

Immunomodulatory effects in workers exposed to naturally occurring asbestos fibers

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Received December 30, 2016; Accepted February 21, 2017

DOI: 10.3892/mmr.2017.6384

Abstract. Natural asbestiform fibers are defined ‘naturally occurring asbestos’ (NOA) and refer to the mineral as a natural component of soils or rocks. The release of NOA fibers into the air from rocks or soils by routine human activities or natural weathering processes represents a risk for human beings. Fluoro-edenite (FE) is a NOA fiber detected in the benmoreitic lava in the area of Biancavilla, South-west slope of Mt. Etna. The aim of the present study was to investigate FE immunotoxicity pathways in a group of 38 occupationally exposed construction workers, in order to find any biological markers of its effect. Subjects underwent respiratory function tests and HRCT total chest scanning. Serum IL-1 β , IL-6, IL-8 and TNF- α were measured. The presence of PPs was significantly greater in subjects exposed than in the control (25 vs. 2). In subjects exposed to FE, IL-1 β and TNF- α values were significantly higher than the controls. The previously observed increase of IL-1 β and IL-18 showed a probable involvement of the proteic complex defined inflammasome by FE fibers.

Introduction

Occupational and/or environmental exposure to some types of asbestos fibers is related with both malignant and non-malignant pulmonary diseases (1-3), of which malignant mesothelioma (MM) and lung cancer are the most typical ones (4). Instead, non-malignant asbestos-related diseases

include pleural plaques (PPs), pleural effusions, diffuse pleural thickening and parenchymal fibrosis (5-8).

Natural asbestiform fibers are defined ‘naturally occurring asbestos’ (NOA) (9-11). The word refers to fibers which are natural components of soils or rocks. The release of NOA fibers into the air by human work activities or natural weathering processes is a potential risk for the general population (9). These fibers have been detected in various parts worldwide, such as Greece, Turkey, Cyprus, Corsica, New Caledonia, Afghanistan, Russia, Montana (USA) and Italy (12-17).

In the 1990s, a greatly increased standardized rate of mortality from MM was observed after epidemiological studies in the municipality of Biancavilla (Sicily, Italy) (17,18). Later studies spotted an asbestiform mineral fibre, called fluoro-edenite (FE), in the lava rocks excavated from a local stone quarry. The derived material had been used locally for about 50 years for building (17-19). The quarry was shut down in 1998 (20). Following some *in vitro*, *in vivo* and epidemiological studies (21-25) the IARC (Lyon, France) classified FE as carcinogenic to humans, but only for MM (26).

The relationship between exposure to asbestos and adverse health effects has been extensively studied (27). These asbestos-related diseases are well documented and approximately 107,000 mortalities are attributable to exposure to fiber worldwide, annually (28-30). Little has been established with regard to FE-related diseases (31-38); however, epidemiological studies have shown that these fibers may cause chronic obstructive lung disease (18,39), PPs and MMs (17,39).

These pathologies are the result of imbalanced inflammatory processes that is the early response to inhaled fibers. Historically, immune system cells have been regarded as the main players in initiating acute or chronic inflammation. However, recent studies have shown that epithelial cells of the respiratory tract and mesothelial cells lining the body cavities are capable of initiating inflammatory events after exposure to pathogenic fibers in the absence of cells of the immune system (40). The aim of this study was to detect FE immunotoxicity pathways in a group of occupationally exposed construction workers (CW), to find any biological markers of its effect.

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Key words: cytokine, interleukin, fluoro-edenite, Biancavilla, fibers, malignant pleural mesothelioma, NLRP3, inflammasome

Materials and methods

Ethics statement. The research protocol received the approval of the Ethics Committee of Catania University Hospital (Catania, Italy) and the written informed consent of all subjects was acquired including them in the study.

Study population. Thirty-eight construction workers residing and working in the area of Biancavilla (Sicily, Italy) were invited to participate in this study. In the same period, 38 construction workers living and working least 40 km away the area of Biancavilla were recruited as control group. Exclusion criteria were thoracic diseases (e.g. asthma, bronchopneumonia, and tuberculosis), previous exposure to asbestos and involvement in construction work in the Biancavilla area for <1 year (the latter only for exposed subjects). A questionnaire was used to obtain information on medical and occupational history, use of medications, smoking and drinking habits. A free medical check, including spirometry and a high-resolution computer tomography (HRCT) chest scan, was given to all workers.

