Differential in vitro cellular response induced by exposure to synthetic vitreous fibers (SVFs) and asbestos crocidolite fibers

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Received 27 September 2005
Available online 13 December 2005

Abstract

In this study, we analyzed the effects of synthetic vitreous fibers (SVFs) on a mesothelial (MeT5A) and a fibroblast cell line (NIH3T3), compared to those exerted by crocidolite asbestos fibers. SVFs (glass wool, rock wools) do not induce significant changes in cell mortality, whereas crocidolite asbestos fibers caused a dose-dependent cytotoxicity. We investigated the correlation between the fiber-induced cytotoxicity and the extent and type of interaction of the fibers with the cell surface, and we observed that SVFs, unlike crocidolite asbestos fibers, establish few and weak interactions. Moreover, after internalization, crocidolite asbestos fibers are often found free in the cytoplasm, whereas glass wool fibers are mainly localized inside cytoplasmic vacuoles. After treatments, we also detected signs of oxidative stress, revealed by an increased reactive oxygen species (ROS) production and by an induction of superoxide dismutase (SOD) activity. The lipoperoxidative damage was characterized by a decrease in polyunsaturated fatty acids (PUFA), an increase in the content of thiobarbituric reactive species (TBARS) and a consumption of vitamin E, as a lipophilic antioxidant.

Furthermore, we investigated the effect of fiber exposure on cell proliferation. and it was found that, unlike crocidolite asbestos fibers, SVFs did not induce a significant increase in DNA synthesis.

Keywords: SVFs; Crocidolite asbestos fibers; Cell viability; Oxidative stress; Proliferation

Introduction

Numerous studies have described the relationship between occupational exposure to asbestos fibers and the development of pulmonary fibrosis (asbestosis) and malignant diseases (mesothelioma and lung cancer) (Robledo and Mossman, 1999; Kuwahara and Kagan, 1995). Asbestos fibers include various types of hydrated mineral silicates and are divided into two groups: the serpentine and the amphibole. Crocidolite (Na\textsubscript{2}[Fe\textsuperscript{3+}]\textsubscript{2}[Fe\textsuperscript{3+}]\textsubscript{3}[OH]\textsubscript{2}Si\textsubscript{8}O\textsubscript{10}) is an iron-rich amphibole fiber and is the most pathogenic type to induce mesothelioma.

The pathogenic mechanisms of asbestos fibers depend on their chemical and physical features (Robledo and Mossman, 1999). Several in vivo and in vitro studies have demonstrated that long asbestos fibers are more fibrogenic and carcinogenic in comparison with short fibers which are efficiently phagocytosed. A frustrated phagocytosis and prolonged interaction of long iron-rich fibers with the cell surface generate reactive oxygen and nitrogen species that result in the oxidation and nitrosilation of proteins and DNA and help to generate an inflammatory response (Kuwahara and Kagan, 1995; Simeonova et al., 1997). For this reason, the antioxidant defense system plays a crucial role against the damage caused by the fibers. Furthermore, in human and rat mesothelial cells, long asbestos fibers induce dimerization and activation of epidermal growth factor receptor (EGFR) and subsequent activation...
of transcription factors regulating genes involved in cell proliferation, carcinogenesis and apoptosis (Pache et al., 1998; Faux et al., 2000; Zanella et al., 1996, 1999).

A number of synthetic vitreous fibers (SVFs), including glass wool, rock wool and slag wool, have been used in recent years to replace asbestos fibers in acoustical and thermal insulation and in many other manufactured products. SVFs represent a family of synthetic, inorganic, vitreous substances classified as fibrous glass, continuous filaments, special purpose fibers and microfibers. They are chemically composed of oxides of silicon, calcium, sodium, potassium, aluminum, boron, magnesium and iron (Foa and Basilico, 1999). Studies have been carried out in order to evaluate the effects of these fibers on the induction of malignant and non-malignant pulmonary diseases during their manufacture, use and removal (Cullen et al., 1997; Oberdorster, 2000; Kim et al., 2001). In vitro studies demonstrated that glass fibers are phagocytized by Syrian Hamster embryo cells and that cytotoxicity and cell transformation are dependent on their length (Hesterberg et al., 1986).

In a recent study of part of our group (Cavallo et al., 2004), some SVFs (glass wool, rock wool) have been characterized for their in vitro toxic effects, evaluating the modifications of the cell surface by scanning electron microscopy and DNA damage by the Comet assay. The results suggested possible cytotoxic and genotoxic effects of these SVFs. In the present study, we extended the previous analysis investigating additional in vitro biological effects of these SVFs on more types of cells, and we compared these responses to those induced by crocidolite asbestos fibers. In particular, we analyzed the cytotoxic effect of the different SVFs and their involvement on cell proliferation and oxidative stress, at different times and using different doses. Mesothelial cells have been selected because they represent a target of asbestos fibers after inhalation and are involved in the release of inflammatory mediators, extracellular matrix constituents, fibroblast chemoattrants and growth factors (Kuwahara and Kagan, 1995; Liu et al., 2000; Fung et al., 1997). Moreover, we used a fibroblast cell line to analyze if a direct fiber interaction in vitro can induce some biological effects correlated with asbestos-induced fibrosis, a process well described as a result of the diffusion of cytokines and inflammatory molecules originating from mesothelial cells and pleural macrophages (Adamson et al., 1993; Schwartz, 1991). Finally, macrophages have been used since they represent the initial direct target of asbestos fibers.

