

Effects of exposure to fluoro-edenite fibre pollution on the respiratory system: an *in vivo* model

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Summary. An increased standardised rate of mortality from pleural mesothelioma among the population of Biancavilla (Sicily, Italy) has been attributed to exposure to fluoro-edenite fibres. Our aim was to establish whether and how these fibres may induce pathological effects using an *in vivo* model.

Lung tissue collected from 60 healthy sheep selected from six flocks habitually grazing near Biancavilla and from 10 control sheep was fixed formalin and paraffin-embedded; sections were stained with haematoxylin-eosin, Masson trichrome and Gomori argentic impregnation. Histochemical studies and immunohistochemical analysis for the localisation of TRAIL, DR5 and MMP13 were also performed.

The lungs of exposed sheep exhibited fibrosis and loss of alveolar architecture with honeycombing of alveolar cavities. Fluoro-edenite fibres were detected close to the alveolar epithelium and interstitia. The parenchyma showed hyaline degeneration and strong PAS-positivity in the interstitium, proteoglycan alterations, reflecting a damaged basal membrane and an involvement of the interstitial matrix. MMP-13 was overexpressed, mainly in fibroblasts and epithelial cells, while positivity for TRAIL and DR5 was detected on alveolar surfaces and in the vascular stroma.

The initial pathological event seems to involve first the alveoli and subsequently the interstitium, giving rise to classic honeycombing. The triggering event at the level of type I pneumocytes would damage the cytoplasmic membrane resulting in loss of cell elements and exposure of underlying capillaries, and eventually in a series of reactions including macrophage activation, possible release of growth factors and metaplastic reconstruction of lung alveoli. Immunopositivity for TRAIL and MMP-13 receptor suggests that apoptotic processes may also be activated by fluoro-edenite.

Key words: Fluoro-edenite, Sheep model, Asbestos exposure, MMP-13, TRAIL, DR5

Introduction

Fluoro-edenite ($\text{NaCa}_2\text{Mg}_5\text{Si}_7\text{AlO}_{22}\text{F}_2$) is a new mineral species recognised in 2001 by the Commission on New Minerals and Mineral Names (IMA: code 2000-049). Epidemiological studies have established a role for it in causing chronic obstructive lung disease (Biggeri et al., 2004). This asbestiform mineral fibre has been found in the benmoreitic lavas, the local stone quarry, and the materials used for buildings and road pavements (Comba et al., 2003; Biggeri et al., 2004) in Biancavilla, a town of eastern Sicily (Italy) where a cluster of mortality from pleural mesothelioma was evidenced by epidemiological survey (Di Paola et al., 1996; Paoletti et al., 2000). These fibres are similar in size and morphology to some amphibolic asbestos fibres (tremolite, actinolite, antophyllite) (Comba et al., 2003). Inhalation of asbestos fibres can cause: chronic inflammation and carcinogenesis.

Given its recent discovery (Gianfagna and Oberti, 2001), few data are available on fluoro-edenite, and those few prevalently compare it to asbestos. Cardile and co-workers (2004b) noted that fluoro-edenite induces functional modifications and affects biochemical parameters in human lung fibroblasts and alveolar epithelial cells *in vitro* and hypothesised a sequence whereby increased production of reactive oxygen species (ROS) could trigger significant DNA damage. Fluoro-edenite fibres also interfere with epithelial cell cycle by reducing the rate of proliferation and increasing the release of the proinflammatory cytokine IL-6, one of the main mediators of asbestos-induced pathophysiological response (Travaglione et al., 2003). Investigations of the ability of fluoro-edenite to induce cyto- and genotoxicity in a mouse monocyte-macrophage cell line via involvement of nitric oxide evidenced that inflammatory

disorders appear to increase the risk for lung cancer (Cardile et al., 2004a). It is well known that hydroxyl radicals generated by asbestos fibres mediate inflammatory fibrosis of the lung and DNA damage that may ultimately result in lung carcinoma and mesothelioma (Manning et al., 2002). Finally, fluoro-edenite fibres generate a significantly greater amount of hydroxyl radicals compared to crocidolite (Rapisarda et al., 2003).