Clinical investigations. Respiratory function tests were carried out using a bell spirometer (Biomedin, Padova, Italy). Equipment, calibration and maneuvers were in conformity with the American Thoracic Society (ATS) guidelines. Forced vital capacity, forced expiratory volume in 1 sec, peak expiratory flow, maximal expiratory flow rate at 25-75% of the vital capacity, total lung capacity and TLCO were assessed and expressed as a proportion of the European Coal and Steel Community reference values, adjusted to individual characteristics (age, weight and height) checked at the time of testing.

Subjects underwent HRCT total chest scanning using an Optima CT 580W (GE Healthcare, Fairfield, CT, USA) without contrast medium. Interstitial and/or pleural abnormalities were recorded. Pleural plaques (PPs) are circumscribed quadrangular elevations with sharp borders and density comparable to tissue, with/without signs of calcification and expressed in terms of frequency and percentage. Parenchymal abnormalities (PA) (subpleural dependent opacity, subpleural curvilinear opacities, subpleural perpendicular lines, parenchymal nodules, honeycombing and ground glass opacities) were expressed in terms of frequency and percentage.

Laboratory tests. Venous blood (10 ml) was collected in the morning, following overnight fasting, to determine red blood cell count, haematocrit, haemoglobin levels, white blood cell count, erythrocyte sedimentation rate, C-reactive protein levels and liver enzyme (aspartate aminotransferase and alanine aminotransferase) levels. For cytokine detection, blood samples were drawn into vacuum tubes with gel and clot activator (Vacuette, Greiner Bio-One, Kremsmünster, Austria) before analysis. After collection, the tubes were left in an upright position for at least 30 min at room temperature but no more than 60 min. Samples were then centrifuged at 3500 rpm for 10 min, then serum was separated and stored at -20°C until analysis. Serum interleukin-1 β (IL-1 β), IL-6, IL-8 and tumor necrosis factors- α (TNF- α) were measured using highly sensitive quantitative sandwich assays (Quantikine ELISA kit, R&D Systems, Minneapolis, MN, USA). The instruments were

Table I. Main features of samples and main results.

Features	Exposed workers	Non-exposed workers	P-value
Gender (male)	38 (100%)	38 (100%)	NS
Age (years)	53.8 \pm 7.9	54.4 \pm 6.5	NS
BMI (kg/m ²)	23.1 \pm 1.8	22.7 \pm 1.7	NS
Smokers	17 (45%)	19 (50%)	NS
Alcohol consumption (g/day)	16.4 \pm 3.5	17.1 \pm 2.9	NS
Working age (years)	21.2 \pm 9.3	22.1 \pm 10.1	NS
Presence of PPs	25 (66%)	2 (5%)	<0.0001
Presence of PAs	3 (8%)	0 (0%)	<0.0001
IL-1 β (pg/ml)	27.51 \pm 8.43	15.28 \pm 5.64	<0.0001
IL-6 (pg/ml)	13.07 \pm 3.25	12.12 \pm 20.15	NS
IL-8 (pg/ml)	18.43 \pm 9.27	17.25 \pm 8.76	NS
TNF α (pg/ml)	21.14 \pm 6.13	9.79 \pm 3.81	<0.0001

BMI, body mass index; NS, not significant.

adjusted and internal quality control was performed using the same lot of the manufacturer's control and calibration material throughout the study.

Statistical analysis. Data were summarized as mean \pm SD for continuous variables and frequencies for categorical variables. Normality was checked by Kolmogorov-Smirnov test and homogeneity of variance by Levene's test. T-test was used for analyzed continuous variance, Fischer's test for categorical variables. Statistical analyses were performed by SPSS ver. 22 (IBM, Armonk, NY, USA) and GraphPad Prism ver. 6.0 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Application of the exclusion criteria did not cause any subject to be excluded from the sample. The main sample characteristics are reported in Table I. All 38 FE exposed CWs had been living in Biancavilla for >35 years, and 74% (n=28) were born there. Besides, all had been working almost exclusively in and around Biancavilla. As to their occupational history, 21 (55%) of the participants revealed they had personally handled and worked with gravel excavated from Mount Calvario quarry until 1998. Furthermore, all had been involved in restoring houses dating back to the 1950s, when the lava from the quarry had been widely used as a building material.

