**Summary.** We previously showed that apoptosis in the lungs of sheep exposed to fluoro-edenite fibres is induced via the receptor pathway. The present study was performed to gain further insights into the mechanisms of activation of programmed cell death induced by the fibres.

Fluoro-edenite fibres are similar in size and morphology to some amphibolic asbestos fibres. They have been found in benmoreitic lavas, in the local stone quarry, in building materials and in road paving at Biancavilla, a town in eastern Sicily (Italy), where epidemiological surveys revealed a cluster of mortality from pleural mesothelioma. Inhalation of asbestos fibres can cause chronic inflammation and carcinogenesis. Since fluoro-edenite has been shown to activate the apoptotic process, we set out to characterise the expression of apoptosis-regulating proteins in fluoro-edenite-exposed lung disease and sought to determine if apoptosis results from fluoro-edenite exposure.

Lung tissue from apparently healthy sheep habitually grazing near Biancavilla was processed for immunohistochemical localisation of bcl-2 and bax. Results showed epithelial and interstitial bax overexpression, especially in cells directly in contact with the fibres, and negative bcl-2 immunoexpression. TUNEL-positive cells were detected in alveoli and connective tissue.

The integrity of alveolar epithelium and alveolar apoptosis are critical determinants in the pathways that initiate fibrogenesis in the lung and fibroblastic foci are usually found close to abnormal or denuded alveolar epithelium.

Our results are consistent with the hypothesis that apoptosis is an important mechanism for removing cells with irreparable fluoro-edenite-induced genetic changes that predispose them to a neoplastic evolution.

**Key words:** Fluoro-edenite, Lung, Bcl-2, Bax, TUNEL

**Introduction**

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 (Paoletti et al., 2000). These 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.

In a recent study of the lungs of sheep chronically exposed to fluoro-edenite fibres, we demonstrated that the initial pathological event seems to involve first alveolar structures, resulting in classic honeycombing, and subsequently the interstitium, inducing fibrosis (Martinez et al., 2006). In particular, the triggering event at the level of type I pneumocytes seems to 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, metaplastic reconstruction of lung alveoli and fibrosis. Sheep lungs exposed to fluoro-edenite also displayed immuno-histochemical upregulation of TRAIL and its receptor (DR5).

A diverse group of signals induce apoptosis, including UV and γ-irradiation, oxidative damage, chemotherapeutic drugs, growth factor withdrawal, and the cytokines tumour necrosis factor α (TNF-α) and tumour growth factor β (TGF-β). The two major mechanisms regulating apoptosis include the intrinsic pathway, mediated by mitochondria, and the extrinsic pathway, induced by death signalling ligands, e.g. TNF- and Fas Ligand (FasL), which bind to their receptors and induce activation of caspase 8 (Charriaut-Marlangue and Ben-Ari, 1995; Adams and Cory, 1998; Ferri and Kroemer, 2001; Green and Reed, 1998). Oxidant-
induced DNA damage is one stimulus that can activate the intrinsic pathway, resulting in outer mitochondrial membrane permeabilisation, followed by release of apoptosis-inducing factors (Charriaut-Marlangue and Ben-Ari, 1995; Adams and Cory, 1998). The mitochondrial pathway is under the influence of regulatory molecules: anti-apoptotic proteins such as bcl-2 and bcl-x as well as proteins that promote apoptosis, such as bax and bad (Sato et al., 1994; Sedlak et al., 1995; Yokohori et al., 2006). These proteins seem to exert their effects by altering the mitochondrial transmembrane potential, thus stimulating or preventing release of apoptogenic factors, and by promoting or inhibiting the cleavage of execution caspase, such as caspase 3, which ultimately leads to apoptosis through nuclear damage (Charriaut-Marlangue and Ben-Ari, 1995; Mignotte and Vayssiere, 1998; Renvoie et al., 1997).

Reactive oxygen radicals are known to be involved in mediating asbestos-induced alveolar epithelial cell injury by causing DNA strand break, lipid peroxidation, and activation of signal transduction pathways (Kamp and Weitzman, 1999). Mounting evidence suggests that apoptosis has an important pathogenic role in lung injury caused by various agents, including asbestos (Berube et al., 1996; Hamilton et al., 1996).

Poly(ADP)ribose polymerase (PARP) is an enzyme that participates in the repair of oxidant-related DNA damage (Hyslop et al., 1988).

Changes in bax and bcl-2 expression may have a large role in inducing susceptibility to apoptosis in pneumocytes and interstitial cells (Guinee et al., 1997).