Materials and methods

Cells and exposure to fibers

MeT5A cells, a human mesothelial cell line, were cultured in Dulbecco’s modified Eagle’s/F12 medium (DMEM/F12) (EuroClone, United Kingdom) supplemented with 10% fetal calf serum plus penicillin (100 U/ml) and streptomycin (100 U/ml), glutamine (2 mM), hydrocortisone (100 ng/ml), insulin (2.5 μg/ml) transferrin (2.5 μg/ml) and selenium (2.5 ng/ml). NIH3T3 murine fibroblast embryo cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% bovine calf serum and antibiotics. THP-1 cells, a human macrophage cell line, were cultured in RPMI 1640 medium (EuroClone, United Kingdom) supplemented with 10% fetal calf serum plus antibiotics. For differentiation, THP-1 cells were plated onto 6-well plates in culture medium containing 100 nM phorbol-12-myristate-13-acetate (PMA) for 48 h before fibers’ exposure.

Crocidolite fibers used in this study are those provided from the Federal Bureau of Mines to the National Institute of Environmental Health Sciences (NIHES) for studies on the biological effects of orally ingested asbestos-related materials. This sample has been chemically and physically characterized in detail (Campbell et al., 1980). Samples of glass wool (GW) and rock wool (RW) were obtained from two Italian manufacturers. Both samples have an alkaline oxide and alkali earth oxide (Na2O + K2O + CaO + MgO + BaO) content greater than 18% in weight. According to the European Commission Directive 97/69/EC, relating to the provisions on classification, packaging and labeling of dangerous substances, the pathogenicity of fibers is determined by a size parameter (the length weighed geometric mean diameter) and biopersistence assays. GW and RW were not classified as “carcinogenic material” by respective manufacturers based on short-term inhalation (T1/2 = 10 days) or intratracheal instillation into rats assay (T1/2 = 28 days). Another sample of rock wool (DRW) was obtained from a Danish manufacturer, and it has a biosolubility greater than Italian fibers. Furthermore, this material was not classified as “carcinogenic material” by the Danish manufacturer. A little quantity of fibrous material (about 1 g) was put on the microscope slide with some drops of water in order to avoid the dispersion of the fibers during the cutting. The fiber length of GW and RW was reduced by cutting the samples with a surgical blade number 22A. All the samples were suspended in distilled water by ultrasonication, and a small amount of each material was filtered onto a polycarbonate membrane. The diameter and length measurements of 300 fibers were carried out by a scanning electron microscope (LEO S 440) at a magnification that allowed every time a precise measure of size fibers in the range 300 x 80 μm.

Arithmetic means (μm) calculated for diameters of the SVFs were: GW 4.3 (standard deviation (SD): 3.5), RW 2.3 (SD: 1.6), DRW 3.7 (SD: 2.5). Arithmetic means (μm) calculated for lengths of the SVFs are: GW 57.3 (SD: 58.6), RW 52.1 (SD: 52.8), DRW 96.9 (SD: 62.8). The percent of respirable fibers with diameter < 5 μm was estimated in 75.2%, for GW; 96.9% for RW and 80.65% for DRW, respectively. The percent of fibers with length > 20 μm was estimated in 24.4% for CR, 81.01% for GW, 77.96% for RW and 91.24% for DRW, respectively. The number of fibers in 1 g of material was estimated as GW 5 x 10^6, RW 18 x 10^5, DRW 4 x 10^5. All fiber stock solutions were prepared at 1% in distilled water.

Cell exposure to crocidolite asbestos fibers and SVFs was performed 24 h after plating at different fiber concentrations (2, 5, 10 and 25 μg/cm²) or at equal fiber number and allowed to proceed for 24 or 48 h. For antioxidant supplementation treatments, cells were preincubated, for 48 h, with vitamin E (Merck, Germany) (15 μg/ml culture medium), before fiber treatments for 24 h (10 μg/cm²). The incorporation of vitamin E in the cell membranes was evaluated as described below (see Vitamin E analysis section).

Cell viability

Cells, grown onto 6-well plates and exposed to fibers for 24 and 48 h, were harvested by incubation in 0.5% trypsin, 0.2% ethylenediamine tetracetic acid (EDTA) for 10 min at 37°C, and cell viability following fiber treatment was measured by Trypan blue exclusion assay. All experiments were performed three times in duplicate.

