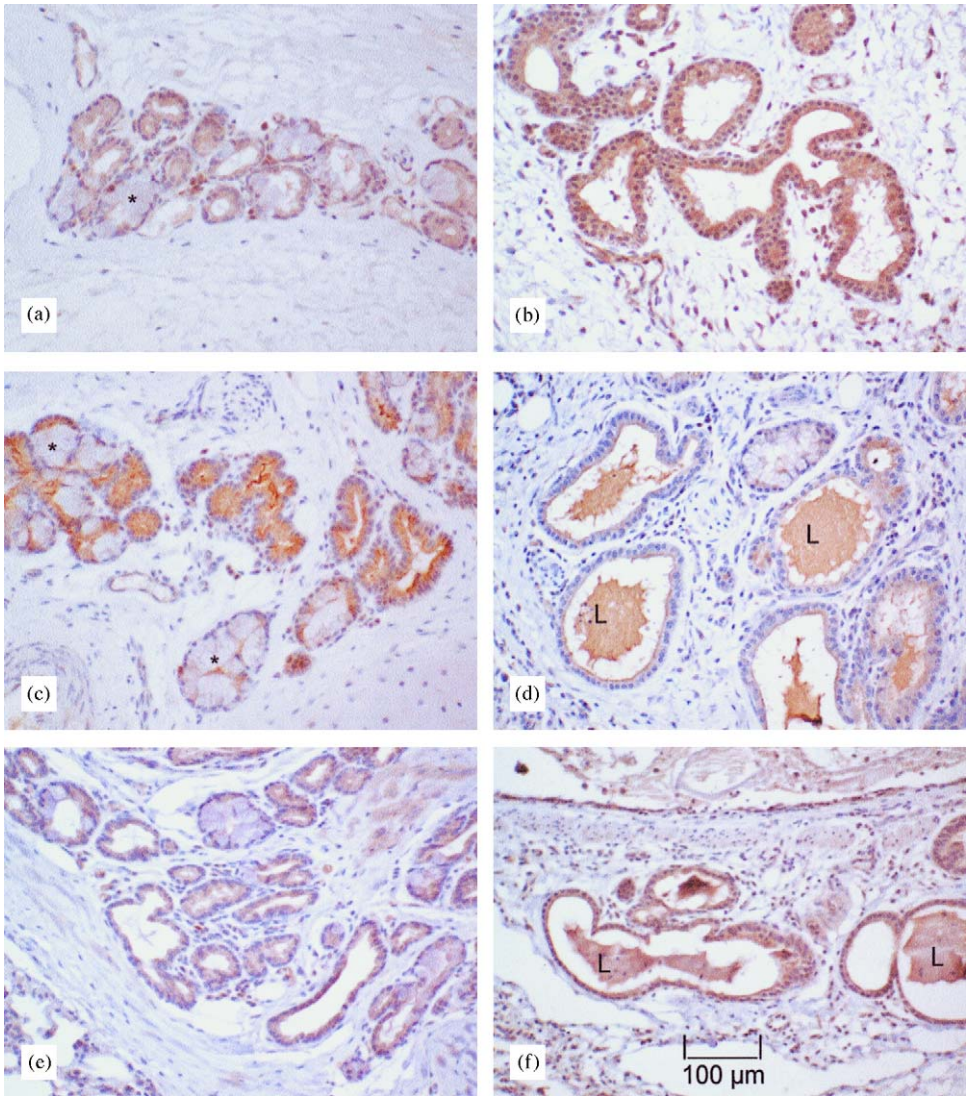

Fig. 1. Immunohistochemical staining of submucosal mucous glands in the bronchi of sheep, brown reaction product indicating the sites of antibody binding. Scale bar, 100 μm. (a) Immunostaining for IL-8 in sham control sheep that were intubated and ventilated but not injured. Light immunostaining is seen in serous cells, while the mucous goblet cells (★) are unstained. (b) Staining for IL-8 48 h after combined burn and smoke inhalation injury. There is moderate to strong staining of virtually all cells lining the dilated gland acini. (c) Immunostaining for IL-1β in a sham control sheep. Serous cells are uniformly stainined, and there is some reaction product in the lumen, while mucous goblet cells (★) are unstained. (d) Staining for IL-1β 48 h after injury. Reaction product is seen in the lumens of glands (L), but very little is seen in the lining epithelial cells. (e) Immunostaining for IL-1α in a sham control sheep. Light staining is seen in serous cells. (f) Staining for IL-1α 48 h after injury. Reaction product is seen in nearly all cells lining glands as well as in the lumens (L).