The present study was directed at establishing whether fluoro-edenite induces pathological effects in the lungs of sheep habitually pastured near Biancavilla, used as an *in vivo* model. Histological changes and cell-fibre interactions at the subcellular level were studied using routine stains, histochemistry and immunohistochemistry. The presence of apoptotic processes was investigated through detection of TRAIL (tumour necrosis factor-related apoptosis-inducing ligand) and its death receptor DR5 and through the expression and localisation of collagenases-3 (Matrix Metalloproteinase, MMP-13), extracellular matrix molecules with an important role in the remodelling of lung architecture in inflammatory disease.

Materials and methods

Animals

Ten female sheep, habitually grazing 3 km from the town of Biancavilla, randomly selected from six exposed flocks (n=60) and 10 control animals from a flock habitually grazing about 30 km from the Biancavilla stone quarry were sacrificed in a slaughterhouse in September-October. Ante- and post-mortem examinations were conducted by a veterinary surgeon to establish the state of health of each subject. The age range of exposed and control animals was 4.0-6.5 years.

Histochemistry

Lung tissue from the right apical lobe and the principal and accessory lung lobes were collected from each sheep and fixed in 10% buffered-formalin; after an overnight wash specimens were dehydrated in graded ethanol and paraffin-embedded, preserving their anatomical orientation. Sections 3-4 µm in thickness were obtained according to routine procedures, mounted on sialane-coated slides and air-dried. Slides were dewaxed in xylene, hydrated using graded ethanol, and stained for histological (haematoxylin-eosin, trichrome, Gomori argent impregnation) and histochemical (PAS, Cationic Iron Colloid according to Murakami et al. 1986) studies.

Immunohistochemistry

For immunohistochemistry, sections were incubated for 30 min in 0.3% H₂O₂/methanol to quench endogenous peroxidase activity, then rinsed for 20 min

with phosphate-buffered saline (PBS; Bio-Optica). High-temperature antigen unmasking was conducted in a microwave oven.

Antibodies for localisation of TRAIL, DR5 and MMP-13, were rabbit polyclonal anti-TRAIL (Santa Cruz Biotechnology, Inc.), rabbit polyclonal anti-DR5 (Novus Biologicals, Inc.) and mouse monoclonal anti-MMP13–Collagenase 3 (Neo Markers, Inc.) used at 1:20 working dilutions. After overnight (4°C) incubation in a humidified chamber, sections were incubated with the secondary antibody; detection was performed with the Streptavidin-biotin method using 3,3'-diaminobenzidine (DAB) as chromogen (LSAB 2 System-HRP, DakoCytomation). Sections were counterstained with haematoxylin and observed with an Axioplan Zeiss light microscope.

Positive controls consisted of tissue specimens with known antigenic positivity. Negative controls were incubated without the primary antibody.

Results

Histochemistry

At light microscopic observation the lung tissue of exposed sheep exhibited secondary focal reactive septal fibrosis that was mild, extensive, or with loss of alveolar architecture. Independently of the stage of the fibrosis, fluoro-edenite fibres with characteristic birefringence were always found in close contact with the alveolar epithelium at the interstitial level (Fig. 1A). Where the bronchiolar mucosa was hyperplastic it tended to show multiseriate changes that sometimes completely occluded the lumen. In some areas of the parenchyma the alveolar epithelium was replaced by cubic epithelium (Fig. 1B); in other areas, pneumocyte loss exposed the capillaries, favouring intra-alveolar microhaemorrhages (Fig. 1C). At the level of the interstitia, endothelial changes often resulted in severe vasodilation.

In the advanced stage of the lesions, lung architecture was disrupted with irregular enlargement of the alveolar cavities, giving rise to honeycombing (Fig. 1D, 2A).

Histochemical examination demonstrated hyaline degeneration of the parenchyma and intense interstitial PAS positivity (Fig. 2B). The cationic iron colloid Murakami technique (Fig. 2C), which specifically evidences acid proteoglycans, showed their reduction in the areas involved by interstitial fibrosis and in the alveoli, whereas in the areas still unaffected by the fibrotic process and alveolar modifications were unchanged.

