BIOLOGICAL AND BIOCHEMICAL CHARACTERISATION OF A "PROLIFERATION FACTOR" FROM QUARTZ DUST-TREATED HUMAN MACROPHAGES

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INTRODUCTION
Silicosis is a chronic fibrotic lung disease caused by inhalation of quartz-containing dusts in coal mine workers, quarriers and stone masons. The disease is characterised by an increase in collagen content of the lungs, leading to destruction of lung parenchyma and to impairment of lung function. Alveolar macrophages are the primary target for the noxious effect of quartz and coal mine dust. In the alveoli dust particles are phagocytised by alveolar macrophages and removed from the lung via the "mucociliary transportation mechanism". In the case of "overload", dust-laden macrophages migrate into the regional lymph node and the interstitium of the lung.

Macrophages produce more than 100 "biofactors" or "mediators" that are involved in various processes of inflammation and immunological defence mechanisms (Nathan et al., 1980; Nathan, 1987). Various authors have reported that human monocytes and macrophages exposed to diverse soluble and particulate agents in vitro, release cytokines which stimulate human fibroblasts to cell multiplication (Austgulen et al., 1987; Bitterman et al., 1982; Glenn and Ross, 1981; Leslie et al., 1984; Schmidt et al., 1984; Seemayer et al., 1987, 1988).

In this paper we present data on the biological and biochemical characterisation of a "proliferation factor" which is produced by human monocyte-derived macrophages in culture following incubation with quartz dust DQ12. The "proliferation factor" (PF) stimulates under serum-free conditions cell replication of "quiescent" human lung and dermal fibroblasts and of human pneumocyte type II (line A-549) as well as collagen synthesis of human and dermal fibroblasts.

MATERIALS AND METHODS

Cell cultures
Isolation of monocytes from the peripheral blood in a Ficoll–Hypaque gradient and cultivation of cells to maturation with characteristics of macrophages has been described in detail (Seemayer and Braumann, 1985, 1988). The cell line WI38 (human embryonal lung fibroblasts) was purchased from Flow Laboratories, Meckenheim, Germany, the cell line MRHF (human foreskin fibroblasts) from Api-BioMerieux, Nurtlingen, Germany, the cell line FH3 (human embryonal skin...
A proliferation factor from quartz-dust treated human macrophages (human pneumocyte type II cells) was kindly provided by Dr G. M. Alink, Department of Toxicology, Agricultural University, Wageningen, The Netherlands. The cell lines WI38, MRHF and A-549 were cultivated routinely in Dulbecco's modified Minimum essential medium with 10% fetal calf serum and antibiotics.

Preparation of dust sample
Fibrogenic quartz dust DQ12 was used as a stimulus for cultures of human macrophages. This is Dörentruper crystal quartz flour (grinding no. 12) with a particle size less than 5 \( \mu \text{m} \). Dust samples were suspended in serum-free RPMI 1640 medium. The samples were subjected to ultrasonic treatment (Sonifier B-12 from Branson Sonic Power Company, U.S.A.) in order to achieve a uniform distribution of the particles and to destroy germs.

Preparation of supernatants from human macrophages in culture
Suspended dust samples were added in a final concentration of 30 \( \mu \text{g ml}^{-1} \) in serum-free RPMI 1640 medium to cultures of human macrophages. After an incubation period of 24 and 48 h at 37°C the culture supernatants were centrifuged for 15 min at 1,000 rpm, filtered through Millipore filters (pore size 0.45 \( \mu \text{m} \)) and treated in a water bath by 56°C for 1 h. The supernatants were then deep frozen at -20°C until used.

Ultrafiltration
Supernatants of quartz-treated human macrophages were ultrafiltered using devices from Millipore and Amicon (Centriprep) with different nominal molecular weight limits (NMWL).

Determination of proliferation of lung-fibroblasts and pneumocytes type II
The proliferation assay has been described in detail elsewhere (Griwatz et al., 1994).

Analysis of collagen synthesis
Whole cell-lysates were hydrolysed in 6N HCl at 110°C for 24 h and analysed for Hydroxyprolin (OH-Prolin) according to the method of Stegemann and Stadler (1967), using Chloramin-T and Dimethylaminobenzaldehyd.

