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Angiogenic effect induced by mineral fibres

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ABSTRACT

Due to the toxic effect of asbestos, other materials with similar chemical–physical characteristics have been introduced to substitute it. We evaluate the angiogenic effect of certain asbestos substitute fibres such as glass fibres (GFs), ceramic fibres (CFs) and wollastonite fibres (WFs) and then compare angiogenic responses to those induced by crocidolite asbestos fibres (AFs). An *in vitro* model using human endothelial cells in small islands within a culture matrix of fibroblasts (Angio-Kit) was used to evaluate vessel formation. The release of IL-6, sIL-R6, IL-8, VEGF-A and their soluble receptors, sVEGFR-1, sVEGFR-2, was determined in the conditioning medium of Angio-Kit system after fibre treatment. ROS formation and cell viability were evaluated in cultured endothelial cells (HUVEC). To evaluate the involvement of intracellular mechanisms, EGFR signalling, ROS formation and nuclear factor-κB (NFκB) pathway were then inhibited by incubating HUVEC cells with AG1478, NAC and PDTC respectively, and the cytokine and growth factor release was analyzed in the culture medium after 7 days of fibre incubation. Among the mineral fibres tested, WFs markedly induced blood vessel formation which was associated with release of IL-6 and IL-8, VEGF-A and their soluble receptors. ROS production was observed in HUVEC after WFs treatment which was associated with cell cytotoxicity. The EGFR-induced ERK phosphorylation and ROS-mediated NFκB activation were involved in the cytokine and angiogenic factor release. However, only the EGFR activation was able to induce angiogenesis. The WFs are potential angiogenic agents that can induce regenerative cytokine and angiogenic factor production resulting in the formation of new blood vessels.

1. Introduction

Environmental or occupational exposure to asbestos has long been recognized as a cause of both benign (pleural fibrosis and pleural plaques) and malignant disease (lung cancer and mesothelioma) (Mossman and Gee, 1989; Mossman et al., 1996). The genotoxic effects of asbestos may be due to a number of mechanisms, of which the formation of reactive oxygen and nitrogen species (ROS/RNS) is thought to be particularly important. Following inhalation of asbestos fibres, ROS and RNS can be generated in the lungs both via Fenton-type reactions catalyzed by iron present on the fibre surface, and via the chronic inflammation induced as a result of prolonged phagocytotic activity of macrophages against the bio-persistent fibres (Tomasetti et al., 2009). The evidence for adverse health effects following exposure to asbestos has prompted a drastic reduction in its use, resulting in the increased use of substitutes composed both of naturally occurring and synthetic materials which are thought to have lower toxicity. A number of man made mineral fibres (MMMFs) including glass wool, rock wool and slag wool have been used in recent years to replace asbestos fibres in acoustic and thermal insulation and in many other manufacturing products. The mineral fibres are structurally similar to asbestos fibres, contributing to the hypothesis that such fibres could cause cancer of the respiratory system. Wollastonite also serves as an asbestos replacement. Wollastonite is a naturally occurring calcium silicate ($CaSiO(3)$) produced in both powder and fibrous forms. The epidemiological evidence for wollastonite is limited, but it does not suggest that workers are at significant risk of an increased incidence of pulmonary fibrosis, lung cancer, or mesothelioma (Maxim and McConnell, 2005). Some reports

Abbreviations: GFs, glass fibres; CFs, ceramic fibres; WFs, wollastonite fibres; NAC, (N-acetylcysteine) and PDTC (pyrrolidine dithiocarbamate); HUVEC, human umbilical endothelial cells; MMMFs, man made mineral fibres; ROS, radical oxygen species; FGF, fibroblast growth factor; PDGF, platelet-derived growth factor; TGF-β, transforming growth factor-β; TNF-α, tumour necrosis factor-α; EGF, epidermal growth factor; EGFR, epithelial growth factor receptor; IL-6, interleukine-6; sIL-R6, soluble interleukine receptor-6; IL-8, interleukine-8; VEGF, vascular endothelial growth factor; sVEGFR-1, soluble vascular endothelial growth factor receptor-1; sVEGFR-2, soluble vascular endothelial growth factor receptor-2; MTT, 3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DCFA, 2′7′-dichlorofluorescein diacetate; NFκB, nuclear factor-κB; MAPK, mitogen-activated protein kinases.