5-Bromo-2′-deoxyuridine (BrdU) incorporation

For BrdU incorporation assay, cells were plated on glass coverslips previously coated with 2% gelatin (Sigma Chemicals Co., St. Louis, MO) onto 24-well plates and exposed to the fibers at a concentration of 5 or 10 μg/cm² for 24 h, serum-starved for 6 h and incubated with 100 μM BrdU (Sigma Chemicals Co., St. Louis, MO) at 37°C for 12 h to allow BrdU incorporation. Cells were then fixed in 4% paraformaldehyde in PBS for 30 min at 25°C followed by a treatment of 0.1 M glycine for 20 min at 25°C and with 0.3% HCl, 0.1% Triton X-100 for 45 min at 25°C to allow permeabilization. After 3 washes in PBS, cells were buffered with 0.1 M Na2B4O7 and incubated with anti-BrdU monoclonal antibody (Sigma Chemicals Co., St. Louis, MO) (1:50 in PBS) for 30 min at 25°C followed by goat anti-mouse IgG-FITC (1:10 in PBS for 1 h) (Cappel Research Products, Durham, NC).
Quantitation of BrdU incorporation was performed by evaluating the percentage of cells showing nuclei positively stained for BrdU on a total of 200 cells, randomly taken from five different fields in each experiment, and the results are expressed as the range of percentages calculated from three different experiments.

**Transmission electron microscopy**

Cells, exposed to crocidolite asbestos fibers and glass wool fibers (5 μg/ cm²) for 24 h, were washed three times in PBS, fixed with 2% glutaraldehyde in PBS for 2 h at 25°C and detached with a cell scraper. Samples were post-fixed in 1% osmium tetroxide in Veronal acetate buffer (pH 7.4) for 1 h at 25°C, stained with 0.1% tannic acid for 30 min and then with uranyl acetate (5 mg/ml) in Veronal acetate buffer, pH 6.0, for 1 h, dehydrated in acetone and embedded in Epon 812. Ultrathin sections (100 nm) were examined unstained or post-stained with uranyl acetate and lead hydroxide.

**Enzymatic antioxidant activities**

Cell membranes were disrupted by putting cells twice in liquid nitrogen, centrifuged at 10,000 × g for 10 min at 4°C. Enzymatic activities were determined, on the supernatants, by a DU-70 Beckman spectrophotometer (Beckman Instruments, Fullerton, CA, USA). Superoxide dismutase (SOD) activity was evaluated according to the method proposed by Spitz and Oberley (1989). In this competitive inhibition assay, superoxide, generated by xantine xanthine–oxidase system, is detected by monitoring the reduction of nitroblue tetrazolium at 560 nm. Total SOD activity was measured at pH 7.8 in phosphate buffer. One unit of activity was defined as the amount of protein that yields 50% of maximal inhibition of nitroblue tetrazolium reduction by superoxide. The results were reported as units of SOD per mg of proteins. Catalase activity was determined on cell supernatants as the disappearance of hydrogen peroxide (10 mM) measured at 240 nm by spectrophotometer in a phosphate buffer at pH 7.4 (Clairborne, 1985). Standard curves were obtained by using bovine catalase (CAT) at different concentrations (1, 2, 5 and 10 units per ml). One unit of CAT is defined as the amount that degrades 1 μM H₂O₂ per min at 25°C. At least two tests were performed on each supernatant, and experiments were repeated twice. Results are reported as mean of two different determinations performed in each sample and expressed as U/mg protein.

**Cu²⁺/Zn²⁺ SOD and Mn²⁺ SOD protein determination by Western blot analysis**

Cells were lysed in RIPA buffer (Harlow and Lane, 1988) supplemented with protease inhibitors. For the analysis of Cu²⁺/Zn²⁺ SOD and Mn²⁺ SOD, proteins were separated on a 12% acrylamide SDS-PAGE and transferred onto nitrocellulose (Amersham Biosciences Europe, Italy). Membranes were blocked with 5% non-fat dry milk (Bio-Rad Laboratories) in phosphate-buffered saline (PBS) containing 0.1% Tween 20 (Sigma-Aldrich) and incubated with an anti-Cu²⁺/Zn²⁺ SOD rabbit polyclonal antibody (1:1000) (SOD-101 Stressgen Biotechnologies Gianford Ave.Victoria BC Canada) or with an anti Mn²⁺ SOD rabbit polyclonal antibody (1:1000) (SOD-110 Stressgen Biotechnologies Gianford Ave.Victoria BC Canada) overnight at 4°C. After washes, a secondary bovine anti-rabbit IgG HRP-conjugated antibody (1:1000) (Santa Cruz Biotechnology Inc.) was added for 1 h at room temperature. Membranes were then washed and specific bands visualized by enhanced chemiluminescence reagent (ECL) (Santa Cruz Biotechnology Inc.). A subsequent hybridization with β-actin was used as a loading control.