of the cytoplasm surrounding the mucus-filled portions of goblet cell cytoplasm, and in a few glands reactions product was seen in amorphous material in the lumens of acini. By 4 h after injury, the earliest interval so far examined, and throughout the first 48 h after injury, glands generally showed moderate to strong immunohistochemical staining for IL-1β in the amorphous material filling their dilated acini and duct lumens. There was no visible staining in the cytoplasm of the glands in some areas, and light staining of a thin layer of apical cytoplasm of most cells in other areas.

Immunostaining for *IL-1*α, (Fig. 1e and f), by contrast, showed light to moderate staining of serous cells in uninjured animals, and more intense cytoplasmic staining of most or all cells lining glands in injured animals, first seen at 4 h after injury. In addition, there was moderate positive staining of the material occupying the lumens of the dilated gland acini.

TNFα showed a highly variable pattern of expression in our experiments, with some animals in both sham control and injured groups showing strong immunostaining for *TNF*α in airway glands, as well as in the residual surface lining epithelium of the bronchi (Fig. 2a and b). In other animals, virtually no positive immunostaining for TNFα was seen. The presence of positive staining for TNFα showed no apparent correlation with the presence of inflammatory cells or other indicators of lung injury.

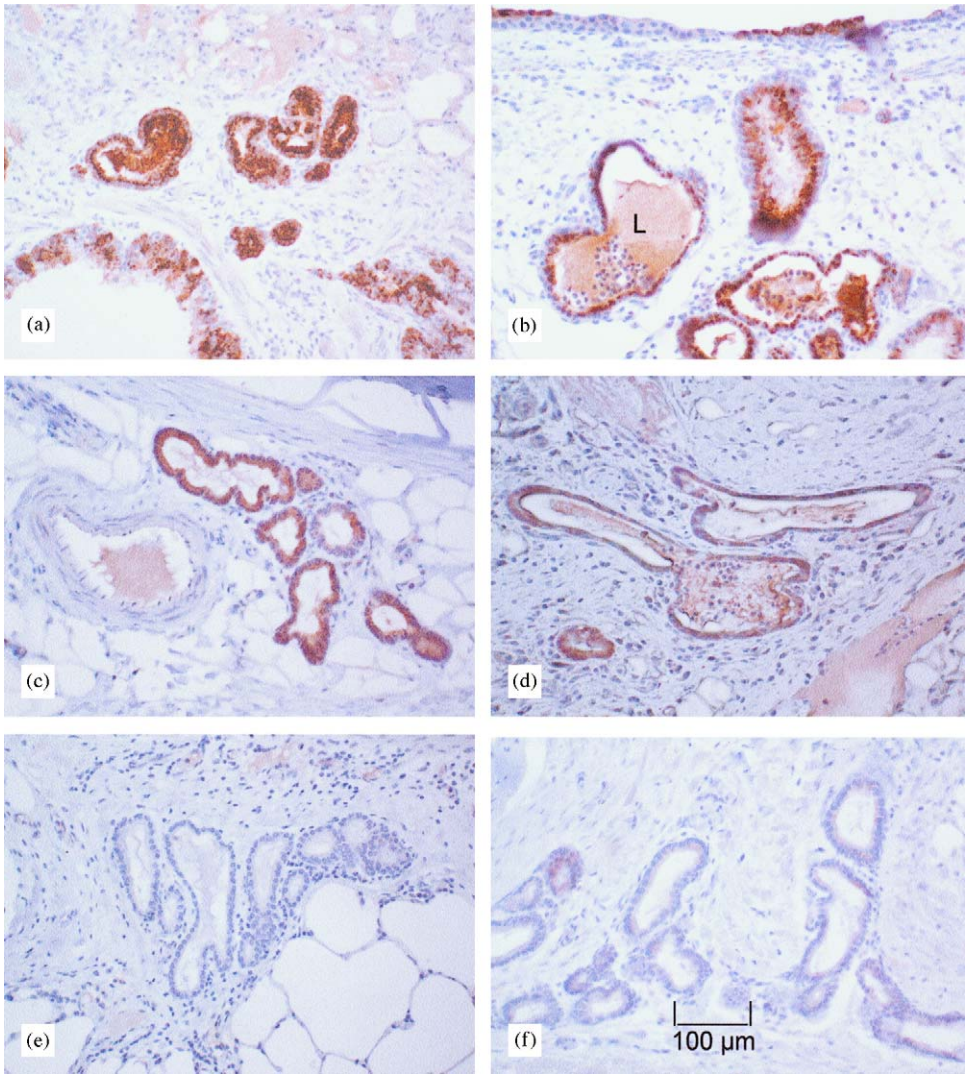

Fig. 2. (a) Immunohistochemical staining for TNFα is seen in some (but not all) sham control sheep. (b) Staining for TNFα is seen in some (but not all) sheep 48 h after injury. Reaction product is seen in gland epithelial cells and in the lumens of glands (L). (c) Immunostaining for VEGF in sham control sheep. Moderate staining is seen of serous cells. (d) Staining for VEGF 48 h after injury. There is moderate staining of most gland epithelial cells as well as some reaction product in gland lumens. (e) Control section 48 h after injury in which nonimmune murine IgG at 0.5 μg/ml was substituted for the primary monoclonal antibody. No significant brown staining is seen. (f) Control section 48 h after injury in which nonimmune rabbit IgG at a concentration corresponding to a 1:500 dilution of serum was substituted for the primary polyclonal antibody. Only very faint nonspecific staining is seen. Scale bar, 100 μm.