Extensive reticular fibre hyperplasia was evidenced by Gomori's silver staining (Fig. 2D).

Immunohistochemistry

Immunostaining for MMP-13 was overexpressed, especially in fibroblasts and epithelial cells. The

interstitia with septal fibrosis showed focal immunostaining. The perivascular venular connective tissue was intensely positive. Increased immunolabelling compared with control tissue was present in the pleural visceral layer and in the submesothelial stroma continuous with the intraparenchymal stroma (Fig. 3A). MMP-13 was also overexpressed in interlobular septa. The subepithelial connective tissue of terminal bronchioles showed increased immunopositivity (Fig. 3B).

TRAIL immunopositivity was noted on the surface of both intact alveoli and of those with damaged walls. The interstitium was positive either in normal and in hypertrophic conditions. The vascular stroma was

immunopositive. Positivity was especially intense in the multilayer epithelium (Fig. 3C).

Like positivity for TRAIL, DR5 receptor immunostaining was evident both at vascular and interstitial levels (Fig. 3D). The bronchiolar epithelium was also immunopositive.

Discussion

Sheep lung is comparable in architecture, volume and respiratory physiological parameters to human lung (Begin et al., 1981). The sheep model of asbestosis is thus an excellent tool to elucidate the pathophysiology of interstitial lung disease (Lee et al., 1997).