**Vitamin E analysis**

Cells were extracted twice in chloroform–methanol (2:1) in the presence of 25 ng of (±)-γ-tocopherol and 25 of (±)-b-tocopherol as internal standards. Tocopherols were derivatized with 50 μl of N,O-bis-(trimethylsilyl)- trifluoracetamide containing 1% trimethylchlorosilane as a catalyst and were analyzed by gas chromatography mass spectrometry with a RTX-5MS column (30 m × 0.25 μm internal diameter, 0.25 mm, RESTEK Corporation, Bellefonte, PA, USA) by a selected ion(s) monitoring technique. The ions selected were 237, 277 and 502 for α-tocopherol; 223, 263 and 488 for γ-tocopherol; and 209, 249 and 474 for δ-tocopherol (Picardo et al., 1996). Analyses were repeated twice in each extract. Results are reported as means of two tests from two different experiments and expressed as μg/mg protein.

**Membrane fatty acid analysis**

Cell lysates were extracted twice in chloroform–methanol (2:1), in the presence of butylated hydroxy toluene (100 μg) as an antioxidant. Phospholipid fraction was purified by thin-layer chromatography, and 100 μg of tricosanoic acid ethyl ester was added as an internal standard. The fatty acids of phospholipid fraction were trans-methylated with sodium methoxide in methanol and analyzed using a combined gas chromatography mass spectrometry system (Hewlett Packard 5890 II gas chromatography combined with 5989 mass spectrometry) on a capillary column (FPA-P, 60 m × 0.25 μm × 0.25 mm, Hewlett Packard, Cupertino, CA, USA). Helium was used as a carrier gas. Oven temperature gradient from 80 to 220°C at 10°C/min was used. The results were obtained after time integration of the chromatogram and final processing of the peak areas and reported as PUFA μg/mg protein (Passi et al., 1991).

**Thiobarbituric reactive species (TBARS) analysis**

TBARS evaluation was performed as described by Jentzsch et al. (1996). 2 × 10⁶ cells, dissolved in 0.5 ml of PBSS, were homogenized in the presence of 25 μl of 10 mg/ml butylated hydroxy toluene and mixed with 100 μl of 0.2 M orthophosphoric acid and 1.25 μl of 0.11 M thiobarbituric acid in 0.1 M NaOH. The reaction mixture was then incubated at 90°C for 45 min in a water bath. Samples were then put on ice to stop the reaction and were extracted once with n-butanol. Samples were then centrifuged, in a Biofuge Status microfuge, Heraeus (Kendo Laboratories, Germany) at 1000 × g for 1 min to facilitate phase separation. Two hundred fifty microliters of the upper butanol phase were placed into a quartz cuvette. Absorption was read at 535 nm and 572 nm to normalize for baseline absorption in a DU-70 Beckman spectrophotometer (Beckman, Fullerton, USA). TBARS equivalents were calculated using the difference in absorption at the two wavelengths. Quantification was made with the aid of calibration curves, obtained by using Malondialdehyde (MDA) stock solution at different concentrations.

**Reactive oxygen species (ROS) detection**

ROS production was detected using 2',7'-dichlorofluorescein diacetate (DCFH-DA, Fluka AG, Switzerland), a substance oxidizable to fluorescent dichlorofluorescein by several different ROS and peroxides. Cells were carefully detached from the plastic flask, washed 2 times with PBS, centrifuged and finally incubated (30 min at room temperature). Membranes were then washed and specific bands visualized by enhanced chemiluminescence reagent (ECL) (Santa Cruz Biotechnology Inc.). A subsequent hybridization with β-actin was used as a loading control.

**Statistical analysis**

Student’s t test was used to determine the statistical significance. Statistical significance was accepted as P < 0.05. Correlation index (r) was used to indicate the correlation between different parameters. Statistical significance was accepted as r ≥ 0.5.

**Results**

Cell viability following exposure to man-made vitreous fibers and crocidolite asbestos fibers

In MeT5A cells, a clear dose-dependent increase in cell mortality was observed after 24 h of exposure to crocidolite...
asbestos fibers. The percentage of cell mortality was 1.1–3.8% in unexposed cells and 4.2–5.9%, 10–11.2% and 11–14.6% at the doses of 2, 5 and 25 μg/cm², respectively. A more pronounced increase in cell mortality was observed after 48 h of exposure to the crocidolite fibers (data not shown). Compared to the cytotoxic effect observed after crocidolite asbestos exposure, parallel exposure of MeT5A cells to glass wool did not show an increase in cell mortality compared to untreated cells (ranging 2–5.3%, 3–7.1% and 4–5.8% at doses of 2, 5 and 25 μg/cm² respectively). Exposure to increasing concentrations of rock wool fibers caused similar results (ranging 2.6–5%, 2.4–8.2% and 2.5–3.8% for rock wool and 2.5–4.2%, 2.7–4.8% and 3–3.8% for Danish rock wool at doses of 2, 5 and 25 μg/cm² respectively).

