Virchows Archiv A Pathol Anat (1993) 422:47–52

Virchows Archiv A
Pathological Anatomy and Histopathology

The fibrosing process in so-called organized diffuse alveolar damage.

An immunohistochemical study of the change from hyaline membrane to membranous fibrosis

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Received February 25, 1992 / Received after revision August 5, 1992 / Accepted August 6, 1992

Summary. On the assumption that some cases of organized diffuse alveolar damage (DAD) result from organization of hyaline membrane, we collected nine autopsy cases of DAD in various stages of the fibrosing process from hyaline membrane to membranous fibrosis and studied changes in the basement membrane and epithelial cells immunohistochemically. In the majority of cases, the following sequence of events was assumed: the hyaline membrane is first formed at the tip of the alveolar septum, a part of the alveolar duct wall where epithelial cells have disappeared. With time it elongates and completely covers alveolar mouths. In the organizing stage, fibroblasts start to permeate through the alveolar duct walls to replace the hyaline membrane completely and to form membranous fibrous tissue. In a few cases, however, fibrous tissue will fill alveolar spaces to form intraluminal diffuse fibrosis. Alveolar epithelial cells and the basement membrane of the alveolar walls are well preserved until the end of the organizing stage when the basement membrane becomes distorted. We believe that membranous fibrosis represents a form of "alveolar duct damage" and that it differs from diffuse fibrosis, which is indicative of diffuse alveolar damage in the true sense.

Key words: Diffuse alveolar damage – Hyaline membrane – Immunohistochemistry – Alveolar epithelium – Basement membrane

Introduction

A descriptive term, diffuse alveolar damage (DAD) was coined by Katzenstein et al. in 1976 to denote a nonspecific tissue reaction following severe acute lung injury (Katzenstein et al. 1976; Katzenstein and Askin 1990). The causes include a variety of infectious agents, inhalants, drugs, ingestants, shock, radiation, and other miscellaneous agents. DAD is considered the morphological equivalent of the clinical entity adult respiratory distress syndrome (ARDS). Morphologically, it is divided into two stages conceptually: an acute and an organizing stage. In the acute stage, the earliest change appears in the interstitium and is associated with intra-alveolar oedema. Degenerative changes in both alveolar epithelial and capillary endothelial cells are seen ultrastructurally. At the end of this stage, the basement membrane has been denuded, oedema has been enhanced and hyaline membrane has formed. Acute changes usually undergo resolution, but in some cases organization ensues. During the organizing stage of DAD, fibroblasts proliferate, mainly in the interstitium with some proliferation of type II cells. Soon the alveolar spaces collapse and a honeycomb structure is produced. Despite a detailed outline of this disease process, the histopathological criteria for the diagnosis of both the acute and organizing stages of DAD are not clear-cut.

The presence of the hyaline membrane seems to be the only constant and characteristic feature of this condition in its early stage. However, the fibrosing process of DAD has not been well documented. Therefore, we decided to carry out an investigation of the fibrosing process using human material.

First, the assumption was made that the hyaline membrane is organized to form membranous fibrous tissue. We then collected nine autopsy cases with either hyaline membrane or membranous fibrous tissue. To identify the alveolar epithelium and basement membrane properly we employed immunohistochemical techniques; anti-human epithelial membrane antigen (EMA) antibody was used for the identification of alveolar epithelium and anti-human collagen type IV for the basement membrane (Fukuda et al. 1987; Hara et al. 1987, 1989).

Materials and methods

From 1301 cases autopsied in the Department of Pathology of Kawasaki Medical School Hospital over the past 10 years, nine cases with either hyaline membrane or membranous fibrous tissue (membranous fibrosis), representing various stages of the fibrosing

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process, were selected for this study. Membranous fibrosis as defined here is a morphological feature, with a sheet of fibrous tissue which covers alveolar duct walls. Membranous sheets of fibrous tissue usually bridge and obstruct the mouths of alveoli and attach only at the tips of the septa, i.e. the alveolar duct walls, and often provoke a collapse of alveolar spaces.