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have implicated a number of these potential asbestos substitutes as causes of lung diseases (Wilson et al., 2001). Epidemiological studies have found elevated risk in workers exposed to mineral fibres (Lipworth et al., 2009; Lee et al., 1995). Despite a high elevation in relative risk of lung cancer among glass fibre production workers, the lack of excess risk among end users and the absence of any dose–risk relations argue against a carcinogenic effect of glass wool, rock wool and refractory ceramic fibres (Lipworth et al., 2009; Lee et al., 1995; LeMasters et al., 2003). In a recent study glass wool and rock wools have been characterised for their *in vitro* toxic effects, evaluating the modifications of cell surface by scanning electron microscopy and DNA damage by the comet assay (Cavallo et al., 2004). A cytotoxic and genotoxic effect was described for synthetic vitreous fibres (Cardinali et al., 2006). Glass fibres were found to be internalised inside cytoplasmic vacuoles, and signs of oxidative stress were observed after cell exposure by an increased ROS production and by an induction of superoxide dismutase (SOD) activity. Oxidative stress and inflammation are two major aetiological factors suggested to play key roles in angiogenesis (Ushio-Fukai, 2006). Exogenous ROS stimulate the induction of a number of cytokines and growth factors by various cell types and promote cell proliferation and migration (Stone and Collins, 2002). Angiogenesis is the growth of new capillaries from the pre-existent microvasculature (Carmeliet, 2005). Tight regulation of the dynamic equilibrium between proangiogenic and antiangiogenic factors is critical to health, as an imbalance in either direction contributes to a wide range of pathological conditions from atherosclerosis to cancer (Folkman, 1995). A large number of proangiogenic factors and their cognate receptors have been identified, including EGF, FGF, PDGF, TGF-β, TNF-α, IL-6, and IL-8. Central to the physiological and pathological regulation of angiogenesis is the VEGF system – its ligands and receptors (VEGFRs). VEGF is the most potent direct-acting angiogenic protein known. It elicits a pronounced angiogenic response in a variety of *in vivo* models (Plate et al., 1992; Cao et al., 1998).

In the present study, we evaluate the angiogenic effect of certain asbestos substitute fibres such as glass fibres (GFs), ceramic fibres (CFs) and wollastonite fibres (WFs) and then compare angiogenic responses to those induced by crocidolite asbestos fibres (AFs). An *in vitro* model using human endothelial cells in small islands within a culture matrix of fibroblasts was used to evaluate vessel formation. The release of IL-6, its soluble receptor sIL-R6, IL-8, VEGF-A and their soluble receptors sVEGFR-1, sVEGFR-2 was evaluated over time in the conditioning medium after treatment with two fibre doses (0.25 and 0.50 cm^2/cm^2 fibres). The ability of mineral fibres to induce ROS formation and cytoxicity was evaluated in cultured human endothelial cells (HUVEC). To evaluate the molecular mechanisms involved in the mineral fibre-induced vessel formation, epithelial growth factor receptor (EGFR) signalling, ROS formation and NFκB pathway were inhibited by incubating HUVEC cells with AG1478, NAC ((N-acetylcysteine) and PDTC (pyrrolidine dithiocarbamate)) respectively, then the release of cytokines and growth factors was analyzed in the culture medium after 7 days of fibre incubation. To further examine the rule of ROS-induced NFκB pathway and EGFR activation on vessel formation, the angiogenic activity induced by fibres was evaluated in Angio-Kit cultured cells in the presence or absence of NAC, PDTC and AG1478.

