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Analysis of fibulin-3 after exposure to asbestos-like fibers

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ABSTRACT

A significantly increased incidence of malignant mesothelioma in Biancavilla (Sicily, Italy) has been ascribed to exposure to fluoro-edenite, a fibrous amphibole extracted from a local stone quarry. Fibulin-3 is a highly conserved glycoprotein proposed as a biomarker for malignant mesothelioma that belongs to the family of extracellular matrix proteins. Previous studies demonstrated high Fibulin-3 plasma levels in workers with pleural plaques exposed to fluoro-edenite.

Therefore, in order to gain insight into the biomolecular mechanisms of fluoro-edenite toxicity, we performed the analysis of Fibulin-3 expression by immunohistochemistry in the lung samples derived from sheep belonging to the area of Biancavilla. Furthermore, an in vitro model of exposed fluoro-edenite fibroblasts was used to perform functional experiments to better understand the modulation of Fibulin-3 expression. The percentage of immunostained area by Fibulin-3 was very much higher in exposed lungs compared with non-exposed ones. The Fibulin-3 protein level was significantly expressed in primary human lung fibroblasts exposed to 50 and 100 μg/ml of fluoro-edenite fibers for 72 h, compared to the unexposed controls. The results from the present study further demonstrate the implication of Fibulin-3 during fluoro-edenite exposure. This would endorse our previous results regarding the use of Fibulin-3 as a possible screening biomarker for fluoro-edenite exposed individuals, thereby contributing to the monitoring of the population at risk. The present study also suggested that the Fibulin-3 overexpression may reflect a defensive response of the tissues after exogenous stimuli and may be implicated in cancer development, especially in the context of fluoro-edenite contamination. However, further studies are necessary in order to make Fibulin-3 a customized screening tool.

1. Introduction

Since 1990, a significantly increased standardized mortality rate from malignant mesothelioma (MM) has been reported in Biancavilla, a town on the southwest slope of Mt Etna in Sicily, Italy (Paoletti et al., 2000; Rapisarda et al., 2003) where a natural, asbestiform fiber, fluoro-edenite [FE; $NaCa_2Mg_5(Si_7Al)O_{22}F_2$] was identified as the main environmental risk factor. FE fibers have been found in inert materials, such as sand and rubble, extracted from a local stone quarry excavated inside the Mt Calvario, lying on the immediate outskirts SE of the town. This material had been widely used for about 50 years by construction firms (Paoletti et al., 2000; Comba et al., 2003). The type of environmental contamination, discovered in Biancavilla suggests that the entire population had been exposed to FE (Biggeri et al., 2004).

FE fibers share size and morphology similarities with certain amphibolic asbestos fibers (including antophyllite, actinolite and tremolite), whose inhalation may induce chronic inflammation and pleural abnormalities (Camaj et al., 2009; Rapisarda et al., 2003; Martinez et al., 2006; Loreto et al., 2008; Rapisarda et al., 2005; Cardile et al., 2007), thus representing a risk factor for MM. FE has been classified as carcinogenic to humans by the International Agency for Research on Cancer (IARC; Lyon, France) (Grosse et al., 2014).

Previous studies demonstrated a higher incidence of chronic obstructive lung disease (Biggeri et al., 2004), inflammation (Ledda et al., 2016a; Ledda et al., 2017) and non-malignant pleural lesions in Biancavilla's general population (Rapisarda et al., 2015a; Rapisarda

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et al., 2015b; Ledda et al., 2016b; Ledda et al., 2016c). Pathogenic mechanisms of lung illness were linked to the activation of different biomarkers including fibulin-3 (Fb-3). High plasma levels of Fb-3 were detected in workers with pleural plaques, occupationally exposed to FE (Rapisarda et al., 2015b). Accordingly, Fb-3 is a highly conserved glycoprotein proposed as a biomarker for MM (Creaney et al., 2014; Kaya et al., 2015; Ren et al., 2016; Pei et al., 2017) that belongs to the family of extracellular matrix proteins. It is also a member of the fibulin family of extracellular glycoproteins (FBLN) encoded by the *EFEMP-1* gene, which maps on chromosome 2p16 (Creaney et al., 2014). FBLN are widely expressed and localized into basement membranes, stroma, and extracellular fibers and provide organization and stabilization to extracellular matrix structures during organogenesis and vasculogenesis (Timpl et al., 2003; Argraves et al., 2003). Fb-3 low expression levels were observed in normal tissue (Kaya et al., 2015). FBLN family suppresses tumor growth and progression through two main mechanisms, by targeting endothelial cells and vascular smooth muscle cells, and by exerting a normalizing action on the tumor stroma to limit tumor growth and metastasis formation (Albig et al., 2006).

Fb-3 is also expressed in condensing mesenchyme, giving rise to bone and cartilage structures. It mediates cell–cell and cell–matrix communication, has variable angiogenic effects (Albig et al., 2006). Fb-3 was detected at different expression levels in several cancer types. However, conflicting data have been generated on its pathogenic role during tumor development. Most recently, we have observed that exposure to FE induces Fb-3 overexpression in both mesothelial cells and in circulating plasma levels from exposed subjects suggesting its role during malignant transformation (Rapisarda et al., 2016). It is not clear whether Fb-3 is originated from the respiratory tract, where pleural plaques have been observed.

