Acute Changes in the Surface Morphology of Hamster Tracheobronchial Epithelium following Benzo(a)pyrene and Ferric Oxide Administration¹

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SUMMARY

Acute changes in the surface morphology of hamster tracheobronchial epithelium were examined by scanning electron microscopy following intratracheal administration of ferric oxide or benzo(a)pyrene absorbed to ferric oxide. Multiple instillation of ferric oxide brought about a loss of ciliated cells and broad areas of abnormal, enlarged non-ciliated cells with roughened or wrinkled surfaces. These were interpreted as areas of epithelial hyperplasia. Multiple intratracheal administrations of benzo(a)pyrene and ferric oxide caused similar changes and, in addition, produced squamous metaplasia and small foci of markedly abnormal protuberant cells. These findings are correlated with a previously reported observation obtained by transmission electron microscopy. The results suggest that scanning electron microscopy is useful in studies of the histogenesis of lung cancer.

INTRODUCTION

Administration of polynuclear hydrocarbon carcinogens with particulate carriers by the intratracheal respiratory route has been effective in the induction of tumors in the respiratory tract of Syrian golden hamsters. Saffiotti et al. (19) have shown that intratracheal instillation of BP² carried on ferric oxide produces squamous metaplasia and squamous carcinomas in the respiratory tract. The hamster model now is being used to define the process of squamous cell differentiation and to clarify the relationship between squamous metaplasia and small foci of markedly abnormal protuberant cells. These findings are correlated with a previously reported observation obtained by transmission electron microscopy. The results suggest that scanning electron microscopy is useful in studies of the histogenesis of lung cancer.

BP with more than 98% purity (Aldrich Chemical Company, Inc., Milwaukee, Wis.) absorbed to ferric oxide (Type R8089; Pfizer, Inc., New York, N. Y.; particle size distribution: 93% by weight < 5 μm in diameter, 80% < 2 μm, and 68% < 1 μm) or ferric oxide alone, were suspended in sterile 0.9% NaCl solution. The suspensions were repeatedly administered by intratracheal instillation to young adult Syrian golden hamsters⁴ of both sexes. Although the BP-ferric oxide mixture was prepared in a 1:1 ratio of 5 mg BP and 5 mg hematite, each instillation contained 2.5 mg BP, as determined by chemical analysis (17), for a total dose of 25 mg of BP.

The specific objective of this study was to evaluate the applicability of the SEM for the visualization of surface morphological changes, such as epithelial hyperplasia and squamous metaplasia, produced by intratracheal instillation of BP-ferric oxide in hamsters. Whenever possible, this evaluation was correlated to the ultrastructural pathology, determined by transmission electron microscopy, reported in the literature.

MATERIALS AND METHODS

BP with more than 98% purity (Aldrich Chemical Company, Inc., Milwaukee, Wis.) absorbed to ferric oxide (Type R8089; Pfizer, Inc., New York, N. Y.; particle size distribution: 93% by weight < 5 μm in diameter, 80% < 2 μm, and 68% < 1 μm) or ferric oxide alone, were suspended in sterile 0.9% NaCl solution. The suspensions were repeatedly administered by intratracheal instillation to young adult Syrian golden hamsters⁴ of both sexes. Although the BP-ferric oxide mixture was prepared in a 1:1 ratio of 5 mg BP and 5 mg hematite, each instillation contained 2.5 mg BP, as determined by chemical analysis (17), for a total dose of 25 mg of BP.

The hamsters were divided into 3 groups. Group A consisted of 35 hamsters, each receiving 5 mg of ferric oxide. Each of the 35 hamsters in Group B received 5 mg of ferric oxide and 2.5 mg of BP. Group C consisted of 20 control hamsters. In all groups, the intratracheal instillation was performed 3 times per week (Monday, Wednesday, and Friday) for a total of 10 instillations. For examination, 3 or 4 hamsters from the 2 treatment groups and 2 hamsters

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³The abbreviations used are: BP, benzo(a)pyrene; SEM, scanning electron microscope; TEM, transmission electron microscope.

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from the control group were anesthetized by an i.p. injection of pentobarbital. The examinations were performed weekly (Mondays) for a period of 10 weeks.

