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Mode of action of fibrous amphiboles: the case of Biancavilla (Sicily, Italy)

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Abstract

Background. The inhalation of fibrous amphiboles can result in pulmonary fibrosis, lung cancer and mesothelioma. Although these fibres have the same disease-causing potential, their different morphologies and chemical composition can determine different biological activities. An unusual cluster of mesothelioma was evidenced in Biancavilla (Sicily) where no inhabitant had been significantly exposed to asbestos.

Objective. We herein discuss the mechanism of action of amphiboles, focusing on the fibres identified in the study area.

Results. Human lung carcinoma cells have been exposed to two different materials: prismatic fluoro-edenite and fibres with fluoro-edenitic composition. Only in the second case, they exhibit features typical of transformed cells, such as multinucleation, pro-survival activity and pro-inflammatory cytokine release. Accordingly, *in vivo* studies demonstrated that the fibrous sample only could induce a mesotheliomatogenic effect.

Conclusions. Fibres with fluoro-edenitic composition behave similarly to the asbestos crocidolite, whose connection with inflammation and lung cancer is well established.

Key words

- fibrous amphiboles
- asbestos
- fluoro-edenite
- Biancavilla
- mesothelioma

INTRODUCTION

Asbestos is a generic term used to identify six well-known silicate minerals belonging either to amphiboles (amosite, crocidolite, anthofillite, actinolite, tremolite) or serpentine (crysotile), two families that have in common a fibrous morphology [1]. Some of these minerals were of significant industrial and economic importance and have been widely used [2], especially in building industry. Occupational exposure to asbestos fibres can cause pulmonary fibrosis (asbestosis), lung cancer and malignant mesothelioma (MM) but also non-pulmonary diseases, including peritoneal mesothelioma, ovarian and larynx carcinoma, as well established in both humans and experimental animals [1]. The International Agency for Research on Cancer Asbestos has classified asbestos as belonging to Group I human carcinogens (IARC, 1987).

Nevertheless, asbestos fibres continue to pose an important health concern due to the long latency period of asbestos-induced diseases. Even if commercial use of asbestos has decreased, old asbestos-containing buildings, the importation of asbestos-containing products, as well as non-commercial asbestos and other elongate mineral particles, represent an important environmental problem. Indeed, there is evidence that the inhalation of these fibres can provoke two types of inter-connected pathogenetic processes: chronic inflammation and carcinogenesis, both involving the lung, after deposition of asbestos particles. *In vitro* and *in vivo* studies demonstrate that, although all types of asbestos fibres have the same disease-causing potential, important determinants for the biological activities of these fibres are their dimensions, surface properties, shape and crystallinity, chemical composition, physical durability, exposure route, duration of the exposure, and dose. For example, amphiboles might be more carcinogenic than serpentine [1].

In this context, to explain the increased pathogenicity of the amphiboles crocidolite and amosite in the induction of human mesothelioma, the "amphibole hypothesis" was advanced: while chrysotile fibres appear to dissolve or fragment over time, amphibole asbestos persisting at sites of tumour development, serve as a chronic stimulus necessary for neoplastic growth [3].

MODE OF ACTION OF FIBROUS AMPHIBOLES: STATE OF THE ART

Although the understanding of the sequence of events, starting with mineral fibres and cells' interaction and leading to a disease development is of primary importance, yet the cellular and molecular mechanisms by which asbestos fibres induce cancer or other diseases remain to be clarified.

Most evidence highlight asbestos fibres' ability to: i)