Age, body mass index, smoking habits, alcohol consumption and working age did not differ between exposed and control. Blood examination tests were within the normal range in all subjects. Functional respiratory tests were within the normal range for all participants. A restrictive ventilatory defect was detected in two subjects (5%) and an obstructive ventilatory defect observed in one (3%) of the exposed group. Control subjects were all in the normal range. TLCO was less in two additional participants of exposed group (data not shown).

The HRCT findings are reported in Table I. HRCT scans revealed low-grade fibrosis in 3 workers (one unilateral and

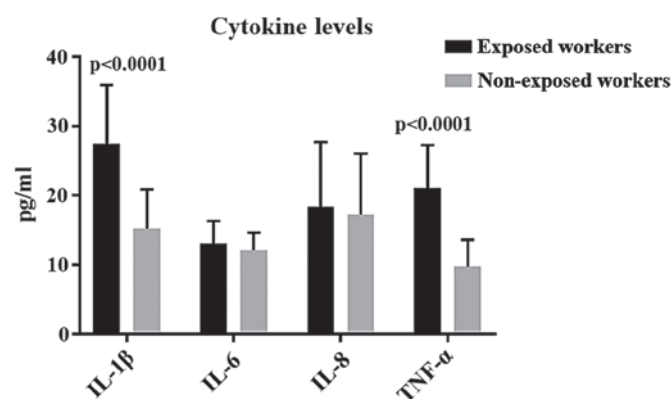


Figure 1. Mean levels of IL-1 β , IL-6, IL-8 and TNF- α detected in fluoroedenite (FE) exposed construction workers (CW) and controls.

two bilateral). In the exposed group, pleural involvement was documented in 25 (66%) subjects, of whom 21 (84%) had bilateral plaques. Calcifications were detected in 5 (17%) participants. In control subjects PPs were detected in 5% of cases (n=2) (Table I).

Cytokine mean levels are reported in Fig. 1. In FE exposed subjects, TNF- α or IL-1 β values were significantly higher ($P < 0.0001$) than the controls, whereas IL-6 and IL-8 values were higher, although not significantly ($P > 0.05$).

Discussion

A number of observations show that altered immune responses are also important in asbestos respiratory toxicity (41). Some of the major cytokines and growth factors involved in the pathogenesis of lung diseases include IL-1, TNF- α , transforming growth factor- β (TGF- β), platelet derived growth factor (PDGF) and IL-8. These agents augment cellular injury and trigger fibroblast proliferation and collagen deposition. Although alveolar macrophages (AM) are thought to be the primary source of these proteins, a previous molecular study suggests that pulmonary epithelial cells are also involved (42). Further evidence suggests that TNF- α is a key cytokine involved in asbestos induced lung toxicity (43,44). TNF- α can increase the levels of alveolar type II cell Macrophage Inflammatory Proteins-1 α (MIP-1 α) mRNA, which suggests that TNF- α can promote pulmonary inflammation through its effects on epithelial cells (45).

In our study, significantly higher levels of TNF- α were detected in FE-exposed subjects, compared to the control ones. Simeonova and colleagues (46,47) found out that asbestos and H₂O₂ both activate NF- κ B and NF-IL-6 in A549 and normal human bronchial epithelial cells which, in turn, stimulate IL-6 and IL-8 gene expression and protein release. A function for reactive oxygen species (ROS) was clarified by their observation that HO \cdot scavengers and N-acetylcysteine (NAC) each decreased asbestos or H₂O₂ induced NF- κ B and NF-IL-6 activity as well as IL-8 and IL-6 protein expression. *In vitro* studies conducted by Travaglion *et al* (48,49), demonstrated that FE interfered with epithelial cell physiology, by reducing the proliferation rate without perturbing the cell cycle and increasing the release of pro-inflammatory cytokines IL-6 and IL-8, from pulmonary epithelial cells.