NIH3T3 cells exposed for 24 h to crocidolite asbestos fibers showed a significant increase in mortality at a dose-dependent rate (ranging 2.9–4.7%, 5.7–8.5% and 9.3–12% at doses of 2, 5 and 25 μg/cm², respectively) in comparison with unexposed cells (ranging from 1.1 to 3.2%). Exposure to either glass fibers or rock wool fibers did not show significant differences in cell mortality, compared to untreated cells (ranging 1.9–3.8%, 1.6–2.8 and 1.4–4.9% for glass wool and 1–3.3%, 1.4–2.8% and 2–3.9% for rock wool at doses of 2, 5 and 25 μg/cm², respectively). A representative experiment performed in triplicate, reporting the percentage of non-viable cells after exposure to the fibers using a maximum concentration, is shown in Fig. 1.

**Cell–fiber interactions**

To analyze the type and extension of the interaction of the fibers with exposed cells, we used phase contrast light microscopy, and we observed that the fiber–cell interactions were dependent on physical features of the fibers such as size, shape and diameter in all cell lines tested. Straight and needle-shaped crocidolite asbestos fibers established numerous interactions with MeT5A cells (evident on 87.6–90.7% of the cells) and maintained these interactions during all washing steps occurring in our experimental procedure (Fig. 2A). Furthermore, the cells interacting with the longest fibers displayed an elongated morphology with cell extensions (Fig. 2A, arrow), whereas the non-interacting cells appeared to have lengthened less. Glass fibers showed physical differences compared to crocidolite asbestos fibers: they appeared typically rod-shaped, longer and thicker (Fig. 2B). In addition, the interactions established with the cells were evident only on 20–33.5% of the cells, but the lengthening of cells along the fibers was still evident (Fig. 2B). Both rock wool fibers (Figs. 2C, D) appeared to interact weakly with the cells. In fact, we observed a further decrease in the number of cells interacting with both rock wool fiber (3.3–14.2%) and Danish rock wool fiber (5–17.6%).

Furthermore, we performed cell–fiber interactions analysis by exposing MeT5A cells at equal fiber number. Increasing fiber number corresponds to a greater number of cells interacting with fibers, but, again, we did observe a higher number of interactions with crocidolite asbestos fibers (95.23–97.82%) compared to SVFs that appear to have only few and weaker interactions with cells (31.81–40.54% for glass wool, 10.76–23.91% for rock wool and 22.9–27.84% for Danish rock wool, respectively). Similar results on cell–fiber interactions were also observed for NIH3T3 (96.46–98.76% for crocidolite asbestos fibers, 27.48–46.1% for glass wool, 11.1–23.79% for rock wool and 29.1–39.17% for Danish rock wool, respectively). A higher percentage of cells interacting with fibers was observed for THP-1 cells (97.5–99.2% for crocidolite asbestos fibers, 60–81.81% for glass wool, 50–77.7% for rock wool and 54.5–85% for Danish rock wool, respectively) compared to either MeT5A or NIH3T3. However, the cell–fiber interactions using SVFs were all lower than when using crocidolite asbestos fibers.

Previous ultrastructural studies focused on the mechanisms of cell interaction and internalization of asbestos fibers using different cell models (Malorni et al., 1990; Jaurand et al., 1979; Fleury et al., 1983; Cole et al., 1991). Therefore, to analyze in more detail the fiber–cell interactions in our experimental conditions, we evaluated, using transmission electron microscopy, both MeT5A and NIH3T3 cells after 24 h of exposure to crocidolite asbestos fibers and glass fibers (Figs. 2E–H). The ultrastructural analysis revealed details of the interaction between cell surface and fibers and of the phagocytosis process. Crocidolite asbestos fibers were observed in plasma membrane invaginations (Fig. 2E), in cytoplasmic vacuoles (Figs. 2F, G) and also free in the cytoplasm (Fig. 2G). Moreover, we observed the preferential localization of the fibers in the perinuclear area (Fig. 2F) in agreement with previous studies that have described the microtubule-mediated transport of crocidolite asbestos fibers to the proximity of the nucleus (Cole et al., 1991). The presence of intracellular fibers free in the cytoplasm may reflect the loss of the vacuole membrane which can occur after the fusion with the lysosomes, as reported (Cole et al., 1991), and which causes the release of hydrolytic enzymes into the cytoplasm which has been postulated as being responsible for the cytotoxic effect of the asbestos fibers. Regarding the glass fibers, unlike crocidolite asbestos fibers,
our ultrastructural observations revealed a reduced amount of fibers internalized by the cells and the preferential localization of the intracellular fibers in cytoplasmic vacuoles (Fig. 2H).

