Use of transmission electron microscopy for studying airway remodeling in a chronic ovalbumin mice model of allergic asthma

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Asthma represents chronic inflammatory process of the airways followed by healing that may result in an altered structure referred to as airway remodeling. The mechanisms underlying airway remodeling remain unknown and so we need to know the minute structural details of the asthmatic airway before mining mechanisms. Histopathological and transmission electron microscopic studies were performed in chronic asthmatic murine lungs and they revealed the damage to bronchial epithelial cells, goblet cell metaplasia with mucus hypersecretion and cellular infiltration in perivascular and peribronchiolar areas. The infiltrated cells were composed mainly of eosinophils, lymphocytes and macrophages. There was a widening of intercellular spaces between bronchial epithelia especially at abluminal regions. Basement membranes were thickened and composed mainly of collagen. There were increased population of smooth muscle and fibroblast and in addition there were more numbers of mitochondria in smooth muscle. This would help us to understand the human asthma in much better way.

Key Words asthma; in situ perfusion fixation; transmission electron microscopy

1. Introduction

Asthma is defined as a chronic airway disease characterized by reversible airflow obstruction, airway hyperresponsiveness, airway inflammation, and structural changes of airway which otherwise called airway remodeling [1]. To demonstrate most of these fundamental features of asthma, one obviously needs various disciplines such as physiology, immunology and pathology. Amongst these pathology has an important role due to its illustrative nature. Indeed the demonstration of pathological features led to novel and exciting hypotheses to understand asthma in different dimensions. For example, pathological observations of mucus plugs and neutrophils in major bronchi in autopsied asthmatic lungs raised a concern in viewing fatal asthma as a different subtype, acute severe asthma, and urged to explore pathogenesis, molecular pathways and therapeutics [2, 3]. The current description of asthma cannot be complete without mentioning the structural changes of airway as contrast to earlier description which merely emphasize the importance of inflammation. Shortly, understanding the structural and minute features of lung may allow us to describe asthma in a holistic view rather than different parts of it. Light microscopy (LM) can reveal most of the gross pathological features of asthma. However, it can’t reveal the minute details of cellular and tissue structures of lung. Electron microscopy (EM) has a lot of potential to identify and characterize these minute features at sub-cellular level. Though EM requires high start-up and maintenance cost, the findings derived from it has extended the knowledge in different dimensions which are crucial in developing better therapeutics. However, due to the availability of EM in central facilities in most of the institutes, it seems that the use of EM by researchers may not be the major concern. To illustrate the minute details of lung, we have used chronic mouse model of asthma and performed EM with lungs. In addition, we used LM to support and add further details on the ultrastructural features.

Before dealing with microscopic features, it is important to describe the mice model of asthma briefly. First of all, it is well accepted fact that experimental animal models are essential and sometimes inevitable tools for clear understanding of the pathophysiology of various complex diseases such as asthma [4]. Though even a single animal model cannot suitably reproduce human asthma completely, mouse models of asthma have been shown to be useful tools to understand the various features of human asthma [5]. The commonly used mouse model of asthma is based on ovalbumin (OVA) sensitization and challenge. OVA, a chicken egg protein, can elicit allergic sensitization if given systemically along with suitable adjuvants such as aluminum hydroxide (ALUM). A series of intraperitoneal injections of OVA+ALUM must be given to attain successful sensitization. To elicit asthmatic features, OVA should be then administered as an inhaled aerosol or intra nasal drops referred to as challenge. It is necessary to perform physiological lung function measurements to make sure that these mice developed asthma features. The term ‘asthma’ in mice is only meaningful with demonstration of airflow obstruction using various sophisticated instruments. We use three methods: single, double chamber plethysmography and flexiVent which determine enhanced pause, specific airway conductance and airway resistance respectively. Since the scope this review is restricted to microscopy, we couldn’t describe these physiological methods in detail. However, readers may refer physiological reviews for details [6, 7]. Generally OVA-sensitization and -challenge leads to pathological features of asthma such as infiltration of various inflammatory cells
such as lymphocytes, eosinophils, macrophages into the airway, mucus hypersecretion and goblet cell metaplasia. However, chronic allergen exposures lead to cause both inflammatory and structural changes of airway such as hypertrophy and hyperplasia of bronchial epithelia, goblet cell metaplasia, basement membrane thickening, sub-epithelial fibrosis, and hypertrophy & hyperplasia of airway smooth muscle. Since there are various pathological features, there is no single technique to demonstrate all. These techniques have been mentioned below.