The abdomen of each hamster was opened on the midline, permitting access to the ventral aorta. The aorta was severed, the animal exsanguinated, and after the chest was opened, the lungs and trachea were removed. The trachea was cannulated to the level of the first cartilaginous ring and the lungs were expanded, at a pressure of 30 cm of water, with Karnovsky's paraformaldehyde-glutaraldehyde phosphate-buffered fixative (9). Prior to use, the osmolality of the fixative, about 2010 mOsmoles/kg, was reduced to approximately 550 mOsmoles/kg by the addition of phosphate buffer. Perfusion continued for at least 2 hr with the lungs completely immersed in fixative. Upon completion of airway perfusion, the trachea was ligated and the lungs were floated in fixative.

The trachea and main stem bronchi were removed from the lungs, washed in distilled water, and dehydrated with increasing concentrations of alcohol. Amyl acetate then was substituted for the alcohol, and the tissue was dried by the critical-point method in carbon dioxide (1). The dried trachea was sectioned longitudinally, cemented to a copper stub, and coated with gold in a vacuum evaporator. To ensure uniform coating the evaporator was equipped with a rotating turntable. The tissues were examined in a JEOL-JSM-2 scanning electron microscope equipped with an X-ray diffraction apparatus, at 5, 15, and 25 kV.

RESULTS

Normal Tracheobronchial Epithelium. In the untreated hamster, the tracheobronchial epithelium consisted of ciliated and nonciliated epithelial cells. Elevations were formed in the epithelium by the underlying cartilage rings, with depressions present between rings. The density of ciliated cells appeared greatest at the edge of elevations formed by the underlying tracheal rings (Fig. 1) and decreased toward the middle of the rings and in the spaces between rings (Fig. 2).

The number of ciliated and nonciliated cells appeared about equal in the trachea (Fig. 3). The cilia were erect and free standing, without mucus droplets. The exact demarcations of ciliated cells usually were obscured by the cilia and in some preparations by the filiform projections or villi protruding between the cilia. These projections appeared taller than the microvilli of nonciliated cells (Fig. 3).

Nonciliated cells in the trachea were polygonally or penta-gonally shaped with raised borders at cell junctions. The surface of the nonciliated cells was covered with discrete microvilli which projected slightly into the lumen.

More ciliated than nonciliated cells were seen near the carina and bronchi (Fig. 4). In the bronchi and lower trachea, the nonciliated cells were oblong, had a rounded surface, and appeared to be squeezed between the ciliated cells. Cell junctions, characterized by the raised border, were discernible but not prominent.

Ferric Oxide Treatment. Three intratracheal instillations of ferric oxide resulted in broad focal areas of nonciliated epithelium which were distributed randomly along the length of the trachea (Fig. 5). Individual cells were large and showed roughened or wrinkled surfaces. These areas, interpreted as epithelial hyperplasia, were still present after 10 intratracheal instillations. A few ciliated cells were present in these areas after 3 instillations but were absent after 10 instillations. A transition from areas of epithelial hyperplasia to more normal appearing epithelium was observed. Some nonciliated cells in the more normal epithelium exhibited raised and roughened surfaces.

Ferric oxide particles, confirmed by X-ray diffraction (Fig. 6), assumed a variety of shapes, sizes, and surface structures, and were present at all times during the treatment. The tracheobronchial epithelium returned to normal 7 weeks after completion of the treatment.

BP-Ferric Oxide Treatment. Three intratracheal instillations of the BP-coated ferric oxide resulted in changes similar to those observed with ferric oxide alone. In addition, there were regions where nonciliated cells protruded into the tracheal lumen (Fig. 7 and 7A) or cells which possessed roughened, wrinkled surfaces. Individual ferric oxide particles frequently were present in areas of cellular change.

Six instillations produced a partial to complete loss of ciliated cells in focal areas of enlarged nonciliated cells. Although still randomly distributed, abnormal areas appeared larger and more numerous than those seen with ferric oxide alone. Many stages were observed in the degeneration of ciliated cells; finally, these degenerated cells were shed, leaving only circular holes in the epithelium. The degeneration was demonstrated by shortened cilia and partial collapse of the cell surface. In most instances, these cells showed depressed margins, and many contained shortened cilia and filiform projections, thereby permitting identification as degenerating ciliated epithelial cells. (Fig. 8).