All nine cases, consisting of six males and three females, aged 25–80 years, had underlying disease: respiratory diseases (lung cancer) in two cases and non-respiratory diseases in the other seven (laryngeal cancer 1, multiple myeloma 1, liver cirrhosis 1, choledocholithiasis 1, nephrotic syndrome 1, dissecting aneurysm 1, multiple injury 1).

The causes of DAD were infectious agents in five cases, shock in two cases, an anti-cancer drug (bleomycin) in one case, and radiation in one other. Clinically, all cases had acute onset of dyspnoea and a diffuse infiltration shadow on their chest X-rays, and terminated in ARDS. Oxygen therapy and assisted ventilation were administered in all cases because of respiratory failure. The survival time after the appearance of symptoms ranged from 12 days to 3 months.

At autopsy, the lungs were fixed by instillation of 20% buffered formalin solution via the airways. Tissues from selected areas were routinely processed and embedded in paraffin. Sections 4 μm thick were stained by haematoxylin and eosin (H & E), elastic van Gieson (EVG), Masson's trichrome, and by Pap's silver impregnation method.

The following antibodies were utilized in the immunohistochemical study; anti-collagen type IV for detection of the basemant membrane and anti-EMA antibody for identification of alveolar epithelial cells. Briefly, 4-μm-thick sections were deparaffinized in xylene, dehydrated in graded alcohol, washed in distilled water, and then rinsed in TRIS-buffered saline (TBS, pH 7.6). All sections were treated with 0.07% trypsin (1:250 Trypsin, Difco, Detroit, Mich., USA) and 0.1% calcium chloride, pH 7.8 at 37° C for 60 min to unmask antigenic sites (Huang et al. 1976; Ordonez and Manning 1988). After washing with distilled water, the sections were treated with 1% hydrogen peroxide in methanol for 30 min to inhibit endogenous peroxidase activity. Then, they were washed with distilled water, rinsed in TBS, and incubated with normal horse serum at a dilution of 1:20 in TBS for 20 min to block non-specific antibody reactions. The sections were then incubated for 60 min with primary antibodies: mouse monoclonal anti-human collagen type IV antibody (Dako, Santa Barbara, Calif., USA; 1:50) and mouse monoclonal anti-human EMA antibody (Dako 1:50). After washing with TBS, the sections were incubated with rabbit anti-mouse IgG (Dako) for 30 min at a dilution of 1:50.

After washing again with TBS, they were incubated with the avidin-biotin complex (Dako) for 50 min at a dilution of 1:50. Following another washing again with TBS, the sections were reacted in a solution of 0.06% 3, 3′-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, Mo., USA) and 0.03% hydrogen peroxide in TBS for 3–6 min. Then they were washed with water, counterstained with haematoxylin, dehydrated, and mounted. All procedures were carried out at room temperature except for the steps of trypsinization.

Results

The histology of the lung in the earliest stage showed interstitial oedema and fibrin deposition along the alveolar duct wall. Sheets of hyaline membrane were attached only at the tips of alveolar septa and most of them covered alveolar mouths entirely (Fig. 1). Alveolar duct spaces and alveolar sacs tended to be dilated, whereas alveolar spaces were somewhat collapsed.

