Electron Microscopic Observations of Influenzal Pneumonitis

TSUYOSHI NISHIMURA, YASUAKI FUJIYOSHI, KOJI IRIE AND AKIRA TANIMURA

Departments of Internal Medicine and Pathology, Kurume University School of Medicine, Kurume, 830 Japan

Received for publication August 16, 1985

Summary: Influenzal pneumonia was experimentally induced in mice. The respiratory system during the acute phase was investigated by light and electron microscopy. Bronchial desquamation and necrosis were noted from the 2nd day to the 5th day and were most noticeable during the 3rd day. An electron dense degenerative substance was demonstrated in the degenerated epithelial cells on the 2nd and 3rd days. On the 3rd day, flattened basal cells appeared in the large bronchus. The basal cells were covered with one layer of cuboidal or flattened epithelial cells, both ciliated and non-ciliated. The epithelial cells had large nuclei and narrowly restricted cytoplasm. On the 5th day, stratified regenerating epithelial cells lined both the large and small bronchi. In the large bronchus, non-ciliated epithelial cells had cytoplasmic projections. Alveolar epithelial desquamation and necrosis, interstitial pneumonia and collapse of the lung were noted from the 2nd to the 5th day. On the 4th day, the collapse of the lung was most noticeable. No regenerative alveolar epithelium was demonstrated in these experiments.

Key words: influenza virus — pneumonitis — desquamation of epithelial cell — disturbance of circulation — collapse of the lung

Introduction

Influenzal pneumonia was epidemic from 1918 to 1919 and in 1958. The virus was first separated by Smith in 1933; and, subsequently, a number of experiments in mice and ferrets have been reported. The pathological changes in patients were also studied by autopsy. A representative work was published by Hers. The ciliated epithelium was first affected, resulting in degeneration and necrosis which caused pneumonitis and atelectasis. In the present investigation, pathological changes in the bronchi and alveolar epithelium during an acute phase of influenzal pneumonia in mice were investigated by light microscopy, scanning electron microscopy, and transmission electron microscopy.

Materials and Methods

Specific pathogen-free (SPF) male mice (Slc: ddy) weighing 18 g, and 26 days old were used in this study. The mice were inoculated, intranasally, with 2.5 ml of saline-diluted (1:2) allantoic fluid containing 10^{-1.7} LD_{50} of influenza virus A/PR8/34 (HON1). Using ether anesthesia the mice were sacrificed after one to five days and their lungs were removed. The lung tissues were prepared by fixing for light microscopy by fixing in 10% formalin and embedding in paraffin. The sections were stained with hematoxylin-eosin. Tissue fragments of lungs were prepared for transmission electron microscopy by fixing in 2.5% glutaraldehyde in 0.1 M phosphate buffer, post-fixing in osmium tetroxide in
the same buffer, dehydrating and embedding in epoxy resin. Ultrathin sections were double stained with uranyl acetate and lead citrate, and examined with S-100 electron microscopy. The specimens for scanning electron microscopy were dehydrated through an ascending alcohol and dried in a critical-point apparatus. Specimens were coated with gold-palladium and observed with a HFS-2 (HITACHI) scanning electron microscope.

Result

The 1st day

A mild infiltration of inflammatory cells consisting of lymphocytes and histiocytes was demonstrated around the large bronchus by light microscopy. Vacuolation and nuclear disruption were present in some of the bronchial epithelial cells. Inflammatory cells, such as lymphocytes and histiocytes, were occasionally observed microscopically in the peribronchial alveolar walls, but the alveolar epithelium was not affected.

In a part of the mucosal epithelium of the large bronchus, large vacuoles were seen with transmission electron microscopy in the cytoplasm, chromatin granules were aggregated in the nucleus, dense degeneration products were seen, the free surface was covered sparsely with short cilia, the intercellular space was enlarged, and inflammatory cell infiltration was noted between epithelial cells. In the mucosa of the small bronchi, Clara cells were occasionally denuded. The head of the ciliated cells were occasionally degenerated or torn up. Lymphocytes were demonstrated in the alveolar capillaries, but the alveolar epithelial cells were intact. No viruses could be demonstrated on the 1st day.

In the large bronchus, cilia observed by scanning electron microscopy, were aggregated in clusters irregularly distributed, or sparse. Mucous substances and bacteria were attached to the cilia. The goblet cells bulged into the bronchial lumen, and a mucous substance was attached to the free surface, which was very rough. The microvilli were decreased in number. In the small bronchus, the cilia were stuck together, and were slightly decreased in number.

