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Different reactivity of primary fibroblasts and endothelial cells towards crystalline silica: A surface radical matter

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ABSTRACT

Quartz is a well-known occupational fibrogenic agent able to cause fibrosis and other severe pulmonary diseases such as silicosis and lung cancer. The silicotic pathology owes its severity to the structural and chemo-physical properties of the particles such as shape, size and abundance of surface radicals. In earlier studies, we reported that significant amounts of surface radicals can be generated on crystalline silica by chemical aggression with ascorbic acid (AA), a vitamin naturally abundant in the lung surfactant, and this reaction led to enhanced cytotoxicity and production of inflammatory mediators in a macrophage cell line.

However in the lung, other cells acting in the development of silicosis, like fibroblasts and endothelial cells, can come to direct contact with inhaled quartz. We investigated the cytotoxic/pro-inflammatory effects of AA-treated quartz microcrystals (QA) in human primary fibroblasts and endothelial cells as compared to unmodified microcrystals (Q). Our results show that, in fibroblasts, the abundance of surface radicals on quartz microcrystals (Q vs QA) significantly enhanced cell proliferation (with or without co-culture with macrophages), reactive oxygen species (ROS) production, NF-κB nuclear translocation, smooth muscle actin, fibronectin, Bcl-2 and tissue inhibitor of metalloproteinase-1 expression and collagen production. Contrariwise, endothelial cells reacted to the presence of quartz microcrystals independently from the abundance of surface radicals showing similar levels of cytotoxicity, ROS production, cell migration, MCP-1, ICAM-I and fibronectin gene expression when challenged with Q or QA.

In conclusion, our *in vitro* experimental model demonstrates an important and quite unexplored direct contribute of silica surface radicals to fibroblast proliferation and fibrogenic responses.

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1. Introduction

Quartz, a well-known fibrogenic material causes significant pulmonary toxicity manifested by edema, acute and chronic inflammation, fibrosis and lung cancer (Ding et al., 2002). Quartz is made of crystalline silica and its toxicity in the lung is due to direct inhalation of fine particles (less than 5 μm diameter) which is very common in mineral workers in both developed and developing countries (Craighead et al., 1998). The lung disease caused by crystalline silica exposure is termed silicosis, a non-curable irreversible disease. The severity of silicosis depends on the structural and surface properties of the quartz particles, namely the size (Cheng et al., 2014), the presence of metals such as magnesium and iron (Castranova et al., 1997) as well as of freshly fractured microcrystals (Castranova, 2004; Fubini, 1998; Donaldson and Borm, 1998). Earlier studies have demonstrated that freshly fractured silica has increased surface reactivity as compared to "aged" silica (Shi et al., 1988; Vallyathan et al., 1988). As a consequence an enhanced production of reactive oxygen species (ROS) and activation of inflammatory mediators can be observed (Ding et al., 2001, 1999; Porter et al., 2002).

Other than by physical fracturation, significant amounts of surface radicals can be generated on crystalline silica by means of chemical aggression by ascorbic acid (AA) (Bavestrello et al., 1995; Fenoglio et al., 2000). In this chemical reaction, exclusive for crystalline silica and not happening with amorphous silica, the quartz surface is partially eroded leading to release of soluble silicates in the medium and to exposition of new surface radicals on the quartz particles (Fenoglio et al., 2000). The AA-dependent modifications on the crystals have been proven to enhance toxicity

Abbreviations: AA, ascorbic acid; Q, unmodified quartz; QA, ascorbic acid-treated quartz.

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in macrophages (Giovine et al., 2003). Moreover, an enhanced inflammatory response in terms of ROS and prostaglandin-E_2 (PGE_2) production, tumor necrosis factor (TNF)-α release and membrane lipid peroxidation as compared to unmodified quartz microcrystals has been observed in cell lines of the monocyte lineage (Scarfì et al., 2007, 2009). These *in vitro* results may be relevant to the comprehension of the *in vivo* onset of silica-induced pathologies in the lung since AA is present in significant amounts in the lung epithelium surfactant (Skoza et al., 1983).

Silicosis seems to be the result of a cycle taking place in the lung involving particle phagocytosis, ROS production, inflammation, cell damage and fibrosis (Lapp and Castranova, 1993; Castranova, 2000; Fubini and Hubbard, 2003). A central role in the onset of this pathology is played by a cross-talk between macrophages and fibroblasts. When silica microcrystals reach the pulmonary epithelium they are phagocytosed by alveolar macrophages which are unable to dissolve completely the particles resulting in an alteration of their functions with the onset of a chronic inflammatory status. This leads to the release of inflammatory cytokines that stimulate fibroblast proliferation and production of large amounts of extracellular matrix (ECM) components, mainly type I collagen, resulting in the formation of permanent fibrotic scars in the lung (Liu et al., 2007; O'Reilly et al., 2005). Nevertheless, also direct fibroblast activation may be possible in the lung through their direct interaction with quartz microcrystals even though this eventuality has been scarcely investigated and mainly with silica nanoparticles (Baroni et al., 2001; Li et al., 2012; Wu et al., 2016).