In our study, FE-exposed subjects showed increased levels of IL-6 and IL-8, although in a non-statistically relevant way. Higher levels of IL-6, IL-8 and TNF- α , have been observed more recurrently overexpressed in the microenvironment of mesothelial cells during neoplastic transformation (50-52). The continuous stimulation of mesothelial cells from FE fibers could account for the increased values of these cytokines, particularly the increase of TNF- α , that is involved in the resistance against fiber toxicity (53). As a matter of fact, significantly greater quantities of PPs were observable in exposed subjects than in the control groups 25 (66%) vs. 2 (5%). PPs can arise even after relatively low exposure to asbestiform fibers and are the most common non-malignant effects (31-33,35,54-57).

Most research on the role of inflammation in asbestos-related diseases has been centred on immune cellular response, including the first cell type accumulating at sites of initial deposition of inhaled asbestos fibres (42). When the injury occurs, mesothelial cells can recruit neutrophils, monocytes and lymphocytes by generating chemokines and cytokines which cause mesothelial cells to release growth factors with paracrine functions (50-52,58-60). New evidence on human mesothelial cells sustains a model where an autocrine loop is perpetrated by fibre-induced inflammasome NLRP3 (NLR family pyrin domain containing 3) priming and activation, with the subsequent augmented pro-inflammatory growth factors transcription activity (61-64).

The inflammasome is a multiprotein complex that links proIL-1 β to its upstream activator. This complex is composed of Pycard, NALP1, caspase-1 and likely caspase-5. It is expressed in myeloid cells and is a component of the immune system (65). The IL-1 super-family of cytokines encompasses at least 11 members, which include IL-1 α , IL-1 β , and IL-18 (66). The inflammasome is responsible for the activation of inflammatory processes (65) and has been shown to induce caspase-1 dependent pyroptosis which is a form of cell death characterized by both apoptosis and necrosis (67).

A recent study (40) showed that exposure to asbestos and asbestiform fibers (i.e. erionite) may trigger and activate the inflammasome via multiple mechanisms (40). These fibers cause dose-related damage to the cell membrane at high concentrations, are phagocytized and can break phagolysosomes. These processes may lead to the activation of ROS via many paths. Inflammasome-related caspase-1 activation leads directly to the maturation and secretion of IL-1 β and IL-18, as well as other inflammatory mediators such as IL-1 α and high mobility group box-1 (HMGB1). These chemokines and cytokines either directly or indirectly lead to acute and chronic inflammation, the latter resulting in various particle and fiber-associated pulmonary and pleural pathologies (40).

Pro-caspase-1 is recruited to the inflammasome complex and processed to active caspase-1, which processes IL-1 and IL-18 into their active forms (65,68). This can account for the high serum levels of IL-1 β detected in FE-exposed workers compared to control ones. Besides, in a previous study carried out on CWs in Biancavilla, high levels of IL-18 were observed which well correlated with the presence of PPs and lung parenchymal fibrosis (31). IL-1 β helps T-cell survival, B-cell proliferation and antibody production, as well as mediating leukocyte transmigration (69,70). IL-1 β roles

in carcinogenicity entails recruitment of myeloid-derived suppressor cells (MDSCs) that prevent natural killer (NK) cells from developing and functioning (71). IL-18 can work synergistically with IL-12 to induce interferon- γ (IFN γ) production by activating T- and NK-cells (69-70,72).

The pro-inflammatory cytokine IL-1 β , which can be inflammasome-dependent, is a critical mediator of inflammation-promoting carcinogenesis (73-76) and fibrosis (77,78). Cell death mechanisms such as apoptosis and necrosis and release of chemokines/cytokines and alarmins such as HMGB1 and TNF- α , may help cancer regress, resist toxicity by fibers and cell growth (62,79), in inflammasome-dependent and -independent pathways (80). The transcription factors, activator protein-1 (AP-1) and NF- κ B that may change inflammation-induced tumor growth into cancer regression (79), are also signals inducing the transcription or priming of pro-IL-1 β .