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interfere with the mitotic apparatus; ii) stimulate host cells' proliferation; iii) induce genetic and epigenetic alterations, as well as cellular toxicity and fibrosis; iv) produce oxidative stress that results in DNA damage and inflammatory cytokines and growth factors' release [4, 5]. In particular, reactive oxygen species (ROS) are known to be involved in mediating asbestos-induced mesothelial cells injury by causing DNA strand break, lipid peroxidation, and activation of signal transduction pathways [6]. However, *in vitro* cell culture studies have provided paradoxical evidence that exposure of mesothelial cells to asbestos causes cytotoxicity or apoptosis rather than malignant transformation [7]. These authors explain their results hypothesizing that one of the principal mechanisms to generate ROS is associated with the contents of asbestos fibre, especially iron [8]. In fact, it has been shown that the iron associated with asbestos participates, via ROS, in the cell toxicity and probably in MM pathogenesis, but the molecular mechanisms largely remain unknown. In this context, some authors have shown that, in asbestos-exposed cells, molecular oxygen mainly located in mitochondria is converted into O_2^- by reduction catalyzed by the iron. This O_2^- is a moderately reactive species capable of generating H_2O_2, which in turn can produce highly reactive hydroxyl radicals (HO•) via iron-dependent catalytic reactions, Fenton reaction and/or Haber–Weiss reaction [6, 8]. Indeed, the chemical properties of asbestos fibres, especially iron content, can contribute to the formation of ROS that is closely related to asbestos-induced pathogenesis [8]. There exist intrinsic defence systems that counteract ROS toxicity in mammalian cells, including the major iron storage protein ferritin. This protein, which is composed of a heavy chain and a light chain, has enzymatic properties, converting Fe^{2+} to Fe^{3+}, as iron is internalized and sequestered in the ferritin mineral core. This function is an inherent feature of ferritin heavy chain (FHC) subunit that exhibits ferroxidase activity, required for iron sequestration. It has been shown that FHC regulates the intracellular iron which catalyses the formation of toxic ROS. In fact, it was demonstrated that FHC works as an anti-apoptotic protein against toxic asbestos and oxidative stress in human mesothelial cells and MM cells [9]. Indeed, MeT-5A human mesothelial cells stably expressing FHC generated less amount of hydrogen peroxide (H_2O_2), one of the main ROS, after asbestos exposure and were more resistant to apoptosis induced by H_2O_2 compared with the cells transfected with the empty vector. These results suggest the contribution of FHC to apoptosis resistance of the MM cells and the potential role of FHC in the pathogenesis of asbestos-induced mesothelioma.

It is worth noting that the events involved in the complex mechanisms of asbestos-induced diseases are not completely independent one from each other and the contribution of each of them might vary depending on the species and on fibre and disease types.

THE CASE OF BIANCAVILLA'S AMPHIBOLE

An epidemiological survey (carried out from 1988 to 1992) on the mortality due to malignant pleural neoplasm in Italy, highlighted an unusual epidemiological cluster of mesothelioma in Biancavilla, a village located in the Etnean Volcanic Complex (Catania, Italy) of eastern Sicily, where no inhabitants had been significantly and professionally exposed to asbestos. The possible cause of such unusual distribution of the pathology was proposed to be the stone quarries located in "Monte Calvario", in the southeast of Biancavilla. All the population, in fact, was exposed to the material derived from stone quarries that had been largely used in the local building industry. Among the minerals present in the benmoreitic lavas extracted from quarries, a new amphibole, initially referred as to an anomalous Na- and F-rich tremolite-actinolite intermediate phase, was discovered. A subsequent crystal-chemistry investigation of the Monte Calvario amphiboles [10] led to the identification of this mineral as "fluoro-edenite", a new end-member of the edenite Þ fluoro-edenite series approved on 30 January 2001 by the CNMMN (Commission on New Minerals and Mineral Names, IMA: cod. 2000-049) [11].

In vitro studies

In this context, we have performed studies aimed at analyzing the effects of the different forms of fluoro-edenite in A549 cells, a tumour cell line from human lung carcinoma with properties of alveolar epithelial cells (*Table 1*). This cell line is largely acknowledged as a suitable model to study the interaction of environmental particulates with lung epithelial cells, epithelia representing the first line of defence against air dispersed harmful substances *in vivo*.

In the first study [4], we have focused on the prismatic form of fluoro-edenite, identified and sampled in the quarry of Mount Calvario. We found that epithelial cells showed a remarkable tropism toward fluoro-edenite material. In fact, the presence of fluoro-edenite in the culture medium induced an active response by epithelial cells, which developed membrane ruffles and filopodia. These structures established a first contact with the fibres, then progressively surrounded and subsequently wrapped around the material, thus engulfing it into the cell cytoplasm through a phagocytic-like process (*Figure 1b*). Surprisingly, the organization of the actin cytoskeleton, which represents the main engine of phagocytosis and one of the key target for a huge number of toxicants, was not handled by the contact with fluoro-edenite and its architecture remained unchanged, appearing well organized in stress fibres both in control and in treated A549 cells (*Figures 1d* and *1e*). However, the prismatic fluoro-edenite was able to interfere with epithelial cell physiology, by reducing the proliferation rate without perturbing the passage of cells through the different phases of the cell cycle. Moreover, in analogy with other asbestos fibres, fluoro-edenite treatment ensued IL-6 secretion, a multifunctional cytokine with immunoregulatory and pro-inflammatory effects. Hence, in this paper we did not evidence any particular effects of the prismatic fluoro-edenite that could be somehow related to cellular transformation. In accordance with these findings, it has been reported that intraperitoneal administration of prismatic fluoro-