2. Materials and methods

2.1. Mineral fibres

Crocidolite asbestos fibres were provided from Union International Contra Cancer (UICC), and these fibres were characterised (chemically and physically) in detail (Governa et al., 1995). The crocidolite samples showed a chemical composition of SiO_2, 48.7%; Al_2O_3, 0.1%; FeO, 36.9%; MgO, 3.8%; CaO, 1.0%; Na_2O, 4.4%; TiO_2, 0.7%; MnO, 0.8%. Samples of glass and ceramic fibres were obtained from two Italian manufacturers. Both samples have an alkaline oxide and alkali earth oxide content greater than 18% in weight (Cardinali et al., 2006) with a chemical composition of SiO_2, 67.0%; Al_2O_3, 4.0%; FeO, 1.0%; MgO, 3.0%; CaO, 7.0%; Na_2O, 16.0%; K_2O, 1.0%; B_2O_3, 6.0%; P_2O_5, 1.0% for glass samples and a chemical composition of SiO_2, 54.0%; Al_2O_3, 48.0%; FeO, 0.1%;TiO_2, 0.1% for ceramic samples. Samples of NYAD wollastonite fibres (Interpace Corp., Wills-Boro, NY) were obtained from an Italian company producing fibre-reinforced cement. The samples were of high purity with a chemical composition of CaO, 47.0%; SiO_2, 50.0%; Fe_2O_3, 1.0%; MnO, 0.1%; MgO, 0.3%; TiO, 0.05% (Governa et al., 1998). The fibres were examined using a scanning electron microscope (Philips XL30, Monza, Italy) equipped with energy-dispersive X-ray analysis (EDAX) apparatus. The length and diameter of 300 fibres were measured by the scanning electron microscope at a tension of 20 kV and magnification of 2000×. The specific surface area of AFs, GFs, CFs and WFs was measured using the nitrogen absorption isotherm technique (Ghio et al., 1992). The samples were degassed at 90 °C for 16 h and then examined with a Sorpty 1750 (Fison Instruments, Milan, Italy), and free space was determined with helium. The fibre amount was expressed as ratio of specific surface area of fibres and surface area of well (cm^2/cm^2). All fibres used were endotoxins free.

2.2. In vitro angiogenesis assay

Blood vessel development was assessed by an Angio-Kit model (TCS Cell Works Ltd., Buckingham, UK/TEMA Research, Bologna, Italy). Human endothelial cells were co-cultured with fibroblasts in a specially designed medium. The endothelial cells initially formed small islands within the culture matrix. They subsequently began to proliferate and then entered a migratory phase during which they moved through the matrix to form threadlike tubule structures. These gradually joined up (9–14 days) to form a network of anastomosing tubules.

Following the manufacturer's instructions, the cultures were treated with 0.25 and 0.50 cm^2/cm^2 of fibres and checked daily to monitor the progress of tubule formation. No medium changes occurred during incubation. After 4–7–14 days of incubation, aliquots of conditioning medium (100 μl) were collected for cytokine analysis. In an other experiment, the Angio-Kit culture cells were exposed to the AFs and WFs (0.25 cm^2/cm^2) in the presence or absence of the inhibitors NAC (100 μM), PDTC (10 μM) and AG1478 (100 μM) and tubules formation evaluated. Next the cultures were fixed in cold formalin 2% and subsequently stained by a tubule staining kit containing anti-CD31 primary antibody (PECAM-1) conjugated

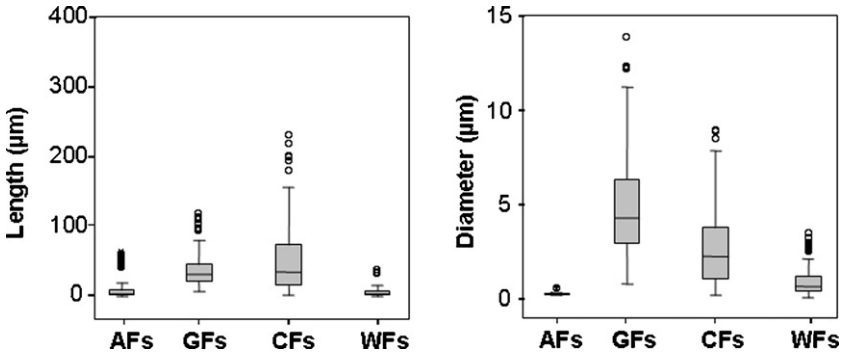

Fig. 1. Box-plot showing length and diameter distribution of crocidolite asbestos fibres (AFs), glass fibres (GFs), ceramic fibres (CFs) and wollastonite fibres (WFs). The fibres were examined by a scanning electron microscope (Philips XL30, Monza, Italy) equipped with energy-dispersive X-ray analysis (EDAX) apparatus. The length and diameter of 300 fibres were measured by the scanning electron microscope at a tension of 20 kV and magnification of 2000×.