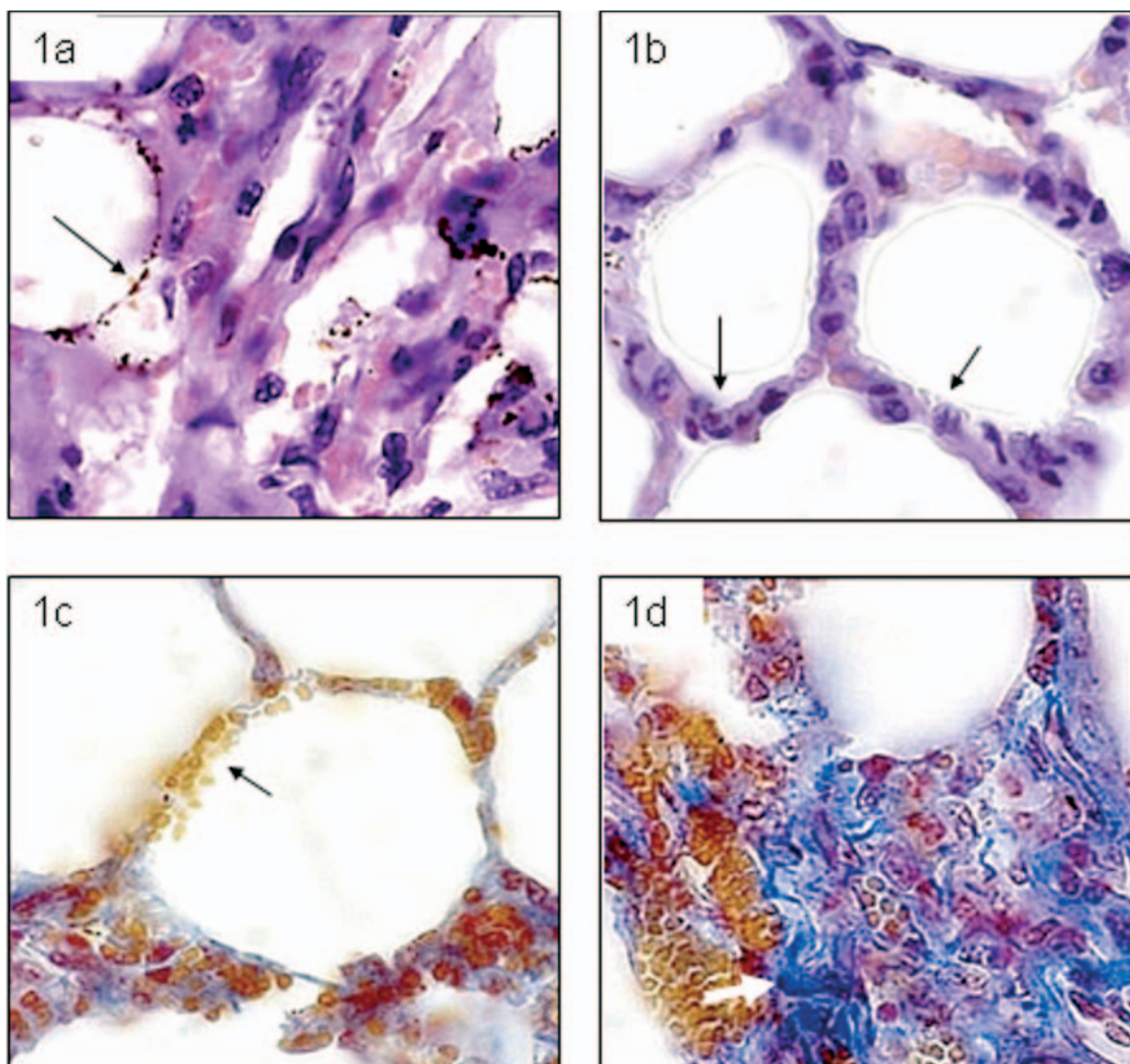


Fig. 1A. Fluoro-edenite fibres in close contact with the lung alveolar epithelium and in the interstitium. Haematoxylin-Eosin. **B.** Cubic metaplasia of alveolar epithelium. Haematoxylin-Eosin. **C.** Alveolar microhaemorrhages due to loss of pneumocytes. Trichrome. **D.** Interstitial fibrosis due to collagen fibre hyperplasia and changes in the alveolar walls. Masson. x 40

We used this model to study the pathological effects of the inhalation of fluoro-edenite fibres through expression of MMP-13 and of TRAIL and DR5 as markers of apoptotic processes.

Inhalation of asbestos fibres can induce two types of interconnected pathogenic processes: chronic inflammation and carcinogenesis, both involving the lung. These mechanisms are linked to the fibres' ability to interfere with the mitotic apparatus, stimulate host cell proliferation, induce the release of free radicals that results in DNA damage, and prolong the release of cytokines and growth factors (Kamp and Weitzman, 1999).

Our microscopic data seem to indicate that the early

pathological event is caused by a direct mechanism initially acting at the alveolar and subsequently at the interstitial level and eventually inducing a classic honeycombing. This sequence could account for the focal septal fibrosis detected in the lungs of exposed sheep. The triggering event at the level of type I pneumocytes would result in an alteration of the cytoplasmic membrane with cell loss leading to exposure of underlying capillaries, followed by a series of reactions including macrophage activation, possible release of growth factors and metaplastic reconstruction of lung alveoli.

It has been postulated that inhaled asbestos fibres penetrate in the lung, reaching the peripheral air spaces