Immunostaining for VEGF (Fig. 2c and d) showed diffuse positive staining of moderate intensity in serous cells in virtually all glands in uninjured sham control animals. At 48 h after injury, immunostaining of somewhat less intensity was seen in the great majority of all gland epithelial cells. In addition, there was light to moderate staining of the amorphous material in the lumens of dilated glands.

4. Discussion

The major purpose of this communication is to describe the presence of several important cytokines within the cytoplasm of the epithelial cells of bronchial mucous glands in sheep. Interleukin-1 beta (IL-1β) and vascular endothelial growth factor (VEGF), a potent mediator of vasodilation and increased vascular permeability as well as of vascular growth, were constitutively expressed in the glands of uninjured animals. In tissue from injured animals, both of these cytokines were seen in amorphous material of the acinar lumens, in gland ducts, and within the bronchial lumens, suggesting that these polypeptide mediators were secreted by the mucous glands after injury. In the case of IL-1β, there was little evidence of resynthesis and storage after injury, although it is possible that IL-1β continued to be synthesized and rapidly secreted without significant intracytoplasmic storage. Only faint immunostaining was seen in uninjured animals for IL-8 or IL-1α, but most of the epithelial cells of airway glands showed intense immunostaining for these cytokines after injury. The changes in the patterns of immunostaining for IL-1α and IL-1β were already well developed at the first

post-injury time point examined, 4 h. Intense immunohistochemical staining for TNF-α was seen in mucous glands of some animals in both the sham control and injured groups. While it is possible that some of the sham animals may have had minor illnesses, or that they might have reacted to instrumentation or intubation with expression of this cytokine in glands, the basis for the extreme variability of TNFα expression remains obscure. There did not seem to be more histological evidence of inflammation in the sections of the lungs of the animals that showed strong TNFα expression in mucous glands.