Table 1
Effects of prismatic fluoro-edenite and fibres with fluoro-edenitic composition, in comparison with crocidolite, in A549 cells

Fibre type cellular effects on:	Prismatic fluoro-edenite	Fibres with fluoro-edenitic composition	Crocidolite
- *actin cytoskeleton*	No change in the actin network, which remains well organized in stress fibres	Dramatic changes in the actin network structure	Dramatic changes in the actin network structure
- *phagocytic-like activity*	Cells develop actin-rich protrusions from the plasma membrane (ruffles and filopodia) to wrap the fibres	Close interaction and strong tropism of cells towards the amphibole material. These events are accompanied by an arising of actin-rich membrane ruffles from the cell surface	Evident close interaction and strong tropism of cells towards the amphibole material thanks to a plethora of actin-rich membrane ruffles stemming from the cell surface
- *antiproliferative capacity*	Dramatic decrease in the number of viable epithelial cells without arrest or interference with the cell cycle	Dramatic decrease in the number of viable epithelial cells without arrest or interference with the cell cycle	Dramatic decrease in the number of viable epithelial cells without arrest or interference with the cell cycle
- *induction of cytokines expression*	Increase of IL-6 secretion in the supernatant	Increase of both IL-6 and IL-8 secretion in the supernatant	More consistent secretion of IL-6

edenite failed in inducing the development of mesothelioma in experimental rats [12].

In contrast, when fibres with fluoro-edenitic composition are concerned, the same authors [12] reported their strong mesotheliomatogenic effect on the peritoneum and, to a much lesser extent, on the pleura. In keeping, when the activity of such a fibrous form was investigated in the same cell line, the A549 cells [13], we found that its effects were very different from those induced by prismatic fluoro-edenite. In particular, the newly characterized fibrous material provoked dramatic changes in the cell morphology, promoting the spread out of cells and the multinucleation [13], which most probably was caused by a failure in the formation of the actin contractile ring at the last stage of cytokinesis (*Figures 1c and 1f*). A close link between actin derangement and multinucleation has been already reported as occurring in cells challenged with renowned actin cytoskeleton-perturbing agents, either promoting the assembly or the breakdown of the cytoskeleton. Importantly, multinucleation has been indicated as a marker of cell transformation.

The above changes in morphology, which were accompanied by a dramatic decrease in the number of viable cells, did not interfere with the passage of the multinucleated cells through the cell cycle [13]. Moreover, cells were not condemned to cell death since no signs of apoptosis or changes in the expression of pro- or anti-apoptotic markers were evidenced. An important aspect that has to be taken into account is the fact that fibres with fluoro-edenitic composition promoted the secretion of IL-6, a multifunctional cytokine with immunoregulatory and pro-inflammatory effects, and IL-8, a potent chemo-attractant for polymorphonuclear leukocytes [13]. Interestingly, these fibres also regulate the expression of phospho-retinoblastoma protein to trigger a network of signals strictly connected with cell proliferation and neoplastic cell transformation [14]. Finally, a study by Cardile and colleagues reveals the involvement of nitric oxide (NO) in the cytotoxic and genotoxic effects caused by fibres with fluoro-edenitic composition in the mouse monocyte-macrophage cell line J774. These effects are in line with those reported for other asbestos and have been ascribed to the permanence of the fibres in cultures for a long period of time, suggesting that inflammatory disorders apparently increase the risk for lung cancer induced by fluoro-edenite [15].