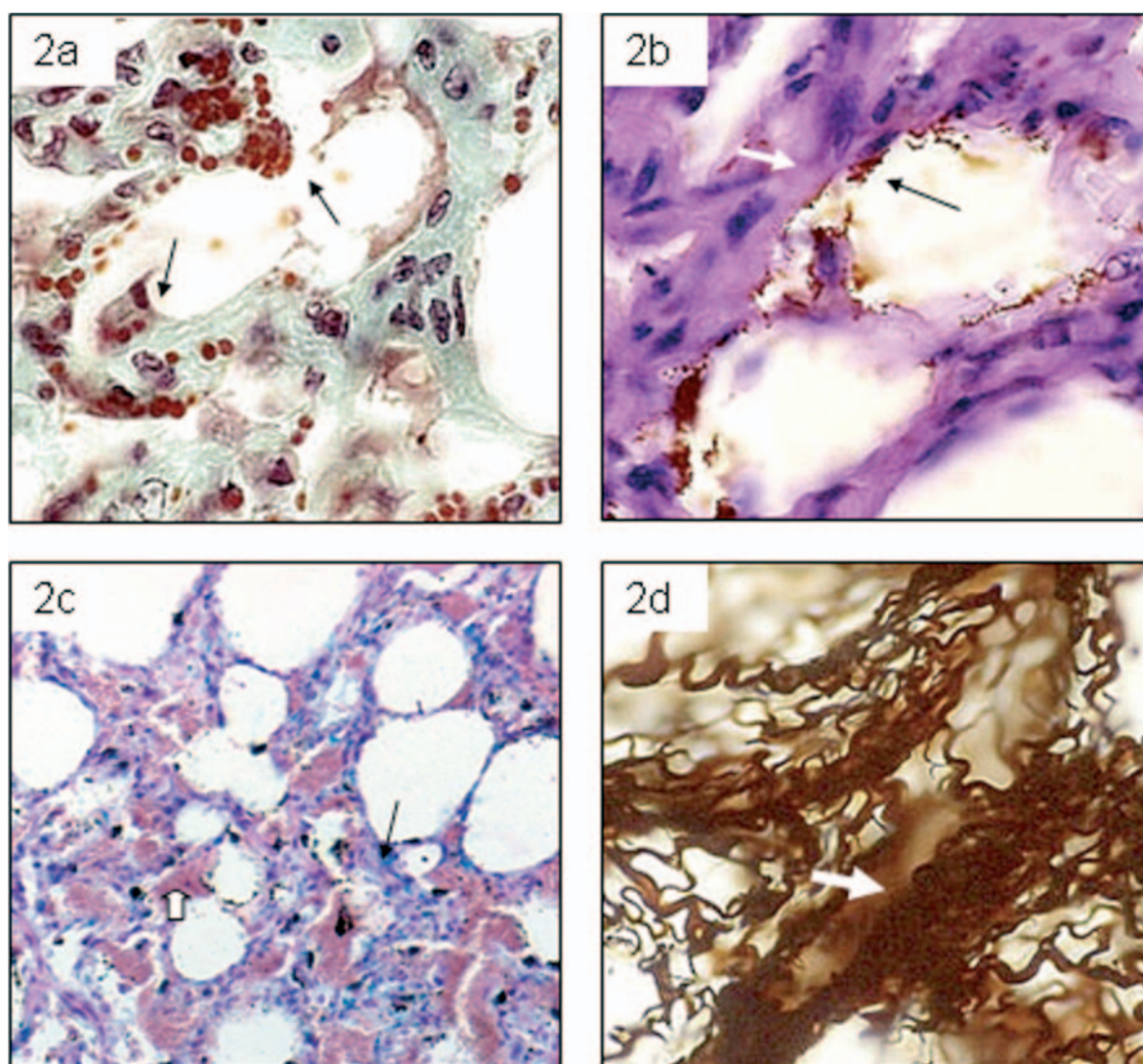


Fig. 2. **A.** Change and confluence of alveolar cavities (honeycombing); perialveolar macrophages. Trichrome. **B.** Intense interstitial PAS positivity. Arrow: fluoro-edenite fibres in an alveolus. **C.** Acid proteoglycans (thin arrow) in interstitial and perialveolar location; dilation of interstitial vessels (white arrow). Cationic iron colloid. **D.** Fibrotic thickening of lung interstitial collagen. Silver impregnation. A, B, x 40; C, x 20; D, x 100

where they are incorporated by, and activate, alveolar macrophages, triggering an inflammatory response. The early phase of this response consists in the accumulation of alveolar macrophages in the alveolar ducts and peribronchiolar regions of the terminal respiratory bronchioles followed by interstitial accumulation of macrophages and fibroblasts, resulting in interstitial thickening. Migrating asbestos fibres and oxidants released by activated macrophages also damage adjacent cells, including type I alveolar epithelial cells, disrupting epithelial integrity and allowing access of growth factors and cytokines to the interstitium. As part of the healing process, type II epithelial cell hyperplasia develops, accompanied by interstitial fibrosis with deposition of

extracellular matrix proteins (Lee et al., 1997).