2. Materials and Methods

2.1 Animals
Male BALB/c mice (8-10 weeks old at starting) were randomly divided into two groups: Sham and OVA (n=6 each). Mice were sensitized by three intraperitoneal injections (i.p. inj.) of 50 µg OVA in 4 mg ALUM (OVA group) or 4 mg ALUM alone (Sham group) on days 0, 7, and 14. Mice were challenged from Day 21 to Day 43 (30 minutes per day, alternative days) with 3% OVA in PBS (OVA group) or PBS (Sham group) using a nebulizer with a flow rate of 9 L/min. After determining airway hyperresponsiveness (AHR) to methacholine on Day 44, mice were sacrificed and lungs were taken for both LM and EM studies.

2.2 Histopathology
Lungs were fixed in 10% neutral buffered formalin, dehydrated and embedded in paraffin. For histological examination, embedded lungs were sectioned on glass slides, deparaffinized, and stained with Hematoxylin and Eosin (H & E), Alcian Blue-Periodic acid Schiff and Masson Trichrome stainings to assess the airway inflammation, goblet cell metaplasia and sub-epithelial fibrosis respectively.

2.3 Transmission Electron Microscopy
Combined in situ perfusion and immersion fixation

Fig. 1 Schematic diagram of in-situ perfusion fixation technique.

Each mouse was anaesthetized by i.p. inj. of sodium pentobarbitone (1-2 mg per mouse), deep anesthesia was confirmed by absence of reflex on tweaking the foot. Thoracic cavity was opened by midsternal splitting incision to expose the heart and posterior vena cava (PVC). 18 gauze cannula was inserted into the apex of the heart to access the left ventricle, cannula was connected to bottle, located 1 meter above the body of the mouse, containing cacodylate buffer via tubing and flow controlling valve (Fig. 1). Initial flush out was performed for 3-5 minutes with PBS after cutting the PVC till achieving complete exsanguination. Then the perfusion was switched over to the cacodylate buffer.
containing glutaraldehyde fixative for 25-30 minutes with a flow rate of approx. 3ml/min. Fixation was done at room
temperature. Hardening of all the exposed organs was felt at the end of fixation. Lungs (hard and fragile) were removed
and immersed into the fixative (2.5% glutaraldehyde) and kept at 4°C for overnight. Fixed lungs were dissected under Dissection microscope (SZX-12, Olympus) and those were cut into many slices of same thickness. Each slice was made
into three blocks and they were post fixed in 1% osmium tetroxide and then processed and embedded in epoxy resin. Ultrathin sections of about 70-80 nm were cut and stained using uranyl acetate and lead citrate. The stained sections were viewed under electron microscope (Morgagni 268D, Fei Electron Optics).

3. Results

3.1 Airway inflammation

Airway inflammation is the main feature in asthma. Airway of Sham mice showed the normal architecture without any sign of inflammation (Fig. 2A), in contrast OVA mice showed the infiltration of various inflammatory cells around the vessels and bronchi (Fig. 2B). There were more of perivascular cell infiltrations than peribronchial infiltration in OVA mice. EM revealed that these cells were predominantly eosinophils, lymphocytes and macrophages (Fig. 2C & D). The presence of eosinophilic granules, peculiar longitudinal grain shaped, indicates these cells are eosinophils (Fig. 2E). These granules have inner electron dense core which is surrounded by electron lucent matrix. In this study, we could find inactive granules since there are intact granules without any loss of core and matrix. In addition, plasmacytes, which are responsible for IgE production, are easily recognized by their characteristic appearance with rough endoplasmic reticulum (Fig. 2F). Macrophages were identified by their pseudopodia (Fig. 2G). Interestingly, nuclear elements were found in the cytoplasm of macrophages (Fig. 2H). indicate the possible phagocytosis of other inflammatory cells such as neutrophils and also this indicate that these macrophages may be healing type which eat most of the post inflammatory nasty substances such as necrotic and/or apoptotic remnants and also initiate the fibrotic process.