**Oxidative stress and lipoperoxidative events in cell membranes in response to man-made vitreous fibers or asbestos treatment**

Considering the similarity between SVFs and crocidolite asbestos fibers, based on fibrous aspects, inhalability, chemical composition and free radical formation, in this work, we analyzed the modification of the pattern of lipophilic and enzymatic antioxidants, the production of ROS and the appearance of the peroxidation markers of cell membranes, in MeT5A, exposed for 24 h to SVFs. The results were compared with that obtained after treatment for 24 h, of the same cells, with crocidolite asbestos fibers. Signs of oxidative stress and peroxidative damages were detected 24 h after the treatment of MeT5A with asbestos or SVFs. The highest content of TBARS (Fig. 3A), which represents the main lipoperoxidative end products, was observed in crocidolite asbestos fibers exposed cells, where their concentration had significantly increased in comparison with the value observed in untreated ones (from 0.018 ± 0.004 to 0.056 ± 0.012 nmol/mg protein, \( P < 0.01 \)). A less noticeable, yet significant effect was observed after the treatment with rock wool (0.049 ± 0.01 nmol/mg protein, \( P < 0.01 \)) and Danish rock wool (0.031 ± 0.006 nmol/mg protein, \( P < 0.01 \)).
protein, \( P < 0.05 \)). Treatment with glass wool did not produce any change in TBARS concentration (0.021 ± 0.005 nmol/mg protein). To strengthen these results, the PUFA content, in the phospholipid fraction of cell membrane, was also analyzed, after 24 h treatment of MeT5A with different fibers. As shown in Fig. 3B, the base concentration of PUFA was 0.99 ± 0.25 \( \mu \)g/mg protein, and treatment with crocidolite asbestos fibers caused the highest peroxidation degree (0.095 ± 0.018 \( \mu \)g/mg protein, \( P < 0.01 \)) followed by rock wool (0.286 ± 0.075 \( \mu \)g/mg protein, \( P < 0.01 \)). A slight decrease of PUFA was also observed after treatment with Danish rock wool (0.85 ± 0.17 \( \mu \)g/mg protein), even though this change was not statistically significant.

The lipoperoxidative process, induced by crocidolite asbestos fibers and SVFs in MeT5A, was associated with decreased amounts of Vitamin E, the main chain breaking antioxidant in the cell membranes, which decreased its concentration, from 78.41 ± 10.39 \( \mu \)g/mg protein in untreated cells to 63.00 ± 12.13 (\( P < 0.05 \)) and 58.24 ± 12.13 (\( P < 0.05 \)) \( \mu \)g/mg protein in Danish rock wool, glass wool, rock wool respectively and to 30.41 ± 5.61 (\( P < 0.01 \)) \( \mu \)g/mg protein in crocidolite asbestos fibers (Fig. 3C).

In order to evaluate if a supplementation of vitamin E could exert a protective role against the observed lipoperoxidative damages, a preincubation of the cells with this antioxidant, followed by the treatment with different fibers, was performed. Indeed, vitamin E supplementation was able to reduce the lipoperoxidative damage, as demonstrated by a decrease of TBARS levels. Preincubation with vitamin E did not influence the TBARS concentration, which in fact resulted not dissimilar to that observed in unsupplemented control cells. Vitamin E addiction significantly reduced 62.95% the production of TBARS induced by treatment with crocidolite asbestos fibers. After treatments with rock wool and Danish rock wool, vitamin E supplementation exerted a protective effect reducing TBARS production of a 11.17% and 3.91% respectively. By contrast, after glass wool exposure, vitamin E was unable to reduce TBARS concentration.

In order to evaluate if the oxidative perturbation of cell membranes, caused by exposure to different fibers, could influence the intracellular redox equilibrium, ROS content and SOD and Cat activity were also evaluated. As reported in Fig. 4A, all treatments produced an increase in the ROS concentration, associated with a consumption of Vitamin E (\( r = -0.86 \)) (Fig. 4B). In untreated samples, the mean fluorescence was 146 ± 7.9, and, in crocidolite asbestos fiber exposed cells, it was 172 ± 4.58 (\( P < 0.005 \)), where the most prominent effect was evident. A significant, even if a less

![Fig. 3. Effect of SVFs and crocidolite asbestos fibers on the concentration of total thiobarbituric acid reactive substances (TBARS) (A), polyunsaturated fatty acids (PUFA) (B) and vitamin E (C) in MeT5A. Cells were exposed for 24 h at 10 \( \mu \)g/cm\(^2\) of the different fibers. All fibers produced a lipoperoxidative effect. *\( P < 0.05 \) and **\( P < 0.01 \) in comparison with untreated cells.](image1)

![Fig. 4. ROS concentration, after treatment for 24 h with crocidolite asbestos fibers or different SVFs (A). The increase in the ROS was associated with a consumption of vitamin E (\( r = -0.86 \)) (B). CTR: untreated, GW: glass wool, RW: rock wool, DRW: Danish rock wool, CRO: crocidolite asbestos. *\( P < 0.05 \) and **\( P < 0.01 \) in comparison with untreated cells.](image2)
noticeable effect, was observed after the treatment with Danish rock wool and glass wool, whose mean fluorescence (MF) values were 164 ± 8.5 ($P < 0.05$) and 162 ± 8.1 ($P < 0.05$), respectively. Rock wool exposure, moreover, did not produce any statistically significant increase in ROS content (154.3 ± 8).