The 2nd day

Light microscopy revealed that most of the bronchi were surrounded by tissues containing inflammatory cell infiltrations, consisting of lymphocytes and histiocytes. The degree of pathologic changes of the bronchial mucosa varied markedly depending on the site. The bronchial epithelial cells generally had few changes. Many of the cells were degenerated and necrotic at the site when the pathological changes were highly advanced. The free epithelial surface was covered with desquamating tissues and an exudate consisting mainly of neutrophils. Bronchiectasis and diffuse pulmonary emphysema were observed.

Transmission electron microscopy demonstrated a marked degeneration of the small bronchial epithelia. In Clara cells, an abundance of lysosomes appeared, mitochondria were swollen, and degeneration products were noted. Inflammatory cell infiltration was noted below the basement membrane. In the ciliated cells of the large bronchi, the cilia were desquamated, shortened, and intracellular degeneration products, vacuolation, and enlargement of the endoplasmic reticulum, and an enlargement of the intercellular space were noted. In the alveolar wall, as seen with transmission electron microscopy, the capillary endothelial cells were enlarged, intracapillary infiltration of lymphocytes and macrophages was marked, and the alveolar wall was thickened. The alveolar epithelial cells were desquamated. Virus particles were observed on the surface of the bronchi.

A comparative study of the large bronchi by scanning electron microscopy revealed that the cilia became short, reduced
in number, were desquamated, and disappeared. The irregularity in arrangement, unevenness in length, and clotting became more serious on the 2nd day than on the 1st day. An erosion was noted on the surface of the cell.

The 3rd day

With light microscopy, the size of the inflamed area had expanded further, as compared to the 2nd day. Mucosal necrosis and desquamation were marked in the bronchi. A single layer of flattened basal cells lined the basement membrane. Abundant cell debris was present in the bronchial lumen. The basement membrane was partially exposed. Numerous inflammatory infiltrates of lymphocytes, neutrophils and histiocytes were noted in the submucosal layer. An inflammatory cell infiltration, consisting mainly of lymphocytes and histiocytes, was restricted to the alveolar septa, which was edematous without fibrous thickening. Highly affected tissue and normal tissue were intermingled. Alveoli were occasionally collapsed and the lung was markedly emphysematous.

Transmission electron microscopy showed a degenerative reaction. The large bronchus was covered with a monolayer of ciliated and non-ciliated epithelial cells varied from a cuboidal form to a flattened form. The cilia were short. The mitochondria varied in size and some were markedly swollen. In the flattened non-ciliated epithelial cells, the cytoplasmic space was small, the nucleus was large, and the number of microvilli was decreased (Fig. 1). Virus particles were present on the surface of the ciliated and non-ciliated epithelial cells.

Transmission electron microscopy revealed virus particles that were in contact with epithelial cilia of the small bronchus. The affected cilia were smaller in number and in size than cilia in control cells (Fig. 2). Cellular debris was present in the lumen of the small bronchi. Degeneration

Fig. 1. Transmission electron micrograph showing flattened basal cells in the large bronchus at 3 days after inoculation. ×4,000

Fig. 2a. Transmission electron micrograph showing virus particles in contact with cilia of the degenerated epithelium at 3 days after inoculation.

Fig. 2b. This is a close-up of the virus particles. ×50,000
Fig. 3. Transmission electron micrograph showing electron dense degeneration products in the degenerated small bronchial epithelium at 3 days after inoculation. ×6,000

Fig. 4. Transmission electron micrograph showing that the interalveolar septa at 3 days after inoculation are thick and hypercellular and contain inflammatory cells. The alveoli contain inflammatory cells and cellular debris. ×4,000

Fig. 5. Scanning electron micrograph showing the large bronchial epithelium at 3 day after inoculation. The cilia are lost or clumped together. ×4,000

was observed in a part of the epithelia of the small bronchi and electron dense degeneration products were seen in the clara cells and ciliated epithelia (Fig. 3). A remarkable inflammatory cell infiltration consisting of lymphocytes and neutrophils, as well as erythrocytes, was found by transmission electron microscopy in the distended capillaries of the alveolar wall. Desquamation and vacuolation of the alveolar epithelium were also noted (Fig. 4).

The scanning electron microscopic findings on the large bronchi on the 3rd day differed from those on the 2nd day as follows; ciliary aggregation, irregular orientation and decreased number of cilia became more remarkable, the free surface of non-ciliated epithelial cells was coarsely uneven. Exudate, inflammatory cells, erythrocytes, and bacterial clots were often attached to the epithelium. The protrusion of goblet cells and ciliated cells became less remarkable (Fig. 5).