The histochemical assays evidenced changes in proteoglycans (PG) that could be related to the action of MMP-13, in particular a reduction in sidecans, which reflects basal membrane damage, as well as in versicans, which reflects an involvement of the interstitial matrix. Loss of PG in the interstitial matrix also contributes to increased microvascular permeability and to impaired mechanical properties of the matrix itself.

Pulmonary fibrosis is characterised by fibroblast/myofibroblast proliferation and extracellular matrix accumulation (Pardo and Selman, 2002). The molecular mechanisms involved in the extensive structural disorganization/remodeling that characterize

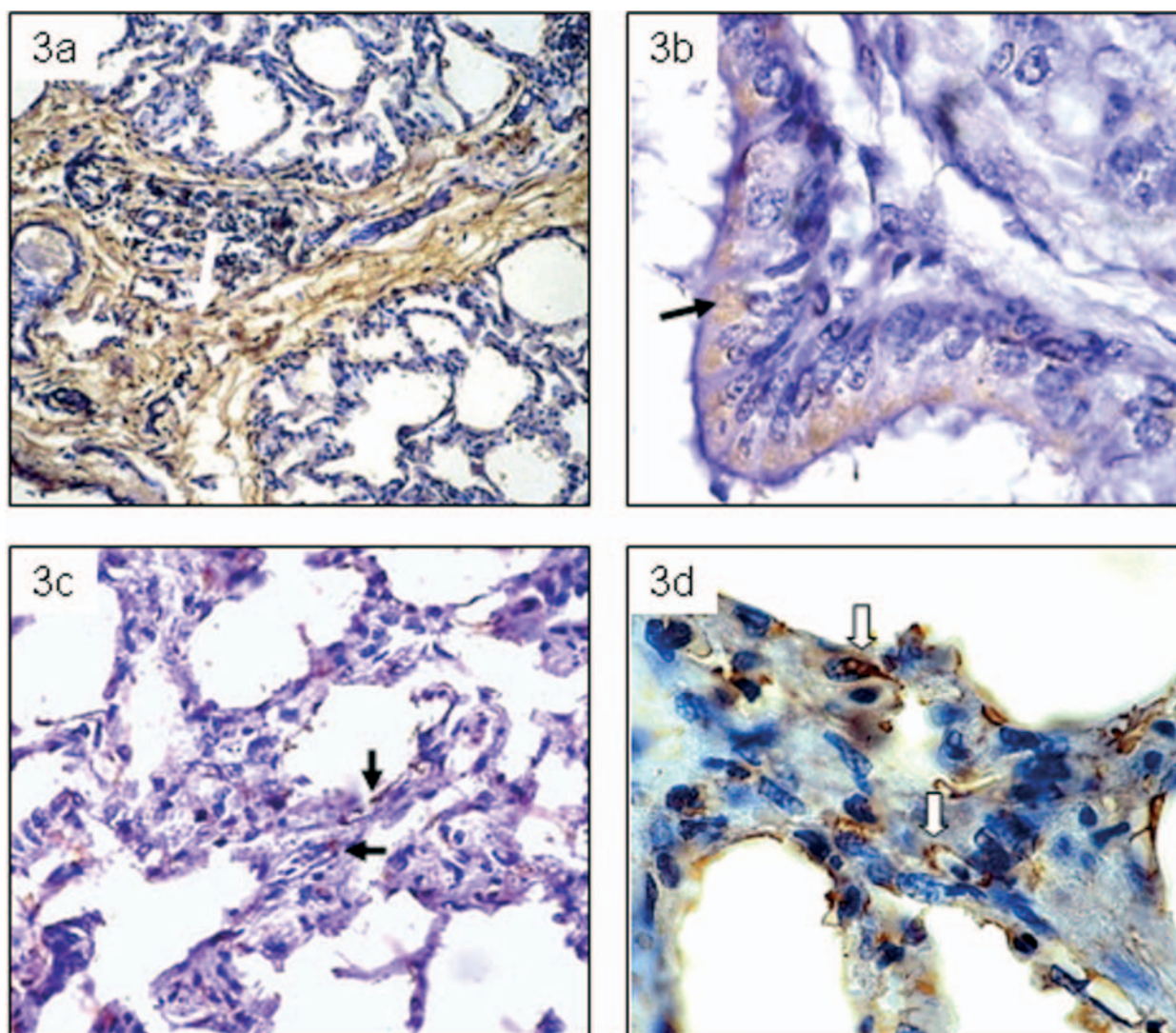


Fig. 3. **A.** Anti-MMP13 immunohistochemical reaction in alveoli and interstitia. **B.** Anti-MMP13 immunohistochemical reaction at the bronchiolar level. **C.** Alveolar and interstitial anti-TRAIL immunohistochemical reaction. **D.** Anti-DR5 immunohistochemical reaction in alveoli and interstitia. A, x 20; B, D, x 100; C, x 40

the fibrotic response also evidenced in our samples, as described in other studies (Pardo et al., 1998; Perez-Ramos et al., 1999; Selman et al., 2000), entail a disequilibrium of some MMPs.

The MMP family consists of 23 human enzymes that collectively degrade extracellular matrix components and induce selective proteolysis of cell surface receptors, adhesion molecules, chemokines, cytokines, and growth factors (Ruiz et al., 2003).

MMPs influence many cellular functions, such as migration, proliferation, apoptosis, and morphogenesis (Vu and Werb, 2000). MMP-13, in particular, seems to be expressed by many different cell types including inflammatory cells, epithelial cells, and fibroblasts (Mariani et al., 1998). Localised increases in this enzyme activity are required for the initial phases of resident cell activation and recruitment (Mariani et al., 1998). Importantly, modifications in MMP expression or activity may participate in enhancing the exaggerated accumulation of extracellular matrix also through disruption of the basement membrane, which seems to play a role in lung fibrogenesis (Raghu et al., 1985; Fukuda et al., 1998; Selman et al., 2001). On the other hand, a considerable body of literature suggests that the integrity of the alveolar epithelium and alveolar apoptosis are critical