RESULTS
In Fig. 1 we present the phagocytosis of quartz dust DQ12 by macrophages in culture. We could demonstrate that supernatants of these quartz dust DQ12 treated human macrophages release a soluble Proliferation Factor which stimulates "quiescent" human lung (FH-27) and dermal fibroblasts (FH-3) to a considerable cell multiplication (Seemayer et al., 1987, 1988).

The process of stimulation of fibroblast proliferation by the PF was also visualised by morphological criteria, such as an increased rate of mitosis and DNA synthesis and by reaches high cell density of fibroblast cultures (Hübner and Seemayer, 1989).
A similar “proliferation stimulating activity” was observed with human lung fibroblasts (WI38) and dermal fibroblasts (FH-3) treated with supernatants of human macrophages incubated with coal mine dust TF-1 (Seemayer and Maly, 1990; Hübner and Seemayer, 1992).

We made further attempts to characterise the PF produced by quartz and coal mine dust treated human macrophages. We found that the PF is still active after incubation for 60 min at 56°C. For estimation of the approximate molecular weight of the PF we utilised ultrafiltration with devices with 10 000 and 30 000 NMWL. Results based on induced cell multiplication of “quiescent” dermal fibroblasts FH-3 and MRHF and on stimulation of DNA synthesis of lung fibroblasts WI38 by the supernatant of quartz dust DQ12 exposed human macrophages larger and smaller than 10 000 and 30 000 NMWL indicated a molecular weight of the factor of more than 30 kDa (Seemayer et al., 1988; Hübner and Seemayer, 1989). In further studies we could demonstrate, that only a continuous presence of the PF induces a high number of DNA synthesising cells of WI38 cells followed by a considerable cell replication. DNA synthesis and cell replication of WI38 cells ceased very rapidly after removal of the PF from the medium (Seemayer et al., 1988; Hübner and Seemayer, 1989).

To elucidate further the characterisation of the PF we performed complementation tests according to Stiles et al. (1979) and Bittermann et al. (1982). By addition of a competence factor such as fibroblast growth factor (FGF) or platelet derived growth factor (PDGF) cell growth of WI38 or MRHF human fibroblasts incubated with supernatant of quartz dust DQ12-treated macrophages was significantly enhanced. Similar results were observed with WI38 cells in presence of supernatant of coal mine dust TF-1 treated human macrophages. Addition of FGF or PDGF remarkable enhanced cell multiplication.

In further studies we were able to demonstrate that beside human fibroblasts, epithelial cells of the alveolar unit, such as pneumocyte type II cells (line A-549), also respond with strong proliferation activity in presence of supernatant of quartz
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Fig. 2. Stimulation of cell proliferation of A-549 cells by a supernatant from quartz dust exposed human macrophages at various dilutions.

dust DQ12-treated human macrophages (Griwatz et al., 1993, 1994, 1995). The stimulating effect of the supernatant led to a markable increase of cell growth of A-549 cells in comparison to control (Fig. 2).

To characterise further the proliferation factor, we incubated supernatants of quartz dust DQ12-treated human macrophages with mercaptoethanol according to Pledger et al. (1977). A strong loss of activity was caused by mercaptoethanol-treatment of the supernatant. Results indicate then presence of disulphide bridges in the molecule of the cytokine which get destroyed by mercaptoethanol (Griwatz et al., 1995). A comparable loss of activity was observed after treatment of the supernatant with trypsin. For determination of the molecular mass of the proliferation factor, supernatants of quartz dust-exposed macrophages were concentrated by ultrafiltration and fractionated by gel-filtration on a column of Sephadex G 150. By gel filtration the proliferation activity eluted in the range of a molecular mass between 75 and 102 kDa (Griwatz et al., 1994, 1995).

Using anion-exchange chromatography, the Proliferation Factor eluted in a linear NaCl-gradient between 210 and 250 mM. Figure 3 shows two of the active fractions in the A-549 proliferation assay, leading to an enhanced cell multiplication. In SDS, polyacrylamide gel electrophoresis of biological active fractions of the anion exchange chromatography revealed two bands with molecular masses of 76 and 79 kDa (Griwatz et al., 1994, 1995).