All these data suggest that fluoro-edenite-induced pulmonary toxicity can result from altered apoptotic mechanisms in alveolar epithelium and lung interstitium that are at least in part regulated by mitochondria.

In this study, we investigated lung tissue from sheep grazing in the vicinity of Biancavilla to determine whether two apoptosis regulatory proteins, bcl-2 and bax, also participate in epithelial and interstitial cell apoptosis. We hypothesised that events in the early apoptotic stages, such as changes in bax and bcl-2 immunoreactivity, may have a role in inducing pneumocyte and interstitial cell susceptibility to apoptosis, suggesting activation of programmed cell death by fluoro-edenite, and that this can be demonstrated by expression of caspase 3 and PARP and by visualisation of DNA strand breaks.

Materials and methods

Animals

Ten ewes habitually grazing 3 km from the town of Biancavilla were randomly selected from six exposed flocks (n= 60); control ewes (n=10) were from a flock habitually grazing about 30 km from the local stone quarry. Animals 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.

Histology

Lung tissue (1 cm³) from the right apical lobe and the principal and accessory lung lobes was collected from each subject and fixed in 10% buffered formalin for 2 h; after an overnight wash specimens were dehydrated in graded ethanol and paraffin-embedded.

Sections were cut 3-4 mm in thickness, mounted on sialane-coated slides and air-dried. Slides were dewaxed in xylene, hydrated using graded ethanol, and stained for histological observations (haematoxylin-eosin).

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 0.01 M phosphate-buffered saline (PBS), pH 7.4 (Bio-Optica, Italy). Sections were irradiated (3x5 min) in capped polypropylene slide-holders with citrate buffer (pH 6.0) using a microwave oven (750 W) to unmask antigenic sites.

Mouse anti-human monoclonal bcl-2 antibody and rabbit-human polyclonal anti-bax antibody, which specifically reacts with a 21 kDa protein that localises to mitochondria (both at 1:100 dilution, Dakocytomation, Italy) were used. A polyclonal and a monoclonal rabbit antibody (both at 1:100 dilution, Dakocytomation, Italy) were used. A polyclonal and a monoclonal rabbit antibody (both at 1:100 dilution) were used for localisation of PARP and caspase 3, respectively (Cell Signaling Technology, Inc. USA).

After overnight incubation (4°C) in a humidified chamber, sections were incubated with the secondary antibody; detection was performed with the Streptavidin-biotin method (LSAB 2 System-HRP, Dakocytomation) using 3,3’-diaminobenzidine (DAB) as the chromogen (Dakocytomation). Sections were counterstained with haematoxylin and observed with an Axiosplan light microscope (Zeiss, Italy).

Positive controls consisted of spleen tissue. Negative control sections were processed like the experimental slides except that they were incubated with PBS instead of the primary antibody.

Staining intensity was assigned a semiquantitative score: 0 = no reactivity, 1 = weak reactivity, 2 = moderate reactivity, and 3 = strong reactivity. Three independent observers (two anatomical morphologists and a histologist) assessed the immunohistochemical reaction, and in the few cases where their evaluations were divergent an agreement was reached after discussion.

In situ detection and measurement of apoptotic cells

For in situ detection of apoptosis at the level of single cells we used terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labelling, TUNEL (In Situ Cell Death Detection Kit,
The method involves the addition of deoxyuridine triphosphate (dUTP) labelled with fluorescein to the ends of the DNA fragments by the catalytic action of TdT. All end labelling experiments were performed in triplicate so that the results for various tissue samples, including rat prostate, could be standardised. Paraffin-embedded lung sections 5 µm in thickness were dewaxed as previously described. Slides were rinsed twice in 0.01 M PBS (pH 7.4), transferred to 0.07 M citrate buffer (pH 6.0; Bio-Optica) and subjected to 750 W microwave irradiation for 1 min for permeabilisation. Sections were then immersed in Tris-HCl 0.1 M, pH 7.5, containing 3% bovine serum albumin (both from Roche) and 20% normal bovine serum (Sigma-Aldrich, USA) for 30 min at 20°C, rinsed twice in PBS and immersed in TdT buffer (Roche Applied Science, Germany). Sections were then covered with TdT and fluorescein-labelled dUTP in TdT buffer and incubated in a dark humid chamber at 37°C for 60 min. All sections were incubated with an antibody specific for fluorescein conjugated to peroxidase with 30 min incubation at 37°C. Staining was visualised with DAB, which stained brown nuclei with DNA fragmentation. Sections were counterstained with the nuclear dye methyl green, 0.5%.

For negative controls, TdT was omitted from the reaction. Positive controls consisted of rat prostate gland after castration.