In the small bronchi, examined by scanning electron microscopy, the number of cilia was markedly decreased, an abundant exudate was attached to the epithelium, most of the remaining cilia were stuck together and the non-ciliated epithelial cells were flattened.

The 4th day

Compared to the light microscopic findings on the 2nd and 3rd days, the inflammatory reaction was observed over a wider area. The alveolar wall and alveoli had many more inflammatory cell infiltrates, and pulmonary collapse was more remarkable. In both the large and small bronchi, necrosis and desquamation were remarkable in the bronchial epithelium
and the basal membrane was partly exposed. In addition, cellular debris had accumulated in the bronchial lumen. The basal epithelial cells were flattened.

With transmission electron microscopy, congestive capillaries were seen in the alveolar wall and the endothelial cells were degenerated and enlarged. An inflammatory cell infiltrate of lymphocytes and histiocytes was remarkable, and alveolar epithelial cells contained distended endoplasmic reticulum and their degenerative desquamation was remarkable. The alveoli became narrower.

**The 5th day**

With light microscopy, the inflammatory cell infiltrate was presented in almost the whole lung field. In both the large and small bronchi, round reserve cells with large nuclei were found among the epithelial cells. Mitosis was sporadically noted. A monolayer of flattened cells partly lined the bronchi. Necrosis was noted in some areas. Histiocytes, lymphocytes, and neutrophils had infiltrated into the tissue surrounding the bronchi. Inflammatory cell infiltration consisting of lymphocytes, histiocytes, and neutrophils together with edema and congestion were widely noted in the alveolar wall. The alveoli were partially collapsed with intra-alveolar hemorrhage and inflammatory cell infiltration.

In a large bronchus, observed by transmission electron microscopy, the bronchial wall consisted of stratified cuboidal cells. The nucleus was oval, the nuclear outline was irregular, and the chromatin granules were distributed evenly. There were fewer mitochondria and less endoplasmic reticulum in the cytoplasm than were found in control cells. There was a moderate amount of ribosomes. The surface layer consisted of cells with microvilli. Cell degeneration was present in some areas. In the degenerated cells, the chromatin granules were coagulated and the cytoplasmic density was increased (Fig. 6). Inflammatory cell in filtrates were noted between the epithelium and below the basement membrane. Ciliated epithelial cells were rarely seen. The cytoplasmic projections in non-ciliated epithelial cells were occasionally seen (Fig. 7).

![Fig. 6. Transmission electron micrograph showing the hyperplastic large bronchial epithelium at 5 days after inoculation. ×4,000](image)

![Fig. 7. Transmission electron micrograph showing the cytoplasmic projection of a non-ciliated epithelial cell in the large bronchus at five days after inoculation. ×4,000](image)
Observed with transmission electron microscopy, the small bronchial wall was composed of multiple layers of cells with nuclei had a somewhat irregular outline. The cells contained few round and small few endoplasmic reticulum and ribosomes. Microvilli without cilia were seen along the bronchial lumen (Fig. 8).

Congested capillaries and degeneration were seen in the alveolar wall, the endothelial cells were enlarged, and changes in mitochondria and other cytoplasmic organelles, vacuolation and exfoliation were noted in the pneumocytes. Decomposition products and infiltrates of lymphocytes and macrophages were seen in the alveolar wall, and the alveolus was narrowed.

With scanning electron microscopy, as on the 3rd day, there were decreased numbers of cilia that were irregular in direction and height in the large bronchi. Non-ciliated epithelial cells had small spherical projections and were protruded into the bronchial lumen, and exudate and erythrocyte on the epithelium were increased compared with those seen on the 3rd day (Fig. 9). Clara cells protrude into the bronchial lumen in the small bronchi and the number of cilia was similar to the 3rd day and less than the control. A quantitative comparison of the number of cilia was not accurate because of the exudate seen on the 3rd day (Fig. 10).

![Fig. 8. Transmission electron micrograph of the small bronchial wall which is composed of multiple layers of cells at five days after inoculation. ×4,000](image)

![Fig. 9. Scanning electron micrograph of non-ciliated epithelial cells with small spherical projections in the large bronchial wall at five days after inoculation. ×2,000](image)

![Fig. 10. Scanning electron micrograph of the small bronchial wall where clara cells protrude into the bronchial lumen at five days after inoculation. ×4,000](image)
Discussion

Viral pneumonia is characterized by bronchiolitis and interstitial pneumonia is accompanied by atelectasis and emphysema. A variety of viruses cause pneumonia. The chief causative agents include respiratory syncytial virus, influenza virus, parainfluenza virus, and adenovirus. When the respiratory tract was affected by such viruses, swelling of cells, necrosis and abnormal cilia occur during the acute phase. The ciliary disorder impairs the mucociliary clearance, which facilitates a secondary bacterial infection. Alveolar parenchymal findings in the acute phase include swelling and desquamation of type I and II alveolar cells and changes in endothelial cells.