The interstitium was oedematous and contained a few inflammatory cells, such as lymphocytes and plasma

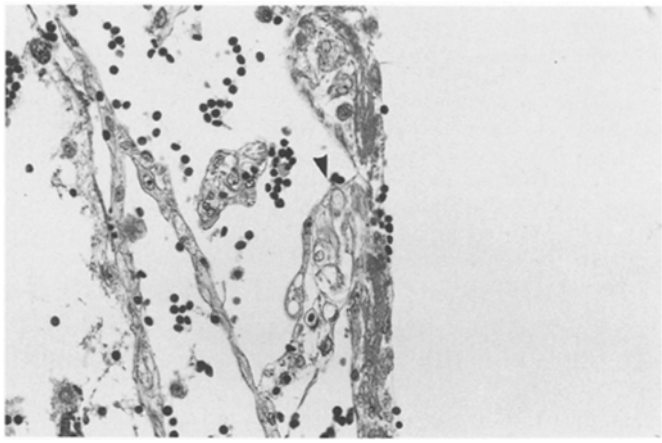

Fig. 1. Hyaline membrane formation in the early stage of diffuse alveolar damage (DAD). Note that it attaches to the alveolar duct walls and covers the alveolar mouths (*arrowhead*). H & E, ×200

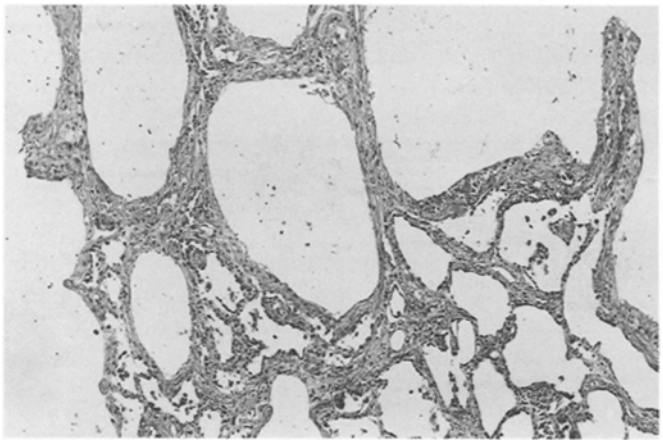

Fig. 2. Membranous fibrosis in organized DAD. The alveolar ducts are markedly dilated, while the alveolar spaces have collapsed. In this photograph no remaining fibrin is discernible. H & E, ×100

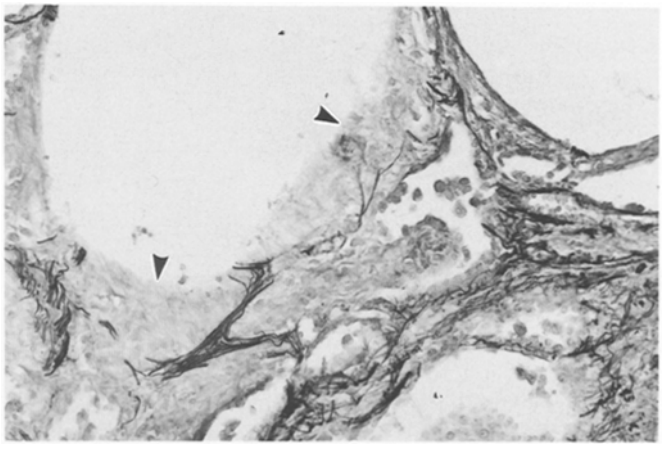

Fig. 3. The organizing stage of DAD. Membranous fibrous tissue is thicker here. Elastic fibres are not present within the fibrous tissue (*arrowheads*). Elastic van Gieson, ×200