determinants in the pathways that initiate fibrogenesis in the lung (Fine et al., 1997; Hagimoto et al., 1997; Adamson et al., 1998; Uhal, 2003). In accordance with other studies (Fine et al., 1997; Hagimoto et al., 1997), we believe that alveolar epithelial cells expressing TRAIL induce epithelial cell apoptosis and lung fibrosis and in turn fibroblasts from human fibrotic lungs induce epithelial apoptosis, and fibroblastic foci are usually found close to abnormal or denuded alveolar epithelium (Uhal et al., 1995, 1998).

The honeycombing due to destruction of the alveolar walls may be explained by the hyperactivity of collagenases and gelatinases on the major extracellular matrix components, causing interstitial fibrillar collagen degradation and contributing to the breakdown of elastic fibres, as also suggested by other authors (Segura-Valdez et al., 2000).

It has been hypothesised that excessive production of oxidants and disruption of normal epithelial cell-matrix interactions caused by MMP activities might initiate apoptotic and/or necrotic pathways in pulmonary diseases (Meredith et al., 1993; Buttke and Sandstrom, 1994; Frisch and Francis, 1994; Buckley et al., 1998). Our data on the expression of TRAIL and its receptor DR5 confirm the link between fluoro-edenite and epithelial cell apoptosis at the site of initial fibre deposition in the bronchoalveolar duct region.

TRAIL is a member of the tumour necrosis factor (TNF) family of ligands, capable of initiating apoptosis through engagement of its death receptors (DR4, DR5) (Wang and El-Deiry, 2003); it selectively induces apoptosis of a variety of tumour and transformed cells, but not of most normal cells (Wang and El-Deiry, 2003), and has been found to play an important role in cell

regulation and in inflammation processes (Hasel et al., 2003; Robertson et al., 2004).

In the lungs of our exposed sheep, expression of TRAIL receptor was most pronounced in areas of inflammatory infiltration and active fibrosis, as borne out by the expression of MMP-13, which may reflect an activation of the apoptotic processes induced by fluoro-edenite.

Given the clinical and prognostic implications of fluoro-edenite exposure, occupational and otherwise, we are continuing our work on the pathological effects of these fibres.

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