All the results reported are suggestive of a putative pro-transforming activity, which is strengthened by the fact that in lung epithelial cells most of the results obtained for fibres with fluoro-edenitic composition were comparable to those obtained with crocidolite, used as a "positive control". Crocidolite is indeed one of the best-characterized amphibole asbestos, with well-depicted cytotoxic properties and accredited carcinogenic capacity [16] and whose connection with severe inflammation and cancer of the lung is renowned. The ability of lung epithelial cells to progress throughout the cell cycle despite the presence of several nuclei in the same cytoplasm, could account for the carcinogenic properties of fibres with fluoro-edenitic composition and of crocidolite. In fact, the uninterrupted proliferation of multinucleated cells inevitably leads to aneuploydia, this nuclear content alteration being largely known to participate to cancer development.

In vivo studies

It is also important to underline that fibres with fluoro-edenitic composition have been found both in a patient died for mesothelioma [17] and in the pulmonary parenchyma of sheep living in Biancavilla area [18]. To strengthen these epidemiological data, an *in vivo* study has been carried out by Belpoggi and coworkers [19]. In this paper, Sprague-Dawley rats (males and females, at the same percentage) were treated "*una tantum*" with i) prismatic fluoro-edentite, ii) fibres with fluoro-edenitic composition or iii) the vehicle. A single