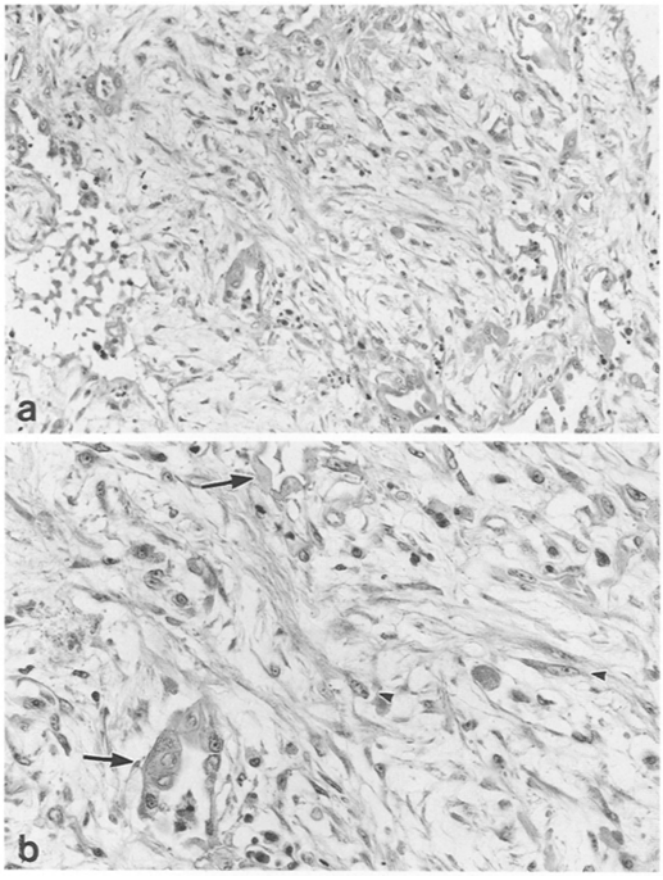

Fig. 4. a "Intraluminal diffuse fibrosis" seen in a case of organized DAD. Note that the fibrous tissue completely obliterates the alveolar spaces in places. H & E, ×100. **b** Higher magnification of **a**. The fibrous tissue consists of fibroblastic cells (*arrowheads*) and collagen bundles. Remaining alveolar epithelia are swollen (*arrows*). H & E, ×200

cells. Type II alveolar epithelia aligned along the alveolar walls were usually swollen.

EVG and Masson trichrome staining failed to demonstrate any collagenous and elastic components in the hyaline membrane at this stage. In the organizing stage, the alveolar ducts were markedly dilated and adjacent alveolar spaces were collapsed (Figs. 2, 3) imparting the appearance of a "honeycomb lung" structure. The hyaline membrane was fragmentary in the beginning and was no longer seen at the end of this stage. Membranous fibrous sheets (membranous fibrosis) were present in exactly the same configuration as the hyaline membrane. In the early phase of the organizing stage, silver impregnated fibres (so-called reticulin fibres) sprouted from the alveolar duct walls into the lower layer of the hyaline membrane. Then spindle and polygonal cells as well as collagen bundles appeared and replaced it. In one case, another fibrosing process was observed, in which fibrous tissue completely filled alveolar spaces (Fig. 4a, b). Fibrous tissue was loose but contained a few polygonal cells resembling fibroblasts, and collagen bundles.

Immunohistochemically, in the early stage of DAD after the hyaline membrane had been formed, EMA positivity remained along the alveolar walls in most

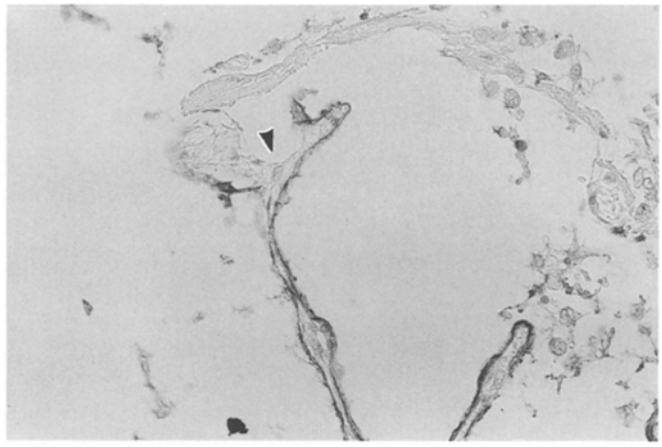

Fig. 5. Epithelial membrane antigen (EMA)-positivity exists along the alveolar walls. The alveolar duct walls, which are overlain by hyaline membrane, lack EMA positivity (*arrowhead*). IP-haematoxylin for EMA, ×200