Therefore, in order to gain insight the biomolecular mechanisms of FE toxicity, we performed the analysis of Fb-3 expression by immunohistochemistry in the lung samples derived from sheep belonging to the area of Biancavilla, where FE is present. Sheep lung model is comparable to the human lung for morphology, architecture, volume and physiological respiratory functional parameters (Begin et al., 1981). Furthermore, an in vitro model of exposed FE fibroblasts was used to perform functional experiments to better understand the modulation of Fb-3 expression.

2. Materials and methods

2.1. Animals

Sixty sheep of both sexes (no. 60), randomly selected from six exposed flocks (no. 360) habitually grazing 3 km from the town of Biancavilla and ten control sheep (no. 10), from a flock (no. 60) habitually grazing about 50 km from the Biancavilla stone quarry, were sacrificed in a slaughterhouse and used for this study as previously described (Musumeci et al., 2015; Musumeci et al., 2016; Ledda et al., 2016d). Ante- and post-mortem examinations were conducted by a veterinary surgeon to establish the state of health of each sheep (according to Community Regulation CE n. 854/04 and council of 29 April 2004). The age range of exposed and control animals was 4.0–6.5 years.

2.2. Histology

Lung tissue (1 cm^3) from the right apical lobe and the principal and accessory lung lobes were rinsed in phosphate buffered saline (PBS; Sigma, Milan, Italy), fixed in 10% buffered-formalin as previously described (Loreto et al., 2008). After an overnight wash, specimens were dehydrated in graded ethanol, cleared in xylene and paraffin-embedded, preserving their anatomical orientation. Sections (4–5 μm in thickness) were cut from paraffin blocks using a microtome, mounted on sialinate-coated slides and stored at room temperature. The sections were then stained with hematoxylin and eosin (H & E) and examined using a Zeiss Axioplan light microscope (Carl Zeiss, Oberkochen, Germany) for general morphological characterization and to highlight the presence/absence of structural alterations. Finally, representative photomicrographs were captured using a digital camera (AxioCam MRc5, Carl Zeiss, Oberkochen, Germany).

2.3. Immunohistochemistry (IHC)

Lung tissue (1 cm^3), 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 3 and 4 μm in thickness were cut, mounted on silane-coated slides (Dako, Glostrup, Denmark), and air-dried. For immunohistochemical analysis, specimens were processed as previously described (Loreto et al., 2014). Briefly, the slides were dewaxed in xylene, hydrated using graded ethanols and were incubated for 30 min in 0.3% H_2O_2/methanol solution to quench endogenous peroxidase activity and then rinsed for 20 min with PBS. The sections were heated (5 min x 3) in capped polypropylene slide-holders with citrate buffer (10 mM citric acid, 0.05% Tween 20, pH 6.0; Bio-Optica, Milan, Italy), using a microwave oven (750 W) to unmask antigenic sites. The blocking step was performed before application of the primary antibody with 5% bovine serum albumin (BSA; Sigma, Milan, Italy) in PBS for 1 h in a humid chamber. BSA was used as a blocking agent to prevent non-specific binding of the primary and secondary antibodies to the tissue sections. Following blocking, the sections were incubated overnight at 4 °C with a rabbit polyclonal anti-EFEMP1 antibody (PA5-26104 Thermo Fisher Scientific, Waltham, MA, USA), diluted 1:100 in phosphate buffer saline (PBS; Sigma, Milan, Italy). Immune complexes were then treated with a biotinylated link antibody and then detected with peroxidase labeled streptavin, both incubated for 10 min at room temperature (LSAB + System-HRP, K0690; Dako, Glostrup, Denmark). The immunoreaction was visualized by incubating the sections for 2 min in a 0.1% 3,3′-diaminobenzidine and 0.02% hydrogen peroxide solution (DAB substrate Chromogen System; Dako, Denmark). The sections were lightly counterstained with Mayer's hematoxylin (Histolab Products AB, Göteborg, Sweden) mounted in GVA (Zymed Laboratories, San Francisco, CA, USA) and observed with an Axioplan Zeiss light microscope (Carl Zeiss, Oberkochen, Germany) and photographed with a digital camera (AxioCam MRc5, Carl Zeiss, Oberkochen, Germany).

2.4. Evaluation of immunohistochemistry (IHC)

The Fb-3 antibody-staining status was identified as either negative or positive. Immunohistochemical positive staining was defined by the presence of brown chromogen detection on the edge of the hematoxylin-stained cell nucleus, distributed within the cytoplasm or in the membrane via evaluation by light microscopy as previously described (Musumeci et al., 2016). Positive and negative controls were performed to test the specific reaction of primary antibody used in this study at a protein level. Positive controls consisted of tissue specimens with known antigenic positivity. Sections treated with PBS without the primary antibodies served as negative controls. Seven fields, area of which was about 600.000 μm^2, randomly selected from each section, were analyzed for morphometric and densitometric analysis. The percentage areas (morphometric analysis) stained with Fb-3 antibody, expressed as % positive, dark brown pixels of the analyzed fields, and the level (high/low) of staining intensity of positive areas (densitometric analysis), expressed as densitometric count ($pixel^2$) of positive, dark brown pixels of the analyzed fields, were calculated using software for image acquisition (AxioVision Release 4.8.2 – SP2 Software, Carl Zeiss Microscopy GmbH, Jena, Germany). Data were expressed as mean ± standard deviation (SD). Statistical significance of results was thus accomplished. Digital micrographs were taken using the Zeiss