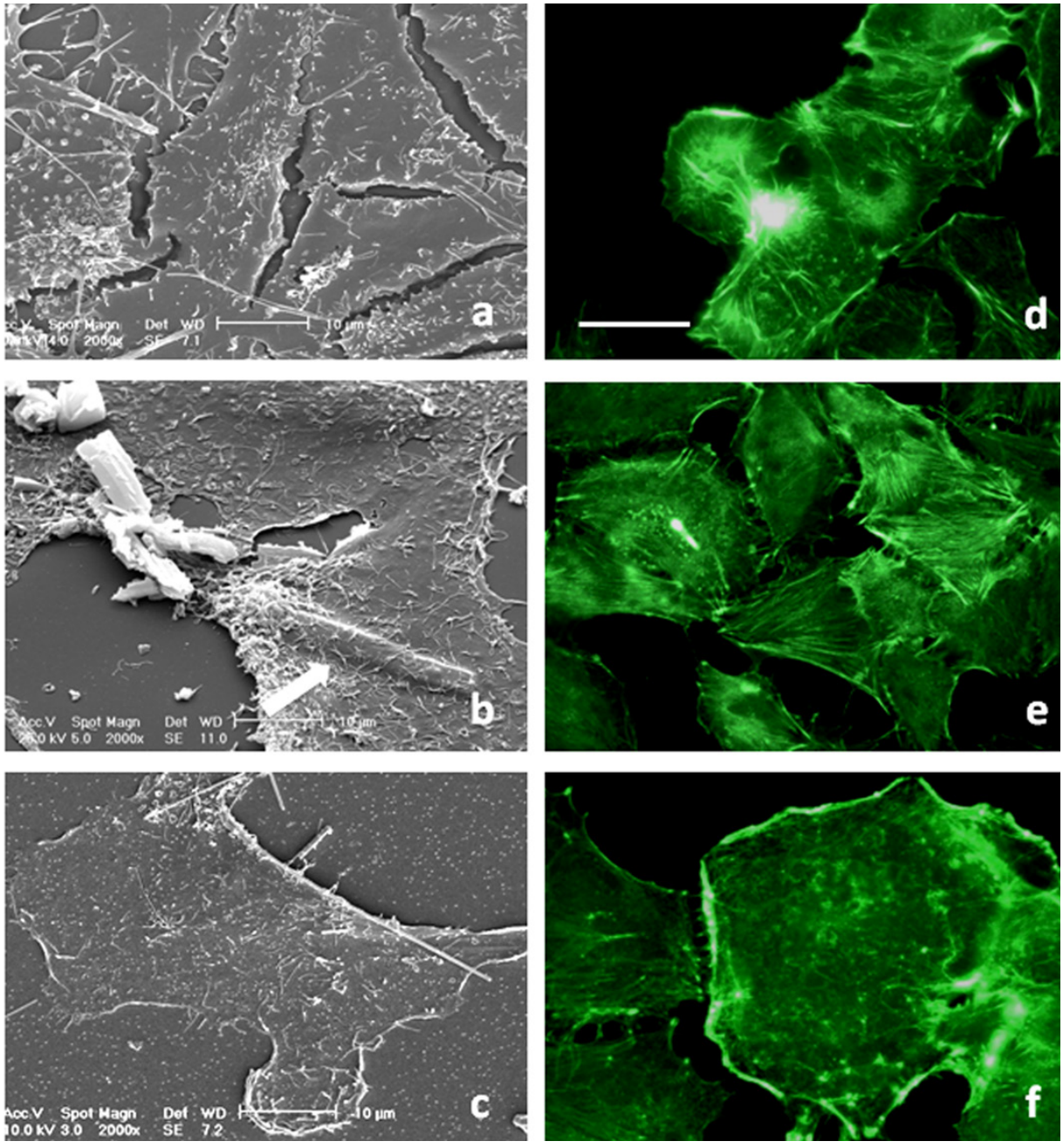

Figure 1
Morphological effects of prismatic fluoro-edenite and fibres with fluoro-edenitic composition on A549 lung epithelial cells.

(a-c) Scanning electron micrographs of (a) control cells, and (b, c) cells treated with (b) prismatic fluoro-edenite or (c) fibres with fluoro-edenitic composition.
(d-f) Fluorescence micrographs of cells stained with FITC-Phalloidin for F-actin detection. (d) Control cells; (e, f) cells treated with (e) prismatic fluoro-edenite and (f) fibres with fluoro-edenitic composition.
Although engulfed by an epithelial cell (arrow), the prismatic fluoro-edenite (b) does not induce morphological changes, in term of cell surface appearance (b) and F-actin distribution (e). By contrast, the exposure to fibres with fluoro-edenitic composition causes a striking modification of the cell morphology, mainly consisting in the enlargement of the cell body and the smoothing of the cell surface (c). These changes were accompanied by a derangement of the F-actin network (f).

dose was used (25 mg), administered by intrapleural or intraperitoneal injection. In line with previous preliminary results [15], these experiments have evidenced that fibres with fluoro-edenitic composition have a mesotheliomatogenic potential, independently from sex. Also, they showed that the injection into peritoneum caused effects much stronger than those observable after injection into pleura. In contrast, treatment with the vehicle only or with the prismatic form of fluo-edenite failed to induce a mesotheliomatogenic response, in accordance

to the criteria reported in Ref. [20]. Strikingly, the effects provoked by fibres with fluoro-edenitic composition are long-term and this proves the bio-persistence of fibres *in vivo*.

CONCLUSIONS

Nowadays, a plethora of evidence suggests a close link between cancer and chronic inflammation. In humans, inflammatory diseases in the lung can be associated with asbestos fibres exposure [21]. Cytokines and growth factors derived from alveolar macrophages are strongly implicated as mediators of asbestos-induced pathophysiological responses. Although best handled by specialized cells like macrophages, inflammation also characterizes the response of epithelial cells to external danger, which hence produce an array of mediators transmitting cellular signals. Moreover, the inflammatory signalling in epithelial cells results in their inappropriate survival and transformation. Hence, the tendency of fibres with fluoro-edenitic composition to act as a transforming agent is also supported by the ability of treated epithelial cells to produce pro-inflammatory cytokines. The inflammatory response is a double-edged sword: it explains acute disease severity but also the attempt of the host cells to obtain the clearance from the causative agent of inflammation. Possibly, surviving cells that continue to release IL-6 and IL-8, could trigger a chronic inflammatory process, a phenomenon known to be tightly related to many types of cancer.

Hence, although several aspects of the fibres with fluoro-edenitic composition cytotoxicity remain to be defined, taken altogether the findings above we can hypothesize the following scenario: cytokines that are produced by epithelial cells in response to a cellular damage, activate neutrophils and macrophages to accumulate in the injured area. The recruited cells then produce ROS ([22], our unpublished results) and additional cytokines in an attempt to remove the unsafe agent. With chronic unresolved inflammation, tissue fibrosis can occur concomitantly with an enhanced risk for cancer, principally arising from enhanced ROS production leading to DNA mutations, lack of apoptosis and enhanced signal transduction that may finally lead to activation of oncogenes.

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Conflict of interest statement

There are no potential conflict of interest of any financial or personal relationship with other people or organizations that could inappropriately bias conduct and findings of this study.

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