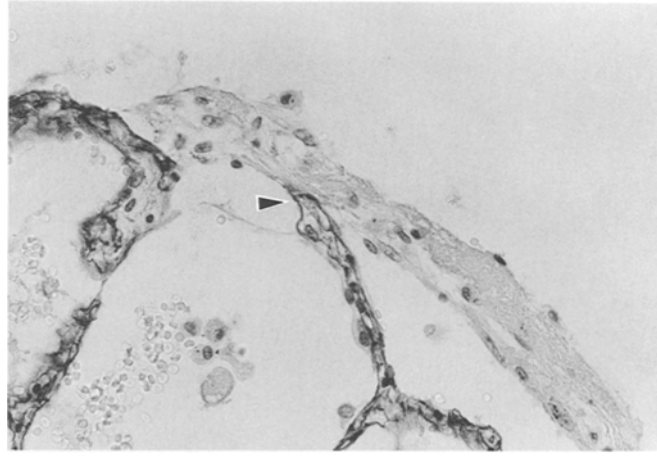

Fig. 6. Collagen type IV immunoreaction. Basement membranes appear to remain intact on the alveolar walls during the early stage of DAD (*arrowhead*). IP-haematoxylin for collagen type IV, ×200

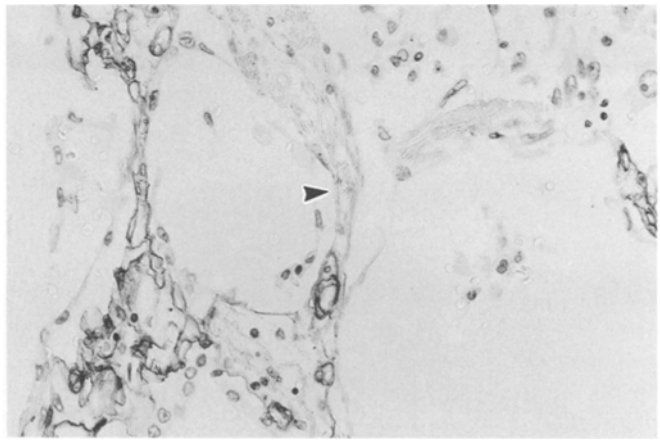

Fig. 7. Collagen type IV immunoreactivity in the organizing stage. Basement membrane appears to be intact except for the alveolar duct walls where fibroblastic cells have seemingly permeated into pre-existing hyaline membrane (*arrowhead*). IP-haematoxylin for collagen type IV, ×100

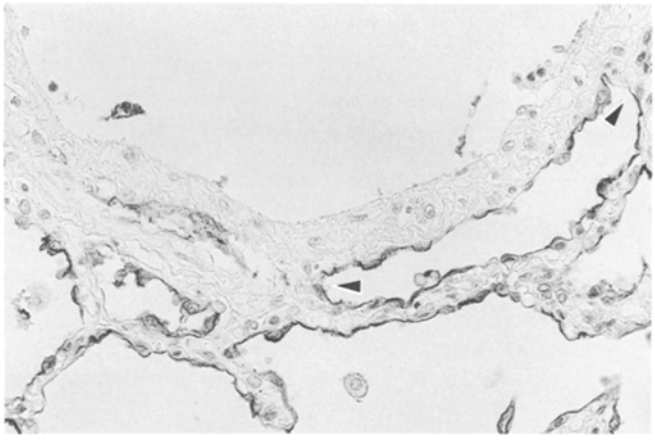

Fig. 8. EMA-positivity in organized DAD. Regenerating epithelia are seen along the undersurface (the alveolar space side) of the membrane but they are not present along the alveolar duct luminal side. Regenerating epithelia always connect with alveolar duct walls and become flat and linear (*arrowhead*). IP-haematoxylin for EMA, ×200