Besides, currently only limited information is available on the direct effect of quartz on endothelial cells, with recent studies mainly approaching the effects of silica nanoparticles (Peters et al., 2004; Liu et al., 2012; Cheng et al., 2014; Smulders et al., 2015). Nevertheless, it has been reported that quartz early lesions *in vivo* also comprise damage to alveolar capillaries in the lung and that this damage can be repaired by proliferation and migration of endothelial cells (Kawanami et al., 1995). Thus, is proven that endothelial cells may come into direct contact with quartz microcrystals, making particle-endothelial interactions potentially relevant to the pathogenesis of silicosis.

The aim of this study was to clarify the role of quartz surface radicals in the damage and/or activation of different cell types involved in the onset of silicosis. To this aim we investigated the cytotoxic effects of AA-treated quartz microcrystals (QA) in human primary fibroblasts and endothelial cells as compared to unmodified microcrystals (Q). The possible indirect cytotoxic effect of Q and QA was also evaluated on both fibroblasts and endothelial cells co-cultured with Q- or QA-stimulated macrophages. Finally, a comparison of the pro-inflammatory effects of Q and QA particles was performed in both primary fibroblasts and endothelial cells by evaluating the different levels of production of pro-inflammatory mediators, of expression of genes relevant to fibrosis, proliferation and inflammation as well as of activation of the pro-inflammatory NF-κB transcription factor.

2. Materials and methods

2.1. Chemicals

All reagents were acquired from SIGMA-ALDRICH (Milan, Italy), unless otherwise stated.

2.2. Cell cultures, quartz particle preparation and analysis

The mouse macrophage cell line RAW 264.7 and Normal Human Primary Dermal Fibroblasts from neonatal foreskin were obtained from the American Type Culture Collection (LGC Standards srl, Milan, Italy). The human vascular endothelial cell line (HECV) was obtained by the Cell Bank and Culture (GMP-IST-Genova, Italy). Cells were cultured at 37 °C in a humidified, 5% CO_2 atmosphere in high glucose Dulbecco's modified Eagle's medium (D-MEM) with glutamax (Euroclone, Milan, Italy), supplemented with 10% Fetal Bovine Serum (FBS, Euroclone) with penicillin/streptomycin as antibiotics. Cell stimulation using different concentrations of sterilized quartz particles (MIN-U-SIL 5: US Silica, Berkeley Spring Plant, $SSA_{BET} = 5.2\ m^2/g$) was obtained by adding 100 μg/ml of distilled water-treated (Q) or ascorbic acid-treated (QA) particles [prepared as described in: Giovine et al., 2003]. In detail, in terms of surface area/incubation volume 100 μg/ml of MIN-U-SIL quartz particles corresponded to 5.2 cm^2/ml of medium.

To measure how much AA could be possibly still bound to QA when the particles were added to the cells we evaluated the adsorption of ascorbic acid (AA) on the quartz microcrystal surface by using ^{14}C radiolabelled AA. During the 5-day incubation of quartz with ^{14}C radiolabelled AA the concentration of total AA was set at 1 mM to maintain a high specific activity (79 Bq/nmol AA) allowing the detection of the AA traces still bound to the microcrystals after washing. The rate of AA adsorption on the microcrystals after incubation with AA for five days was very limited and it amounted to 10.9 ± 1.2 nmol/m^2 (54.8 ± 6.0 pmol/mg) of quartz dusts. This quantity corresponded to a 5.4 nM concentration of bound AA on the 100 μg/ml quartz dusts used in the following experiments with the various cell types; no release of radioactivity was measured in the cell medium once the quartz particles were added to the cells. Thus, during the experiments with the various cell types the traces of AA were bound on the quartz surface and not free.

2.3. Cell viability

Experiments were performed in quadruplicate on 96-well plates. RAW 264.7 macrophages were plated at a density of 25,000 cells/well, while fibroblasts and HECV endothelial cells were seeded at 10,000 cells/well and allowed to adhere overnight. Then Q or QA particles were added at 100 μg/ml final concentration for 24 and 72 h at 37 °C. Depending on the experiment, cells were incubated alternatively in complete medium or in medium with 2% FBS. For co-culture experiments fibroblasts or HECV were seeded at 250,000 cells/well in 6-well plates in complete medium and allowed to adhere overnight. Then 0.4 μm pore size cell culture inserts, namely transwells (Becton Dickinson Labware, Franklin Lakes, NJ, USA) to avoid cell-to-cell contact but to allow the sharing of the same medium, were added to the wells and 2×10^6 RAW 264.7 cells were seeded onto them. After 2 h Q or QA particles were added at 100 μg/ml final concentration in the cell culture inserts and further incubated for 3 days. At the end of the experiments cell viability was assayed both by MTT (0.5 mg/ml final concentration) and SYTOX green DNA-staining (0.5 μM final concentration) (Life Technologies, Milan, Italy) tests as already reported (Pozzolini et al., 2003). Data are means ± S.D. of three independent experiments performed in quadruplicate.

2.4. ROS detection

Experiments were performed in quadruplicate on 96-well plates. Primary fibroblasts as well as HECV were plated at a density of 10,000 cells/well, and allowed to adhere overnight. Cells were then washed once with Hank's Balanced Salt Solution (HBSS) and incubated for 30 min at 37 °C with 10 μM 2′,7′-dichloro-dihydro-fluorescein diacetate dye (Life Technologies). After incubation with the dye, cells were washed with HBSS, incubated at 37 °C for 15 min and then challenged with 100 μg/ml Q or QA particles for 4 h. The plates were finally read on a Fluostar Optima BMG using 485/520

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