In 1937, Straub who experimentally induced an influenza virus infection in mice stated that pulmonary collapse was characteristic of pneumonia due to influenza viruses. Bronchial epithelial cells became necrotic leaving only the basal layer intact. These changes are well advanced by the third day (Spencer, 1972).

In the present experiments on the 1st day, a mild inflammatory cell infiltration was demonstrated in the tissue surrounding the bronchi. At the transmission electron microscopic levels, vacuolation, degeneration products and aggregations of chromatin granules were found in some of the epithelial cells of the large bronchus, but the small bronchi had few of these changes on the 1st day. Goblet cells and ciliated epithelial cells were similarly affected. The priority could not be determined. Necrosis was most noticeable on the 3rd day in the small bronchi which contain Clara cells. Transmission electron microscopy revealed a marked reduction in the number of cilia, and degeneration.

On the 3rd day, the large bronchus was lined with one layer of possibly basal cells, flattened or cuboidal cells with large nuclei which remained after desquamation of the epithelial cells. The cilia were markedly decreased in number and in height. Virus particles which appeared to be in contact with or close to the ciliated cell surface were demonstrated in the lumen of the large and small bronchi on the 2nd and 3rd days.

Possible granular osmiophilic bodies, which were described as a microscopy of virus particles by Harford, Hemlin and Parker (1955), appeared in the cytoplasm of bronchial epithelial cells on the 2nd and 3rd days in this experiment. They could not have originated from the proliferation and maturation of virus particles and have been considered to be nonspecific cell inclusion bodies which occur in degenerating cells. No mature virus particles could be found in the cells.

An electron microscopic study by Dourmashkin, 1970 and Blaskovic, 1972 revealed that a pathogenetic virus first sticks to the cilia of the epithelium and then enters into the cell following the fusion of the cell membrane and the viral envelope. According to Liu, 1955, a nuclear protein antigen could be demonstrated by a fluorescent antibody technique in the cell as early as 2 hours after inoculation. The antigen was first found in the nucleus and then moved to the cytoplasm. The virus began to bud at 12 to 18 hours after inoculation (Tamura, 1978). Prior to the budding, proteins nominated by the virus, such as HA, NA, and M, were incorporated into the protoplasm of the infected cells, to replace the major constituents of the protoplasmic membrane (Hay, 1974). Degeneration of the epithelial cells began within 24 hours after inoculation (Tamura, 1978).

Plummer and associates (1964) demonstrated an abundance of mitochondria and endoplasmic reticulum in the cytoplasm of the non-ciliated epithelial cells. Tamura demonstrated fibrillary inclusions in the nuclei of the alveolar epithelial cells, bronchial epithelial cells, and endothelial cells.
Tamura stated the inclusions were probably composed of viruses. Plummer found filament strands indicative of viral ribonucleoprotein.

Convalescence from pneumonia in experimental animals was characterized by hyperplasia of bronchial epithelium and alveolar epithelium. Regenerating epithelial cells and epithelial stratification were noted in both the large and small bronchi on the 5th day of this experiment, but subsequent changes could not be observed because all the mice died on the 6th day. As seen in bronchiolitis caused by an oxidant, regeneration of epithelial cells began with cell division and differentiation of non-ciliated epithelium into ciliated epithelium in the bronchiols (Evans, 1975, 1976).

In the present study, atelectasis and pulmonary emphysema were most remarkable on the 4th day, when inflammatory cell infiltration in the alveolar wall was most profound, together with degenerative swelling of capillary endothelial cells and desquamation of alveolar epithelial cells, shrinkage of alveoli, and stromal thickening. Soto considered impaired O$_2$ diffusion due to A-C block as the cause of death. Type II alveolar epithelium has been considered to produce surfactant (Adamson, 1973; Clements, 1970; Morgan, 1981). The impairment of type II cells is considered to be a main cause of collapse of the lung and death (Stinson, 1976). Impairment of the endothelial cells causes severe hemorrhage and capillary congestion. Hemorrhage and capillary impairment are common findings in influenza pneumonia and are very significant. Stevens, 1976, emphasized the importance of microvascular components in lethal influenza pneumonia. Though inflammatory cell infiltration of the peribronchial tissue and alveolar interstitium were most prominent on the fourth day, pulmonary edema and congestion were most severe on the fifth day. Thus it can be concluded that disturbance of circulation is the cause of death.

References