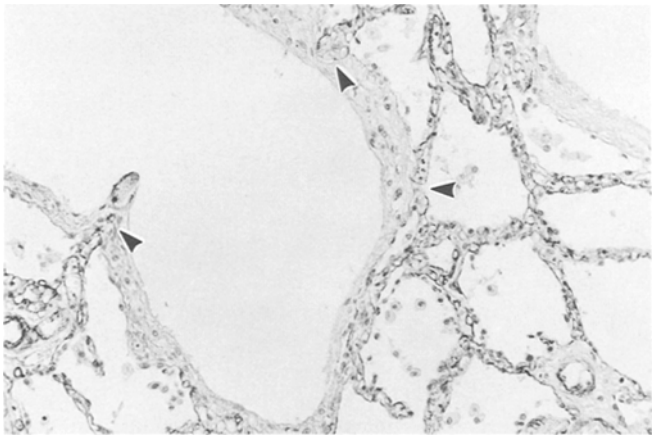

Fig. 9. Basement membrane in the organizing stage of DAD. Note that it is present along the alveolar walls and the capillaries, except for areas of the alveolar duct walls where it has disappeared (*arrowhead*). IP-haematoxylin for collagen type IV, ×100

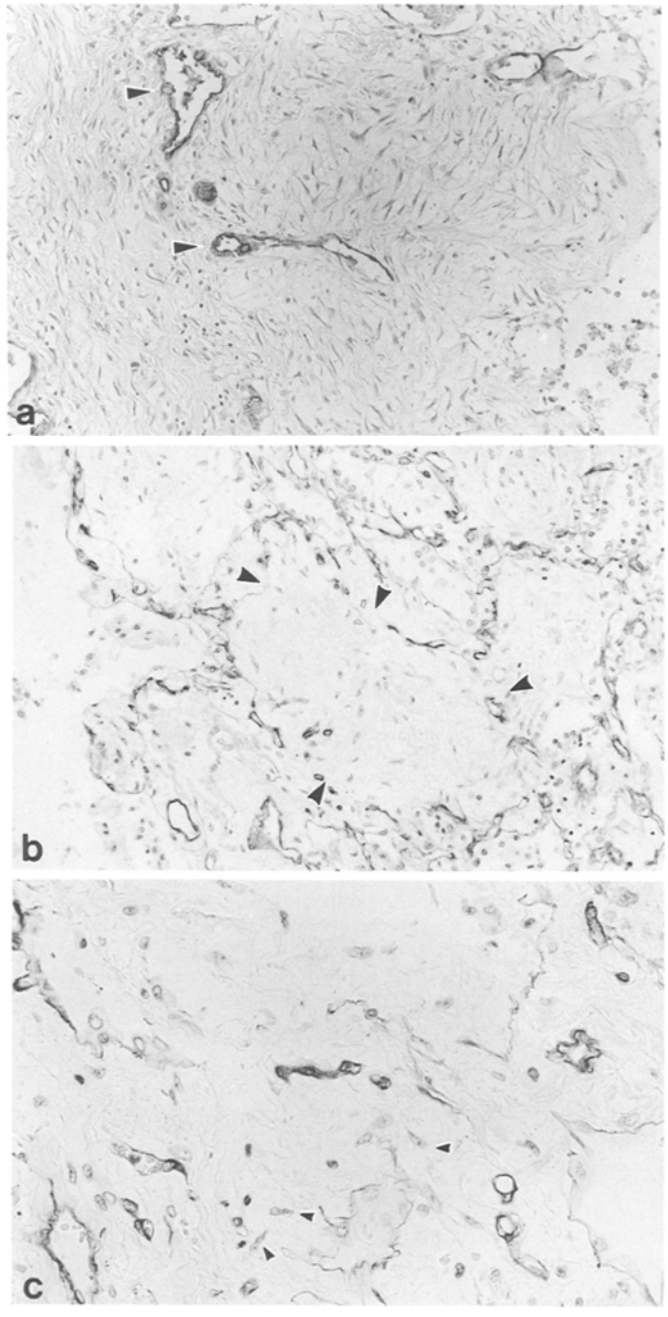

Fig. 10. a Area of intraluminal diffuse fibrosis. Note that EMA-positive cells have disappeared along the alveolar walls, but partially the remaining alveolar epithelial cells show EMA immunoreactivity (*arrowheads*). IP-haematoxylin for EMA, ×100. **b** Area of intraluminal diffuse fibrosis. The alveolar spaces are filled with fibrous tissue. The original framework of the alveolar structure is clearly demonstrated by its basement membrane. Note that the subepithelial basement membrane is focally destroyed (*arrowheads*). IP-haematoxylin for collagen type IV, ×100. **c** Magnification of **b**. Fibroblastic cells have entered alveolar spaces through a defective portion of the basement membrane (*arrowheads*). IP-haematoxylin for collagen type IV, ×200

parts, but it was absent on the alveolar duct walls in most cases (Fig. 5). The hyaline membrane was never covered by epithelial cells at this stage. In contrast, collagen type IV immunoreactivity was preserved throughout this stage. No defects were noticed along the alveolar walls or alveolar duct walls (Fig. 6). However, the linearity of collagen type IV immunopositivity became disrupted and irregular, and was lost focally especially at the alveolar duct walls (Fig. 7). In the organizing stage, after the hyaline membrane had been replaced by the collagenous component, EMA-positive cells were seen along the undersurface (the alveolar space side) of the membranous fibrous tissue. The remaining epithelial cells on the alveolar wall were swollen and cuboidal. Those cells seemed to grow on the alveolar walls and to proceed along the undersurface of the membrane centripetally through junctional points. The cells, forming a fringe on the undersurface of the membrane, tended to be rather flat and linear (Fig. 8). EMA-positive cells were never observed on the luminal aspect of the membrane. Type IV collagen, which was intact along the alveolar wall surface initially, became irregular, especially at the tips of alveolar walls (Fig. 9). A basement mem-

brane structure with collagen type IV immunoreactivity was not seen in the membranous fibrous tissue (Fig. 9). In one case, an area was observed in which EMA positivity disappeared completely along the alveolar walls, and basement membrane remained along the walls in the organizing stage (Fig. 10a–c).