Preliminary results demonstrate that supernatants of quartz-dust exposed human macrophages not only enhance the cell multiplication but also stimulate the collagen synthesis of human fibroblasts (Fig. 4). Importance has also been attributed to tumour necrosis factor alpha (TNF-α) in fibrosis, especially in
Fig. 3. Proliferation activity on A-549 cells by two active fractions from DQ12 supernatant after elution from anion exchange chromatography (Q-Sepharose) at 210–250 mM NaCl (20 mM Tris–Cl, pH 8.5).

Fig. 4. Stimulation of collagen-synthesis (measured as OH–Prolin) and cell multiplication by a supernatant of DQ12-treated macrophages (DQ12-SN).
A proliferation factor from quartz-dust treated human macrophages pathogenesis of silicosis and pneumoconiosis (Piguet et al., 1990; Driscoll and Maurer, 1991). An important factor for TNF-α production and release by monocytes is endotoxin, a major lipopolysaccharide constituent of the cell wall Gram-negative bacteria (Carswell et al., 1975). Because endotoxin contamination by bacteria of mineral dusts cannot be ruled out, we examined suspensions of coal mine dusts and quartz dust DQ12 for the presence of endotoxin utilising the very sensitive Limulus amoebocytes lysate test (LAL). Furthermore, we analysed supernatants of human macrophages treated with quartz dust DQ12 and with coal mine dusts for the presence of TNF-α by the cytotoxicity bioassay with L-929 cells (mouse fibroblasts). In addition, we investigated the effect of pure TNF-α on human pneumocyte type II cells (line A-549) alone and in combination with the Proliferation Factor from quartz dust DQ12 treated with human macrophages. Our results revealed that just a few samples of coal mine dusts from the Ruhr Valley contained endotoxin and only endotoxin-containing dusts stimulated human macrophages to produce TNF-α (Griwatz and Seemayer, 1994, 1995). While TNF-α led to a slight stimulation of cell proliferation of A-549 cells at low concentrations (1 ng ml⁻¹), a strong inhibition of cell proliferation of A-549 cells in presence of TNF-α was accompanied by morphological transformation of the epithelial cells to more spindle shaped cells. It is remarkable that strong stimulation of cell proliferation of A-549 cells by supernatants of quartz dust treated macrophages was completely inhibited in the presence of TNF-α (50 ng ml⁻¹) (Griwatz and Seemayer, 1994, 1995).

DISCUSSION

Results presented demonstrate that human macrophages in culture obtained by cultivation and differentiation of blood monocytes release a soluble factor following incubation with quartz dust DQ12 or coal mine dusts from the Ruhr Valley. This factor stimulates “quiescent” or only moderately replicating human lung and dermal fibroblasts as well as pneumocyte type II cells (line A-549) to a considerable cell multiplication (Seemayer, 1989; Seemayer and Maly, 1990; Griwatz et al., 1993, 1994, 1995). Therefore, we designated the factor as a Proliferation Factor. According to the dual control model of growth regulation (Stiles et al., 1979) the Proliferation Factor was classified as a “progression factor” because a continuous presence was required for cell multiplication and an enhanced growth was observed by addition of competence factors such as FGF and PDGF (Seemayer and Maly, 1990; Seemayer, 1989; Hübner and Seemayer, 1989, 1992).

Bittermann et al. (1982) reported that human alveolar macrophages incubated in vitro with soluble and particulate agents, release an alveolar macrophage derived growth factor (AMDF), exhibiting activity as a progression factor for human fibroblasts. The molecular weight of AMDGF of 18 kDa differs from the MW of the proliferation factor in the range of 75–102 kDa. Our described cytokine with proliferation activity differs by its molecular mass from known factors (Wahl, 1988). An explanation could be that activated macrophages produce a binding protein which forms a biological active complex, as described by Blum et al. (1989) for Somatomedin C. Leslie et al. (1989) described a cytokine in bronchioalveolar lavage fluid (BALF) of normal rats which stimulated cell proliferation of rat
primary pneumocytes type II cells. When BALF was fractionated by gel filtration on a column Sephadex G150 a broad peak of activity eluted with an apparent molecular mass of approximately 100 kDa. Results presented suggest a complex interaction of cells of the lung, particularly of the alveolar unit in fibrotic lung diseases, especially silicosis.

REFERENCES


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