Numerous focal areas of enlarged, broad, nonciliated epithelial cells showing rough surfaces with raised and rounded cell margins were present (Fig. 9). These areas appeared to be in a transitional stage between basal epithelial cell hyperplasia and squamous metaplasia (Fig. 10 and 10A). In the regions of squamous metaplasia, surface cells appeared to be sloughing from the underlying cells, with cell margins that curved toward the tracheal lumen. The degree of cell distortion was determined with the help of stereo-pair photographs (Fig. 11). The underlying cells in these areas presented surfaces totally different from those of luminal nonciliated epithelial cells (Fig. 12).

A similar pattern of epithelial hyperplasia and squamous metaplasia was present after 10 instillations of the BP-ferric oxide mixture and persisted 7 weeks after the final intratracheal instillation. These changes, although focal in nature, involved approximately one-half of the surface of the trachea. Because of a low density of microvilli on cell surfaces which resulted in less available surface for the reflection of electrons, many of the cells in the regions of hyperplasia appeared black at low magnification.

Ferric oxide particles usually appeared as clumps in the trachea and bronchi and were identified readily by X-ray diffraction (Fig. 13). In contrast to the individual particles or small groups of particles observed earlier in
the experiment, the clumps were numerous and large. Numerous holes, marking the location of degenerated ciliated cells (Fig. 8), were present with white blood cells near or on top of the holes (Fig. 14).

Large clusters of cells protruding well into the tracheal lumen were visible 1 week after the last treatment (Fig. 15). The cells comprising the clusters were large and displayed the normal surface structure of nonciliated epithelial cells, but the cell margins were not well defined. The groupings were found in areas of epithelial hyperplasia or beginning squamous metaplasia and differed markedly from other previously observed areas where individual cells were discrete with normal cell margins or, subsequently, where cell surfaces were rounded.

Abnormal cells of this type, seen 4 weeks after the last treatment, were in contrast to those observed 1 week after the last treatment in that, although large, they possessed a variegated surface. They were not numerous, were randomly located, and were observed singly (Fig. 16) or in clusters (Fig. 17). The usual microvilli were absent, but were replaced with either short, blunt projections or long, coiled, interdigitating ridges (Fig. 17). Some cell surfaces at the base of the enlarged cells also presented a variegated surface structure, differing from the more normal surface structure of adjacent cells (Fig. 17).

**DISCUSSION**

The results of this study indicate that the SEM is a useful, appropriate tool for the visualization of preneoplastic surface changes in the respiratory epithelium. Previous studies (7, 11, 19, 20) showed that intratracheal instillations of BP carried on ferric oxide produces epithelial hyperplasia and squamous metaplasia in the respiratory epithelium of the Syrian golden hamster. In this study, multiple instillations of ferric oxide produced a loss of ciliated cells with broad focal areas of epithelial hyperplasia. Similar changes were observed with the administration of BP-coated ferric oxide. In addition, focal areas of squamous metaplasia and small foci of protuberant cells with abnormal surface structure were observed.

Interpretation of the epithelial hyperplasia and squamous metaplasia rests upon a comparison of SEM photographs and published TEM results (5, 7, 8). In ferric oxidetreated animals, there was widespread focal replacement of the columnar epithelium with polygonal basal cells. The transmission electron micrographs showed that the surface of the hyperplastic cells was broad, somewhat rounded, and possessed scant microvilli. Transmission micrographs of tracheal epithelium following multiple instillations of a BP-ferric oxide mixture showed areas of squamous metaplasia and adjacent epithelial hyperplasia (7, 8). The squamous metaplastic cells were flattened at the surface of the epithelium, and some edges were separating from underlying cells and turning toward the tracheal lumen. Undifferentiated cells, in foci of epithelial hyperplasia, possessed protruding surfaces with variable numbers of microvilli.

The experimental protocol used in this experiment and the TEM experiment (7) was identical, and comparable results were obtained in terms of time of onset and nature of the changes. However, the large protuberant single cells or cell clusters observed by SEM were difficult to interpret, as such cells have not been discussed in TEM studies. The size of the cells and their unusual surface structure indicate abnormalcy, and they could represent atypical hyperplastic epithelial cells. While possibly malignant, they could not be judged so, and proper interpretation requires further correlation with either light or transmission electron micrographs. Should they slough from the epithelium, these types of abnormal cells could influence exfoliative cytology interpretations (12).