As regards SOD (Fig. 5A), all treatments produced a significant induction of its activity, which increased from 6.9 ± 0.49 U/mg protein, in untreated cells, to 8.38 ± 0.61 ($P < 0.05$) after exposure to crocidolite asbestos fibers, 14.16 ± 1.53 ($P < 0.01$) after treatment with rock wool, 11.81 ± 0.75 ($P < 0.01$) and 14.24 ± 1.44 ($P < 0.01$) U/mg protein, after treatment with Danish rock wool and glass wool, respectively. When cytosolic Cu$^{++}$Zn$^{++}$SOD and mitochondrial Mn$^{++}$SOD were evaluated by Western blot (Fig. 5B), after treatment with different SVFs, no significant difference was seen in the expression of both proteins, by contrast, treatment with crocidolite asbestos fibers induced a down-modulation of Cu$^{++}$Zn$^{++}$SOD and an up-modulation of Mn$^{++}$SOD.

As regards Cat activity, the enzyme that neutralizes hydrogen peroxide, no statistically significant modifications were observed after all treatments (5.63 ± 0.73 GW, 5.12 ± 0.69 DRW, 4.81 ± 1.09 RW, 4.53 ± 0.56 CRO) with respect to the control (5.51 U/mg protein) (Fig. 5C), although a trend to reduce the activity of catalase was seen with all fiber treatments.

Cells proliferation in response to fiber exposure

To investigate the possible proliferative effect resulting from the interaction of the fibers with the cells, we analyzed cell cultures exposed for 24 h to crocidolite asbestos fibers and SVFs by the BrdU incorporation assay. Cell cultures exposed or unexposed to the fibers were incubated with BrdU, and the positive nuclei were visualized by immunofluorescence with anti-BrdU monoclonal antibody. In unexposed MeT5A cells, the percentage of BrdU-positive nuclei appeared to range from 57.6% to 83%. An increased BrdU incorporation was observed after exposure to crocidolite asbestos fibers, reaching a percentage of positive nuclei ranging from 90.7% to 93% in cells which appeared to clearly interact with the fibers as assessed by parallel phase contrast microscopy (Fig. 6D, arrows). No evidence of increased BrdU uptake, compared to control cells, was observed in cells interacting with glass, rock wool and Danish rock wool fibers (range 56–87%, 59–82%, 56.5–75.8% respectively) (Figs. 6F, H, L, arrows). Similar results were obtained in NIH3T3 fibroblasts. In fact, cells exposed to crocidolite asbestos fibers displayed an increased number of BrdU-positive cells compared to unexposed cells, whereas glass fibers exposure did not seem to influence BrdU incorporation (data not shown).

These results suggest that, while crocidolite asbestos fibers appeared to exert a proliferative effect on the cells which can depend on fiber–cell interactions, the SVFs did not seem to induce a significant increase in proliferation of the cells interacting with them compared to the unexposed ones.

Discussion

Numerous SVFs have been used to replace asbestos which is known to induce fibrosis and malignant diseases, and it has been demonstrated that the pathogenic mechanism of asbestos depends on the chemical and physical features of the fibers (Robledo and Mossman, 1999; Foa and Basilico, 1999; Oberdorster, 2000; Mossman et al., 1990; Wylie et al., 1997; Hart et al., 1994).

In this study, we analyzed the effects of different types of SVFs (glass wool, rock woods), which are not classifiable as carcinogenic (IARC, 2002), on a mesothelial (MeT5A) and a fibroblast cell line (NIH3T3) comparing their effects with those exerted by crocidolite asbestos fibers. We were interested in correlating the possible cytotoxicity of these fibers with the peculiar interaction between fibers and cell surface and possible subsequent lipoperoxidative events. Our results on cell viability showed that the SVFs did not induce an increase in cell mortality compared to untreated cells, whereas crocidolite asbestos fibers exposure caused a dose-dependent increase in cell mortality, in agreement with previous studies (Dong et al., 1994). These
results are also consistent with the previous observations of part of our group showing no or little DNA damage and cell surface alterations using the same SVFs (Cavallo et al., 2004).

To investigate the mechanisms involved in the response of SVFs compared to crocidolite asbestos fibers, we first analyzed the interactions of the fibers with the cell surface and the following phagocytosis. By phase contrast light microscopy, we observed that SVFs, unlike crocidolite asbestos fibers, appeared to establish only few and weak cell surface interactions in both MeT5A and NIH3T3 cells. Furthermore, by transmission electron microscopy, we analyzed the fiber internalization process of crocidolite asbestos fibers and glass wool. The ultrastructural examination revealed that, while crocidolite asbestos fibers are often found free in the cytoplasm, glass wool fibers, on the contrary, are phagocytosed and distributed mainly on cytoplasmic vacuoles. Previous studies suggested that the cytotoxicity induced by crocidolite asbestos fiber could be exerted by the release of hydrolytic enzymes into the cytoplasm after lysosome fusion with vacuoles containing the fibers (Cole et al., 1991). Therefore, our observations indicated that the decreased cytotoxicity induced by SVFs, in comparison with that exerted by crocidolite asbestos, could be a product of...
the membranes surrounding the internalized SVFs, which could protect the cells from the action of hydrolytic enzymes.

