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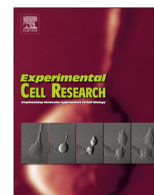

Research Article

Angiogenesis correlates with macrophage and mast cell infiltration in lung tissue of animals exposed to fluoro-edenite fibers

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ABSTRACT

Angiogenesis plays a crucial role in progression of pleural malignant mesothelioma. A significantly increased incidence of pleural mesothelioma has been attributed to exposure to fluoro-edenite, a fibrous amphibole extracted from a local stone quarry. In this study, we have investigated the expression of CD68-positive macrophages, tryptase-positive mast cells and CD31 positive areas, as expression of microvascular density, in lung tissue of sheeps exposed to fluoro-edenite fibers *vs* controls, by immunohistochemical, morphometric and Western blot analysis. The result have evidenced a significant increase in the expression of CD68-positive macrophages, tryptase-positive mast cells as well as a significant increase in microvascular density evaluated as CD31 positive areas in lung tissue of of sheeps exposed to fluoro-edenite fibers *vs* controls. These data confirmed the important role played by tumor microenvironment components, including macrophages and mast cells, in favour of angiogenesis in pleural mesothelioma induced by fluoro-edenite exposure.

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1. Introduction

Pleural malignant mesothelioma arise from the mesothelial cells and grows aggressively disseminating in the thoracic cavity and producing malignant pleural effusions. A significantly increased incidence of pleural mesothelioma in Biancavilla (Sicily, Italy) has been attributed to exposure to fluoro-edenite (FE), a fibrous amphibole extracted from a local stone quarry [1–3]. FE is a mineroil fiber of volcanic origin which is closely related to edenite, a much widely represented material. FE has been considered as an agent responsible for the development of pleural mesothelioma in rats injected with FE fibers into their peritoneal cavities [4]. FE has been assessed as carcinogenic to humans in its amphibole form, and many studies have investigated the mechanisms of toxicity of this fiber [5–7].

Angiogenesis is involved in the progression of malignant mesothelioma [8,9]. Vascular endothelial growth factor (VEGF) is the principal angiogenic cytokine involved [8,10,11], modulating also through an increase in vascular permeability the development of pleural effusion and ascites [12].

Tumor microenvironment is emerging as a crucial aspect in the progression of solid and hematological malignancies. In this context, macrophages and mast cells have been demonstrated to have a role in enhancing angiogenesis in cancer through the release of pro-angiogenic factors and through a complex cross-talk within the tumor microenvironment [13]. Several studies have been established associations between tumor associated macrophages, mast cells and microvascular density in different malignancies [14–17].

Therefore, in this study we have examined the expression of CD68-positive macrophages, tryptase-positive mast cells and CD31 positive areas, as expression of microvascular density, in lung tissue of sheeps exposed to fluoro-edenite fibers *vs* controls by immunohistochemical, morphometric and Western blot analysis.

2. Materials and methods

2.1. Animals

Sixty sheep of both sexes (n=60), randomly selected from six

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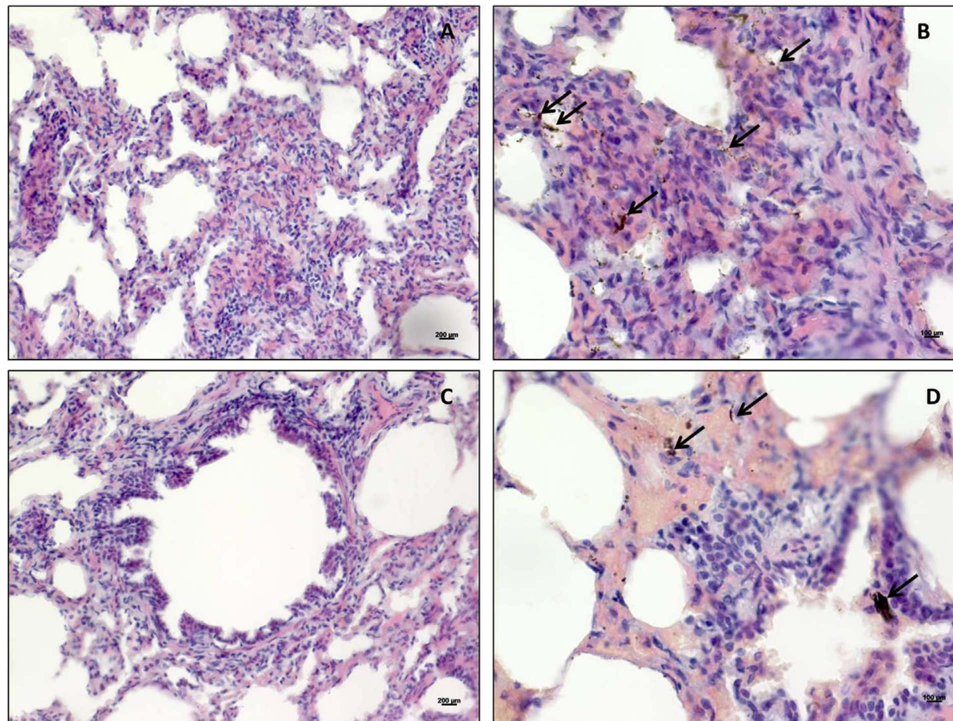

Fig. 1. *Haematoxylin & Eosin staining. (A, C),* control lung tissue. (*B, D),* exposed lung tissue of sheep in which fluoro-edenite fibers (black arrows) are in close contact with lung bronchioles and interstitium. Original magnification: A, C, 100 × ; scale bar: 200 μm; B, D, 200 × ; scale bar: 100 μm.

exposed flocks (n=360) habitually grazing 3 km from the town of Biancavilla, and ten control sheep (n=10), from a flock (n=60) habitually grazing about 50 km from the Biancavilla stone quarry, were sacrificed in a slaughterhouse and used for this study as previously described [18]. 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), fixed in 10% buffered-formalin as previously described [19,20]. After an overnight wash, specimens were dehydrated in graded ethanol, cleared in xylene and paraffin-embedded, preserving their anatomical orientation. Section (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 or absence of structural alterations. Finally, representative photomicrographs were captured using a digital camera (AxioCam MRc5, Carl Zeiss, Oberkochen, Germany).

2.3. Immunohistochemistry

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 3–4 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 [21]. 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 phosphate-buffered saline (PBS; Sigma, Milan, Italy). 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 mouse monoclonal Anti-Mast Cell Tryptase antibody (ab74506; abcam, Cambridge, UK), ready to use; mouse monoclonal Anti-CD31 antibody (ab187377; abcam, Cambridge, UK), diluted 1:200 in phosphate buffer saline (PBS; Sigma, Milan, Italy); and mouse monoclonal Anti-CD68 antibody (ab31630; abcam, Cambridge, UK) 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).

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