The evidence favouring the production and secretion of pro-inflammatory cytokines by bronchial mucous glands is based entirely on immunohistochemistry at this point. Clearly, a stronger case could be made if evidence could be found of mRNA production by gland epithelial cells, or if quantitative increases in the amount of cytokine protein content could be demonstrated after injury. Future studies will attempt to acquire evidence along these lines. A pattern seems to be emerging from these histochemical studies, however, that leads us to suggest that mucous glands may have important secretory functions beyond production of viscous mucus, including signaling and regulation of other cells and tissues, and perhaps stimulation and/or enhancement of inflammatory responses in injured tracheal and bronchial tissue. While our experiments have been limited to acute reactions to inhaled toxic fumes, it is reasonable to speculate that secretion of pro-inflammatory cytokines by airway mucous glands might play a role in chronic airway diseases [7,8].

The immunohistochemical findings have been distinct and reproducible, with the exception of the positive staining for TNFα in only a minority of animals. When the concentration of the primary antibody was reduced stepwise, immunostaining of glands persisted at greater antibody dilutions compared with staining of most other tissue components. Although rabbit IgG does exhibit a tendency to adhere nonspecifically to mucus, careful titration of nonimmune rabbit and mouse IgG showed no nonspecific staining of airway glands at concentrations comparable to those of the primary antibodies described here. Furthermore, in untreated animals, immunostaining for cytokines generally was seen in serous cells, not in the highly acidic mucus within the goblet cells. Different patterns of reaction to injury were seen for different cytokines, which again would not be expected as a consequence of nonspecific charge-related adhesion of primary antibodies to gland contents.

The literature has long described secretion of mucus as a consequence and concomitant of acute inflammation of mucosal surfaces [9]. Stimulation by autonomic nerves and by bradykinin has been shown to lead to secretion of mucus by airway glands in the short term [10]. In chronic bronchitis and chronic asthma, hyperplasia of airway mucous glands is a prominent feature [11]. However, there has been very little suggestion of a pro-inflammatory function of the mucous glands themselves. The presence of IL-8 in mucous glands has been described in a canine model in which the trachea was exposed to a supernatant of *Pseudomonas aeruginosa* [12], and in bronchi from patients with cystic fibrosis (CF) [13]. In addition, using primary cultures of human submucosal gland serous cells [14], synthesis and secretion of IL-8 has been demonstrated [13,15,16].

One essential function of airway mucus is thought to be the removal of microparticulate foreign substances that enter the airways with the inspired air. The constant movement of mucus from the airways into the pharynx by ciliated cells effectively removes inhaled bacteria and other microscopic particles and very likely protects the host against infection by the aerosol route [17,18]. Low concentrations of gaseous toxins such as ozone have been shown to reduce the threshold dose for infection of animals by aerosols of bacteria, supporting the concept that active clearance of bacterial aerosols is important [19]. In this context, secretion of cytokines that can initiate and sustain acute inflammatory exudation would seem to be salutary, since limited exudation of neutrophils and plasma proteins would add antibacterial activity to the moving layer of mucus in the bronchi. In the case of smoke inhalation injury, however, disruption of the mucociliary escalator by wholesale loss of ciliated cells in the upper trachea is an early feature of the reaction to injury, at least in sheep [20]. Without a continuous sheet of ciliated cells to remove secreted mucus, the secretions of glands tend to accumulate in sheep after smoke inhalation injury, to restrict air flow and to be forced into smaller airways and lung tissue during mechanical ventilation [3].

Several practical questions are suggested by the findings reported here. If airway mucous glands do secrete pro-inflammatory cytokines, what controls the synthesis and release of these cytokines? Are these functions controlled by the same stimuli that regulate secretion of mucus [21,22], or by other mechanisms? Can pharmacological alteration of cytokine secretion by glands significantly affect the outcome of reactions to injury in the lung, or the courses of any diseases of the lungs? Are specific cytokines stored in structurally distinct granules within the epithelial cells of glands? Do permanently distinct cell types function as mucus-secreting and serous cell types, or is there some plasticity that allows cells to switch their secretory phenotype? Investigation of these questions will be of interest in the future.

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