Considering that the pathogenic effect of asbestos and SVFs, after the interaction, is also mediated by a chemical effect, we proceeded to analyze possible biochemical modifications. Our results showed that the exposure of MeT5A to crocidolite asbestos fibers or SVFs determined the development of a lipoperoxidative damage associated with a significant induction of SOD activity and production of ROS in the intracellular environment. For their surface properties, crocidolite asbestos and SVFs are able, in aqueous media, to exhibit surface oxidizing adsorptive sites that permit the reduction of O$_2$ to O$_2^-$ (Shukla et al., 2003). This free radical, in turn, is enzymatically reduced to H$_2$O$_2$ by SOD activity, and this induction was higher after treatment of cells with SVFs, which produced an oxidative stress less pronounced than that induced by crocidolite asbestos fibers.

In order to evaluate if the increase of SOD activity, observed after treatment with crocidolite asbestos fibers and different SVFs, was associated with an increased protein expression of both cytoplasmic Cu$^{++}$Zn$^{++}$ and mitochondrial Mn$^{++}$SOD, both proteins were analyzed by Western blotting. After treatment with SVFs, mitochondrial or cytoplasmic SOD proteins were not modified, suggesting that the observed increase in the total enzymatic activity could be ascribed to post-translational modifications. By contrast, after crocidolite asbestos treatment, the increase in the SOD activity was associated with a down-regulation of Cu$^{++}$Zn$^{++}$SOD and with an up-regulation of Mn$^{++}$SOD. This result is in agreement with previous studies which attributed a crucial role to SOD and in particular to the mitochondrial component (MnSOD) in protecting the cells against the cytotoxic effects of crocidolite asbestos (Kinnula et al., 1996; Kinnula, 1999).

H$_2$O$_2$, produced by SOD activity, exerts a key role in the cell, modulating different biochemical responses. In association with transitional metal pool, released from different fibers, H$_2$O$_2$ stimulates the production of hydroxyl radical. This compound, in turn, is responsible for a sequence of events, represented by the observed decrease in PUFA concentration associated with the consumption of vitamin E and the increase of TBARS, the end products of lipoperoxidation (Minotti and Aust, 1989; Gutteridge, 1994). The significant reduction of lipoperoxidative damages, due to vitamin E supplementation, underlined the key role of Fenton reaction in the mechanism of membrane injury induced by different fibers mainly by crocidolite asbestos (Fig. 7).

The catabolites of arachidonic acid participate, together with H$_2$O$_2$, in the induction of NF-$\kappa$B. This transcription factor, in turn, exerts a pleiotropic effect, promoting the survival pathway, inducing gene expression for different growth factors, stimulating cell proliferation and enhancing the expression of different enzymes such as Mn$^{++}$SOD (Faux and Howden, 1997). The described cellular oxidative response could be correlated with the fiber used. In particular, the structural characteristics of SVFs permit a different possibility of interaction with the biological substrate as it has been demonstrated with the microscopic analysis performed in this study.

In order to elucidate if cell–fiber interactions and lipoperoxidative events on exposed cells might exert a proliferative effect, we performed a BrdU incorporation assay, and, as assessed in previous proliferation studies, in vivo and in vitro (Cullen et al., 1997; Adamson, 1997; Adamson et al., 1993; Donaldson et al., 1995; Goldberg et al., 1997; BeruBe et al., 1996), we observed that SVFs exposed cells did not show increased cell proliferation rates, whereas there was a significant increase after exposure to crocidolite asbestos fibers. These findings paralleled the observation that crocidolite asbestos fibers, unlike SVFs, induce an up-regulation and ligand-independent phosphorylation of EGFR (Pache et al., 1998; Zanella et al., 1999; Faux et al., 2000), stimulating cell proliferation and transformation by the activation of mitogen-activated protein kinase (MAPK) and transcription factors (NF-$\kappa$B, AP-1) with relative increase of expression of the proto-oncogenes (c-jun, c-fos) (Robledo and Mossman, 1999; Pache et al., 1998; Faux et al., 2000; Zanella et al., 1999; Shukla et al., 2003).

Taken together, our present results suggest that the lack of effect of SVFs, in contrast to crocidolite asbestos fibers, on cell viability and proliferation could be ascribed to the different chemical composition of SVFs as well as their weakness and lack of interaction with the cells and provide...
evidence for a slight effect of SVFs on lipoperoxidative events at the cell membranes which may influence the intracellular redox equilibrium. Even if the carcinogenic potential of SVFs has not been completely clarified, presented results underline a role of SVFs in inducing oxidative stress events. This redox unbalance, in turn, could be responsible for possible mutagenic effects which have to be taken in account in prospective studies.

Acknowledgment

This work was partially supported by grants from Ministero della Salute, Italy.

References