The induction of caspase-1 dependent pyroptosis, a type of cell death characterized by both apoptotic and necrotic features, is another important function of inflammasomes (81). This progression features nuclear DNA fragmentation, plasma membrane rupture and release of inflammatory mediators such as IL-1 α , IL-1 β , IL-33 and HMGB1 which play significant roles in inflammatory processes (72,81). Consequently, the inflammasome and inflammation can promote proliferation and maturation of target cells and immune suppression in tumors (82). It is assembled in response to a wide range of conserved exogenous molecules including asbestos (83). Multifaceted inflammasomes are critical in sensing and responding to a variety of extracellular and intracellular stresses through several pathways and may be important in both pulmonary defense and promulgation of chronic inflammation leading to pathologies (40).

In the lungs, highly inflammatory cytokines of the IL-1 family, including IL-1 β , are key factors to inflammation. For instance, IL-6, a pleiotropic inflammatory cytokine frequently released in tandem with IL-1 β , is a key growth-promoting and anti-apoptotic factor that is also involved in immune regulation and inflammation (84,85). IL-6 is frequently expressed in malignant respiratory epithelial cells, and high circulating levels in serum are related to a poor prognosis in lung cancer patients (86,87). Its potential use as a biomarker and prognostic indicator is bolstered by the fact that it is not detected in the serum of healthy individuals and patients with benign lung pathologies (40). Comar *et al* (88) observed significant high levels of IL-6 in MM patients. This is in line with what we observed with regard to the increased level of IL-6 in the exposed group compared to the controls, although not significantly; this may derive from mesothelial involvement, although without cancer lesions.

Asbestiform fibers can trigger and activate the NLRP3 inflammasome in mesothelial and epithelial cells, as well as in cells of the immune system via multiple pathways. Inflammasome priming and activation may play vital roles in both early lung and pleural injury as well as in inflammation, tumor initiation and promotion. TNF- α , a protein implicated in both proliferation of mesothelial cells and prevention of asbestos-induced injury (89,90), it may also stimulate MM cell survival through the induction of genes encoding NF- κ B-dependent anti-apoptotic molecules (91,92) and activate chemo-resistance. TNF- α receptors are regulated

by both TNF- α and IL-1 α in human mesothelial cells (58) that synthesize and release both IL-1 β (93) and IL-1 α after injury (67,68). The fact that the chemotactic and autocrine growth factor IL-8 (94-97) is generated by human mesothelial cells in response to TNF- α and IL-1 released by macrophages or after exposure to asbestos (98), supports the concept that inflammasome-mediated mature IL-1 β release is essential to the production of other cytokines and chemokines critical to the development of MMs (40).

In our study, the observation of significantly high levels of TNF- α in exposed subjects could be accounted for with the resistance action against the stimulus deriving from inhaling FE fibers. As described for asbestos, it is conceivable that FE may promote the NLRP3 inflammasome in macrophages, monocytes and lung epithelial cells, leading to IL-1 β secretion (91,92). Increased production of TGF- β , a hallmark of inflammation and the fibrotic process, triggers the activation, proliferation and *trans*-differentiation of epithelial cells and resident fibroblasts into collagen-producing myofibroblasts. This has been put down to inflammasome-induced secretion of mature IL-1 β (98). IL-1 β also triggers the secretion of neutrophil-attracting CXC chemokines, resulting in a further influx of neutrophils that amplify cell damage and oxidant release (99). Unlike macrophages and monocytes, NADPH oxidase-derived ROS are neither required for inflammasome priming nor activation by human neutrophils, but are necessary for exporting mature IL-1 β from the cell (100). IL-1 β secretion by AMs is enhanced by fibrogenic agents that also raise production of PDGF, a growth-promoting cytokine and chemo-tactic factor (101).

A recent immunohistochemical study, conducted in lung tissue of sheep exposed to FE fibers showed a significant activation of AM and mast cells (102). An *in vitro* study on mesothelial Met-5A and monocyte-macrophage J774 cells exposed to FE caused induction of the heat shock protein 70 (Hsp70), stimulated formation of ROS and NO \cdot (measured as nitrite). Exposure of cells to FE induced lactate dehydrogenase activity and decreased cell viability (103). It is conceivable that FE may trigger inflammatory processes that, like in the case of asbestos, may lead to the activation of inflammasome.

In this study, as well as in a previous one (31) on IL-18, where high IL-1 β serum levels have been detected, both cytokines are primarily involved in the inflammasome activation process. Our results suggest that it is necessary to go on with research on immune-modulators involved in the pathogenic mechanisms responsible for FE related diseases.

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