3.2 Goblet cell metaplasia

Sham mice showed almost no or occasional PAS positive cells in the airway (Fig. 3A). On the other hand, OVA mice showed more PAS positive cells (Fig. 3B) indicate the goblet cell metaplasia. Airway of mice is populated mainly with ciliated cells and Clara cells and less mucous cells. EM revealed that OVA mice showed more goblet cells with lot of mucous granules (Fig 3C & D). Ciliated cells were recognized by its columnar shape, large basal nucleus, electron lucent cytoplasm and obviously the presence of cilia, Clara cells were by its apical projection towards its lumen and
presence of electron dense serous granules and mucous cells were by the presence of mucus which can be recognized by membrane bound vacuoles containing electron lucent substance. Similar to other studies [8, 9] we also found the mucous granules in Clara cell indicate the conversion of Clara cell to mucous cell.

Fig. 3 LM and TEM of mucous cell metaplasia. AB-PAS staining of lungs of Sham control mice (A, 10X) showed no Goblet cell metaplasia whereas OVA mice (B, 20X) show marked Goblet cell metaplasia (Br: bronchiole, v: blood vessels). Inset showed the magnified mucous cell (60X). TEM of bronchi showed mucous cells characterized by the presence of electron lucent mucus containing secretory granules (sg) and ciliated epithelial cells (cec) were compressed towards the side (C, 880X, c, cilia). Clara cells (cc) were also seen with mucus electron dense as well as electron lucent secretory granules at the apical projection (D, 880X).

3.3 Widening of intercellular spaces between bronchial epithelia

Fig. 4 TEM of intercellular space widening. Sham control mice (A, 880X) showed thin and regular intercellular space compared to OVA mice which showed marked widening of intercellular space between adjacent bronchial epithelial cells (B, 880X). Arrows indicate the junction. High power magnification showed the cytoplasmatic elongations of adjacent bronchial epithelia in OVA mice (C, 10500X).

The adjacent bronchial epithelia are interconnected by tight junctions and these junctions control the paracellular permeability and maintain the epithelial barrier function. In Sham mice, these intercellular spaces were thin and regular (Fig. 4A). However, in OVA mice these spaces were widened especially at abluminal regions compared to apical regions (Fig. 4B). Higher magnification revealed the cytoplasmatic prolongations of the adjacent epithelial cells (Fig. 4C).

3.4 Basement membrane thickening

Basement membrane is the connective tissue structure which supports the airway epithelial layer. LM cannot visualize this structure easily unless otherwise there is a thickening. MT staining showed that Sham mice had less collagen compared to thickened collagen layer in OVA mice (Fig. 5A & B). Sham mice showed thin basement membrane underneath the basal cell (Fig 5C). OVA mice showed thickening of the basement membrane (Fig. 5D, 4634.4 ± 359.7 vs. 881.4 ± 142.6; OVA vs. Sham, mean ± sd). This thickened basement membrane predominantly composed of collagen (Fig 5E).
3.5 Smooth muscle alteration

Fig. 6 TEM of airway smooth muscle. Airway smooth muscles (asm) in Sham control mice (A, 880X) were thin whereas OVA mice (B, 880X) showed the increased in the size. These smooth muscles from OVA mice also showed the presence of more number of mitochondria (m) (C, 1950X).

Normally airway smooth muscle present mostly in proximal bronchi and this is responsible for most of the bronchoconstriction. The presence of smooth muscle can be recognized by LM but detailed sub-cellular structures can be readily recognizable with EM. While normal mice showed a single layer of smooth muscle (Fig. 6A), OVA mice showed hyperplasia and hypertrophy of smooth muscle (Fig. 6B). Interestingly, there were more numbers of mitochondria in these cells (Fig. 6C).