Discussion

The concept of DAD seems to be well recognized and the term is frequently used in the field of lung pathology. To date, however, its pathogenesis is not understood and the fate of the hyaline membrane has not been described in detail.

Recently, Sugihara et al. (1989) and Manabe et al. (1990), workers in our laboratory, studied the fibrosing process in hyaline membrane disease. Unfortunately, however, the structure of the basement membrane was not investigated. To elucidate its pathogenesis in more detail we collected nine autopsy cases with either hyaline membrane or membranous fibrosis in the lung and did not re-examine their cases. We reconstructed the possible sequence of the fibrosing process histopathologically and identified the alveolar epithelia and basement membrane immunohistochemically. Our results support the views of the Sugihara and Manabe groups on the fibrosing process of the hyaline membrane, and suggest both that the epithelial cells of the alveolar duct walls are the primary site of injury and that the epithelial cells of the alveolar walls are generally spared. Denudation of cells over the alveolar duct wall evoked hyaline membrane formation, and organization of the hyaline membrane started at the tips of the alveolar wall, which comprise the alveolar duct wall and the edge of the alveolar mouths. Silver impregnated (reticulin) fibres and fibroblastic cells emigrated into the hyaline membrane and finally replaced it with collagenous tissue. In an immunohistochemical study with anti-type IV collagen antibody the basement membrane was at first intact after the epithelial damage. Later, linear EMA-positive cells were noted over the undersurface of the membranous fibrous tissue sheet and facing the retained alveolar spaces without apposition of the basement membrane. In general, it is said that type II alveolar epithelial cells increase in number after alveolar epithelial injury, and are important in repair (Kawanami et al. 1982). This histological change thus probably represents regeneration of cells arising from alveolar epithelium near the alveolar duct surface. In one case, membranous fibrous tissue was seen in association with intraluminal diffuse fibrosis in which fibrous tissue filled alveolar spaces. In these areas, no EMA-positive cells were present along the original alveolar walls or over the apposed fibrous tissue, suggesting that the cells were totally destroyed in areas of intraluminal diffuse fibrosis.