Atypical epithelial hyperplasia and squamous metaplasia are associated with bronchial carcinoma in humans (2) and hamsters (19). Exposure of the respiratory epithelium to chemical carcinogens causes such changes (2–4, 7, 15, 18, 19). The various stages of tracheal epithelial alteration, such as basal cell hyperplasia and squamous metaplasia, can be differentiated by light microscopy and transmission electron microscopy (8). However, examination of a large area of the respiratory tract by transmission electron microscopy is not feasible. Please (16) has estimated that it would require 7.5 years of continuous microscope use to examine 1 sq cm of ultrathin sectioned material at ×1500. The area shown in Fig. 10 is of comparable magnification, and illustrates the utility of the SEM. The SEM permits visualization of the whole trachea, shows the extent of change, and provides an estimate of the surface area involved in the change. At the same time, at high magnifications, the examination of small areas of the epithelium and even of individual cells is possible. Furthermore, stereophotographs and X-ray diffraction permit visualization of cell distortion and identification of metallic elements. The SEM has been of value in studies of the respiratory tract (6, 10, 13, 14, 21). In parallel with light microscopy, the TEM, and exfoliative cytology, the SEM can be used to obtain a better understanding of the events leading to cancer of the lung, especially of the relationship between hyperplasia, metaplasia, and anaplasia.

**REFERENCES**

6. Holma, B. Scanning Electron Microscopic Observation of Particles...
Fig. 1. Normal tracheal epithelium. Ciliated cells form patches or clusters over the ridges created by underlying cartilage rings. × 77.

Fig. 2. Normal tracheal epithelium. Broad areas of nonciliated cells in space between cartilage rings. Two ducts from subepithelial glands are present. × 77.

Fig. 3. Normal tracheal epithelium. Nonciliated cells are hexagonal or polygonal with raised borders at cell junctions. Ciliated cells are not numerous and have filiform projections or villi protruding between the cilia. × 3000.

Fig. 4. Normal bronchial epithelium. Ciliated cells are numerous and partially obscure the rounded, oblong, nonciliated epithelial cells. × 2310.
Fig. 5. Tracheal epithelium following 3 instillations of ferric oxide. Broad focal areas of enlarged nonciliated, epithelial cells with roughened or wrinkled surfaces. × 770.

Fig. 6. Tracheal epithelium following 10 instillations of ferric oxide. Roughened surfaces of nonciliated cells. Note ferric oxide particle (arrow). × 2310.

Fig. 7. Tracheal epithelium following 3 instillations of BP plus ferric oxide. Nonciliated cells appear rounded and protrude into tracheal lumen. × 450. Inset (Fig. 1) is magnified further in Fig. 7A. × 4500.

Fig. 8. Degenerating epithelial cell. Cilia are absent, and cell margins are depressed from surface height of surrounding cells. × 3000.

Fig. 9. Tracheal epithelium after 6 instillations of BP plus ferric oxide. Broad areas of nonciliated epithelial cells with raised and rounded cell margins. × 1000.
Fig. 10. Tracheal epithelium after 9 instillations of BP plus ferric oxide. Focal area of squamous metaplasia with raised cell margins. × 300. *Inset* (Fig. 10) is magnified further in Fig. 10A. Note surface structure of underlying cells. × 1000.

Fig. 11. Tracheal epithelium 1 week after 10 BP plus ferric oxide instillations. Stereo pair of photographs. Degree of cell distortion in area of squamous metaplasia can be easily determined. × 770.
Fig. 12. Tracheal epithelium 1 week after 10 BP plus ferric oxide instillations. Distorted squamous epithelial cell from Fig. 11. Note surface character of underlying cells. x 2310.

Fig. 13. Clump of ferric oxide particles, identified by X-ray diffraction. x 2000.

Fig. 14. Tracheal epithelium 4 weeks after 10 instillations of BP plus ferric oxide. Numerous holes (arrows) mark the former location of ciliated cells. Note ferric oxide particle (F) and white blood cells (W). x 770.

Fig. 15. Tracheal epithelium 1 week after 10 instillations of BP plus ferric oxide. Large groups of cells protruding into tracheal lumen. x 610.

Fig. 16. Tracheal epithelium 4 weeks after last BP plus ferric oxide instillations. Single enlarged, protruding nonciliated cell with protruding surface. x 3500.
Fig. 17. Tracheal epithelium 4 weeks after last BP plus ferric oxide instillations. Cluster of enlarged nonciliated cells. \( \times 700 \). A, \( \times 2310 \) (A, inset, is further magnified at upper right, \( \times 7700 \)); B, \( \times 3850 \); C, \( \times 7700 \).
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