4. Discussion

Various studies have described the histopathologic features of asthma by LM. However, there are few reports to describe the ultrastructural features by EM. This could be because of the requirement of sophisticated instrument, trained hands, laborious work and interpreting capacity. In this study, we have described the minute details of lung which occurs in chronic model of asthma. Airway inflammation in asthma is the infiltration of various kinds of inflammatory cells around the airway rather than in the parenchyma. Generally this inflammation is described as perivascular and peribronchial inflammation. This is the contrast feature compared to chronic obstructive pulmonary
inflammation may not require EM, however very often there is confusion in differentiating eosinophils from neutrophils since book descriptions may not be found frequently. Immunohistochemistry also can differentiate eosinophil from neutrophil but it can’t reveal the activeness of eosinophils. However, EM not only can recognize eosinophils due to the peculiar feature of eosinophilic granules but also reveal the activeness of the granules. As shown in Fig. 2E, these granules have two major parts: core and matrix. Intact granules indicate inactive state and granules with loss of core and/or matrix indicate the active state. Generally it has been believed that eosinophil degranulation is absent in mice [12]. However, it has been demonstrated that the activation is more in airway lumen of asthmatic mice [13] and similarly we had found partial active granules in our sub-acute model of asthma (our unpublished observations). However, we could find the intact or inactive granules in chronic model of asthma (Fig. 2E). But these mice also showed higher IL-5 levels. This indicate that IL-5, eosinophil survival factor, may be responsible for the persistence of these inactive eosinophils in chronic model. EM can easily recognize the plasma cells by its peculiar feature of RER arrangement surrounding the nucleus. These plasma cells synthesize more IgE which leads to cause mast cell degranulation and airway obstruction [14].

Airway inflammation is mostly commonly accompanied by goblet cell metaplasia. This can be recognized by PAS staining in which goblet cell shows the presence of magenta colored mucin containing granules. On the other hand, EM can describe minute details related to this feature such as cilia, microvilli, electron dense secretary and electron lucent secretary granules. These minute structures can differentiate the type of epithelial cells and often the functional status of the cells. In mice, it has been shown that Clara cells are being converted to mucus hypersecreting cell type [8, 9].

Infiltrated inflammatory cells injure the structural cells such as airway epithelia. This injury is predominantly mediated by the toxic proteins secreted by these inflammatory cells. For example, eosinophil secretes eosinophil peroxidase, major basic protein, eosinophil cationic protein and eosinophil-derived neurotoxin [15]. Chronic allergen exposures lead to reduce defensive mechanisms of airway epithelia and cause the damage to those cells [16]. Also we found earlier that bronchial epithelia of asthmatic mice have shown mitochondrial swelling and loss of cristae [11]. It has been observed that increased numbers of mitochondria and altered mitochondria in bronchial epithelium in murine model of asthma and asthmatic children [17-18]. This might lead to cause apoptosis at earlier stages; this is evident by reduction in cytochrome c oxidase in bronchial epithelia, increased cytosolic cytochrome c in lung cytosols and also there is an involvement of death receptor pathway to induce apoptosis in asthmatic bronchial epithelia [11, 19]. These indicate the possibility of involvement of both intrinsic and extrinsic pathways of apoptosis in asthmatic bronchial epithelia. This might be lead to shrinkage of these epithelia. This could support our observation of widening of the intercellular space between subsequent bronchial epithelial cells. Interestingly, the widening was prominent in the abluminal regions rather than the apical regions. However, denudation was not a frequent finding in this chronic model of asthma; indicate that this would have occurred at earlier stage of the disease. In evident to this we have found ciliated epithelia in BAL slides of asthmatic mice compared to normal mice (our unpublished observations). Also it has been reported that epithelial clumps have been found in sputum and BAL fluid of human asthmatics [20].

It is known that epithelial injury is known to activate the epithelial mesenchymal trophic unit (EMTU) in the airway [21]. In this process growth factors are getting released by both bronchial epithelia and fibroblast. Due to this activation the attenuated fibroblast layer which is located beneath the basement membrane is transformed into myofibroblast and start secreting both actin and collagen [21]. This leads to more accumulation of collagen beneath the epithia, called sub-epithelial fibrosis. Though it is called sub-epithelial fibrosis the collagen bundles were observed even between and underneath the smooth muscle. There is hypertrophy and hyperplasia of smooth muscle which is located underneath the fibroblast. In addition, there is increased numbers of mitochondria indicate the possibility mitochondrial biogenesis [22]. However, the functional status of these mitochondria is not clear.

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