The basement membrane (type IV collagen) of the original alveolar walls generally remained intact during the process of either type of fibrosis. However, it became disrupted at the points through which fibroblastic cells entered the alveolar spaces, in later stages. The change in the basement membrane in these cases resembled that in paraquat lungs (Hara et al. 1989). The early stage of paraquat lung is characterized by occupation of the alveolar spaces by oedema and fibrinous exudates, after which intra-alveolar exudate is soon replaced by fibrous tissue (intraluminal diffuse fibrosis). As Hara et al. observed, the basement membrane of the alveolar walls usually retained the structure of its original framework with some disruption. It still remains to be elucidated whether the change in the basement membrane is the cause or the result of the permeation of fibroblastic cells from the walls. It is not known whether fibroblasts can go through the basement membrane without leaving holes as neutrophils or macrophages do.

We speculate that both fibrosing processes – intraluminal membranous fibrosis and diffuse fibrosis – are the result of epithelial assault and that they differ only in their severity and extent.

It should be noted that DAD includes two different morphological changes; one resulting from the damage to alveolar duct walls and the other from that to alveolar walls. They may therefore be anatomically designated as diffuse alveolar duct damage and DAD, respectively. Here, the term "diffuse" does not imply "generalized" and "bilateral", but rather indicates an extensive involvement of a certain anatomical compartment such as an alveolus. Diffuse alveolar duct damage is characterized by the presence of hyaline membrane in its early stage and by membranous fibrosis along the alveolar duct wall in its later stage. In contrast, DAD is characterized by intra-alveolar oedema with loss of alveolar epithelia in the early stage and by complete filling of alveolar spaces with connective tissue in the later stage. Diffuse alveolar duct damage prevails over DAD in general. The existence of two different fibrosing processes may imply a difference in their pathogenesis. The former, with injury of the alveolar duct walls alone, may suggest the entrance of some stimulating factors through the airways, whereas the latter, with diffuse involvement of the alveolar wall, may suggest vascular spread of the causative agents or extensive spread of these agents through the airways. Membranous fibrosis may develop de novo as Manabe and co-workers indicated and is sometimes seen in association with intraluminal polypoid fibrosis. In such cases, membranous fibrosis and intraluminal polypoid fibrosis may be related to each other in that both fibrosing processes start from the alveolar duct walls. These findings, therefore, may suggest that the same or a similar pathogenetic mechanism is operative in their development.

In the present study, all cases were commonly accompanied by interstitial fibrosis of various degrees, although interstitial fibrosis was comparatively much milder than intra-alveolar fibrosis and was limited to the areas adjacent to those where the latter change was evident. As we have reported (Kobashi et al. 1990), damage of the alveolar duct walls may evoke simultaneous intraluminal and interstitial fibrosis. In the wall of a pulmonary abscess, for example, the lack of alveolar epithelial cells on the alveolar duct walls contributes to the development of fibrosis of both types.

In summary, our study confirmed that so-called organized DAD in which pre-existing hyaline membrane is organized does exist. However, confirmation of our results should be substantiated by other studies, including animal experiments. The term DAD does not seem to be appropriate for this type of lesion; most cases are actually the result of alveolar duct damage. We therefore prefer the pathogenetic term "diffuse alveolar duct damage".

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