Ten fields from randomly selected slides were observed under a light microscope. Each field was photographed with a digital camera (Canon, Japan) at x60 magnification. On each photomicrograph three observers blinded to sample identity counted the number of total epithelial and mesenchymal cells as well as the number of these cells exhibiting a positive TUNEL reaction. The proportion of positive cells in the total number of epithelial and mesenchymal cells was calculated for each photomicrograph and a mean value was obtained for each sheep. The results were expressed as a percentage.

**Statistical analysis**

Data analysis was performed using the SPSS-PC software (SPSS Inc., USA). Data were tested for normality with the Kolmogorov-Smirnov test. All variables were normally distributed. Comparisons between two means were tested with Student’s t test. A value of $P \leq 0.05$ was considered significant. Cohen’s kappa was applied to measure the agreement between the three observers and averaged over all three to evaluate overall agreement using the following grading: 0-0.2 (slight), 0.21-0.40 (fair), 0.41-0.60 (moderate), 0.61-0.80 (substantial), and 0.81-1.0 (almost perfect).

**Results**

**Histology**

At light microscopic level, lung tissue from exposed sheep exhibited secondary focal reactive septal fibrosis that varied between mild, extensive, and/or with loss of alveolar cytoarchitecture. Independent of the degree of fibrosis, fluoro-edenite fibres were consistently found in close contact with the alveolar epithelium and within the interstitium (Fig. 1A,B).

In the advanced stage of the lesions, lung architecture was disrupted with irregular enlargement of the alveolar cavities giving rise to honeycombing.

**Immunohistochemistry**

In exposed sheep lung focal bax overexpression was...
Fluoro-edenite exposure activates lung cell apoptosis

Fig. 2. A. Bax staining in unexposed sheep lung. x 20. B. Bax immunohistochemical reaction in alveoli and interstitium and fluoro-edenite fibres in exposed sheep lung. x 20. Inset: black arrows indicate the fluoro-edenite fibres. C. Bax immunoreaction in exposed bronchiolar mucosa. x 20.

Fig. 3. A. Bcl-2 immunostaining in unexposed sheep lung. x 40. B. Negative bcl-2 immunoreaction in alveoli and interstitium and fluoro-edenite fibres (black arrows) in exposed sheep lung. x 20. Inset: black arrows indicate the fluoro-edenite fibres. C. Negative bcl-2 immunostaining at the bronchiolar level in exposed lung. x 20.
demonstrated in the alveolar epithelium and interstitium, particularly in alveolar epithelial cells in close contact with the fibres (Fig. 2B) and in the mucosal epithelium lining the terminal bronchioles (Fig. 2C). Interstitial immunopositivity was mostly detected in the areas of reactive fibrosis at the sites of fibre penetration. After penetration into bronchial and alveolar cavities, several fibres reached the interstitium, giving rise to a fibrotic reaction that was proportional to the degree of penetration and led to more intense immunostaining in these areas.

All sections of exposed sheep lung were negative for bcl-2 immunolabelling (Fig. 3B,C).

In exposed lungs, caspase 3 and PARP immunoreactivity was markedly increased in the areas where fibres were embedded and was mainly localised to the pulmonary and bronchial epithelium and to the fibrotic interstitium (Fig. 4B-5B).

Interobserver agreement, measured as kappa coefficient, was 0.84 (almost perfect).

In situ detection of DNA fragmentation

Lung sections were examined for evidence of apoptotic cells. DNA fragmentation was visualised in situ using the TUNEL assay.

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Fig. 4.A. Caspase 3 immunohistochemical reaction in unexposed sheep lung. x 20. B. Caspase 3 immunohistochemical reaction in alveoli and interstitium in exposed lung. x 20. Inset: the same section at lower magnification.

Fig. 5.A. PARP immunohistochemical reaction in unexposed lung. x 20. B. PARP immunohistochemical reaction in exposed alveoli and interstitium. x 20.
Microscopic observation of exposed lungs evidenced TUNEL-positive cells both at the level of septal interstitia and in alveolar epithelium. Light microscopic studies demonstrated TUNEL-positive cells in the alveolar wall (Fig. 6D), in interstitium and in bronchiolar epithelium (Fig. 6B).

**Fig. 6.A** TUNEL reaction in unexposed sheep lung bronchiolar structure. x 30.  
**Fig. 6.B** TUNEL reaction. Apoptotic cells at the bronchiolar and interstitial levels in exposed lung. x 20.  
**Fig. 6.C** TUNEL reaction in unexposed lung alveolar structures. x 40.  
**Fig. 6.D** TUNEL reaction in exposed lung. Alveolar apoptotic cells. The open arrow points at nuclear changes; solid arrows indicate apoptotic bodies. x 100.
Positive lung cells exhibited morphological evidence of apoptosis, i.e. cell surface blebs, altered nuclear shape, apoptotic bodies and a parallel loss of nuclear volume, despite preserved integrity of the plasma membrane (Fig. 6D).

The percentage of TUNEL-positive cells was significantly greater (P<0.01) in exposed compared with control lungs (Fig. 7).

Interobserver agreement, measured as kappa coefficient, was 0.81 (almost perfect).

Discussion

Epidemiological studies have shown that exposure to fluoro-edenite correlates directly with increased pulmonary morbidity and mortality. However, the mechanisms by which the fibres may cause cellular and molecular toxicity are poorly understood. To gain further insight into the mechanisms and to follow up on our previous studies of lung apoptosis activation by fluoro-edenite, we investigated programmed cell death in vivo by analysing immunoexpression of bax protein and bcl-2 oncoprotein (which are involved in the early stages of apoptosis); caspase 3; PARP, and DNA strand breaks (which are the end result of this process after the molecular “point of no return”) in lung tissue.

Apoptosis is an integral part of the cell cycle. It is involved in the morphogenesis of embryonic tissue and in the homeostasis of adult organs and tissues, removing unneeded cells in many organ systems (Huppertz and Kaufmann, 1999).

The apoptotic cascade has been divided into three sets of stages. *Initiation stages* include induction of the cascade, for instance by ligand-receptor interactions leading to the first proteolytic event. *Execution stages* begin with the activation of execution caspase such as caspase 3: this is called the “point of no return” since, once activated, these proteases degrade a variety of proteins (including those involved in DNA maintenance and repair such as PARP), resulting in irreversible cell damage. These complex events lead to apoptotic death, with collapse of the nucleus and of the cell itself (Huppertz and Kaufmann, 1999).

Reactive oxygen radicals are additional powerful triggers of DNA single-strand breakage and induce PARP activation (Szabo, 1998). PARP participates in DNA repair during moderate sublethal oxidant stress and in vitro studies indicate that asbestos fibres are capable of activating PARP in pulmonary rat cells (Dong et al., 1995; Ollikainen et al., 2000).

Apoptosis occurs if a cell is given sufficient time to organise a series of intracellular events that inevitably lead to its destruction. It follows that the apoptotic cascade is under the intrinsic control of individual cells. Apoptosis can therefore be regarded as an injury-limiting mode of cell disposal (Huppertz and Kaufmann, 1999).

Studies of asbestos-induced pulmonary toxicity have shown a direct genotoxic effect by induction of DNA strand breaks and apoptosis in relevant lung target cells. Moreover, asbestos-induced neoplastic transformation may result in a situation where DNA damage overwhelms DNA repair in the face of a persistent proliferative signal (Upadhyay and Kamp, 2003). Uhal and co-workers (1995) suggested that apoptosis might be a mechanism of cell death in fibrosing lung diseases.

Excessive apoptosis may promote lung injury and fibrosis, while failure of normal apoptotic mechanisms may contribute to cancer formation and resistance to chemotherapy (Upadhyay and Kamp, 2003). A recent study demonstrated that asbestos-induced mesothelioma is highly resistant to therapy in part because of its resistance to apoptosis, due to increased expression of the antiapoptotic protein bcl-2 and decreased expression of the proapoptotic protein, bax (Narasimhan et al., 1998). Our data demonstrating poor bcl-2 immunoreactivity and bax upregulation, suggesting an increase in the concentration of bax homodimers that enable apoptosis, agree with the findings of Narashimhan and colleagues (1998), who documented nearly absent bcl-2 expression and uniform bax expression in malignant pleural mesothelioma cell lines. Plataki and co-workers found increased expression of proapoptotic and reduced expression of antiapoptotic molecules in epithelial cells from diseased lungs that may be responsible for inadequate and delayed re-epithelisation, which in turn contributes to fibroblast proliferation. Some evidence also suggests that apoptosis is directly involved in loss of alveolar wall cells (Yokohori et al., 2006). Recent studies have suggested a role for epithelial apoptosis as a key profibrotic event in lung fibrogenesis (Li et al., 2004). Nonetheless, it is broadly accepted that tumour growth also results from cell escape from apoptotic death (Niehans et al., 1997).

Our data are consistent with the hypothesis that apoptosis is an important mechanism for removing cells with irreparable fluoro-edenite-induced genetic changes that predispose them to a neoplastic evolution.
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References


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