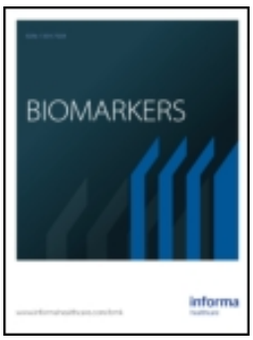

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RESEARCH ARTICLE

Serum growth factors in asbestosis patients

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Abstract

Various growth factors, including platelet-derived growth factor (PDGF) and transforming growth factor (TGF)-β, have been implicated in the pathogenesis of asbestos-induced disease. PDGF and TGF-β levels were determined by enzyme-linked immunosorbent assays in the banked serum samples of a cohort of workers with asbestosis, and the relationships of the growth factor levels to the subsequent development of cancer and to the radiographic severity and progression of asbestosis in the cohort were examined. Serum levels of PDGF and TGF-β were found to be unrelated to the development of cancer, and serum levels of PDGF were found to be unrelated to the severity and progression of asbestosis. However, serum levels of TGF-β were found to be statistically significantly related to disease severity ($p = 0.01$), increasing approximately 2.4-fold from ILO radiographic category 0 to category 3, and they were marginally related to disease progression ($p = 0.07$), in multivariate analysis controlling for other contributory factors including cumulative asbestos exposure. This suggests that serum TGF-β may be a useful biomarker for asbestos-induced fibrotic disease.

Keywords: *PDGF; TGF; pneumoconiosis severity; pneumoconiosis progression*

Introduction

The risk for developing cancer and non-malignant pulmonary disease as a result of past exposure to asbestos continues to be a major concern in occupational health both in the USA (Becklake et al. 2007, O'Reilly et al. 2007) and globally (Lin et al. 2007). Although the precise molecular mechanisms for asbestos pathogenesis remain unclear, some progress in the understanding of these disease processes has been made in recent years, particularly implicating the role of altered expression of proteins involved in cellular growth signal transduction pathways (Mossman et al. 1996, Robledo & Mossman 1999).

Identification of these proteins has allowed the application of a molecular epidemiological approach to the study of populations with asbestos exposure and the development of potential predictive biomarkers to identify individuals at the highest risk of disease. For example, we have found that expression of the protein products of various oncogenes and tumour suppressor genes (e.g. *ras*-p21, *EGFR*, p53) in serum can be predictive of the subsequent development of cancer in cases of asbestosis (Brandt-Rauf et al. 1992, Partanen et al. 1994, Husgafvel-Pursiainen et al., 1997, Li et al. 2005). In this particular cohort, the combination of biomarkers was found to have a relatively high positive predictive value (0.76), negative predictive value (0.66) and specificity (0.85); however, the sensitivity was less robust (0.51), indicating the need for the development of additional biomarkers (Li et al. 2005).

The expression of several growth factors has been suggested to play a role in asbestos pathogenesis, including platelet-derived growth factor (PDGF) and transforming

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growth factor (TGF)-β (Brody 1993, 1997). In preliminary studies, we found that serum levels of these proteins may be elevated in asbestos-exposed individuals. For example, among asbestosis cases, elevated serum PDGF was found more frequently in radiographically advanced cases of disease (Brandt-Rauf et al. 1992), and among firefighters, elevated serum TGF-β was found more frequently in those with a history of asbestos exposure and/or radiographic evidence of fibrotic lung disease (Ford et al. 1992). However, these studies only involved limited numbers of workers and relied on semiquantitative techniques based on immunoblotting for biomarker determination, and so the significance of the findings was uncertain. The present study involves quantitation of serum levels of PDGF and TGF-β by enzyme-linked immunosorbent assays (ELISAs) on multiple banked serum samples from a high-risk cohort of workers with diagnosed asbestosis to determine the relationship of these potential biomarkers to the status of the pneumoconiosis and the occurrence of cancer.

Materials and methods

In 1978–79 a cohort of 115 cases of compensable asbestosis was assembled at the Finnish Institute of Occupational Health in Helsinki (Oksa et al. 1997, Oksa 1998). On return visits from 1980–88, clinical evaluations were performed (including obtaining chest radiographs) and serum samples were collected on many of these cases, aliquoted and stored frozen at -70^{0}C. For the purposes of this study, suitable clinical information and serum samples were available on 82 of these cases for a total of 218 chest radiographs (two to three per case) and 187 serum samples (one to five per case). This group consisted of 74 men and eight women with an average age of 65 years at the end of sample collection in 1988. They had an average of 20 years of employment in asbestos-related industries in job categories with high likelihood of asbestos exposure (i.e. asbestos mining, asbestos insulation, asbestos spraying, asbestos cement manufacturing). All the patients had asbestosis and relatively high cumulative exposure to asbestos with an average estimated cumulative exposure of 523 fibre-years ml^{-1} (range 14–1750 fibre-years ml^{-1}). They included 16 never-smokers and 66 current or ex-smokers.

This group was obviously a high-risk cohort for having severe and progressive pneumoconiosis and for the subsequent development of cancer. Cancer incidence in this group was followed up until 31 December 2001, from the Finnish Cancer Registry, a national registry with complete coverage of diagnosed cancers in the country (Teppo et al. 1994). At that time, among these 82 subjects there had been 33 cancers (20 lung cancers, three mesotheliomas and 10 others including prostate, bladder, pancreas, rectum, gallbladder, melanoma of the skin and non-Hodgkin's lymphoma).

For evaluation of asbestosis, posterior–anterior chest radiographs were arranged in random order and classified for degree of disease by the consensus of three readers (two radiologists, one of whom was an ILO B-reader, and an internist/occupational medicine physician) who were blinded to the subjects' identities, using the ILO 1980 classification in effect at the time of the study. The distribution of the subjects by their baseline radiographic subcategory was: one case 0/0, 11 cases 0/1, 25 cases 1/0, 29 cases 1/1, six cases 1/2, three cases 2/1, five cases 2/2, one case 2/3 and one case 3/3; or aggregated by major radiographic category: 12 cases 0, 60 cases 1, nine cases 2 and one case 3. The distribution of the subjects by their worst radiographic subcategory was: one case 0/0, eight cases 0/1, 19 cases 1/0, 21 cases 1/1, three cases 1/2, nine cases 2/1, 10 cases 2/2, six cases 2/3 and five cases 3/3; or aggregated by major radiographic category: nine cases 0, 43 cases 1, 25 cases 2 and five cases 3. Of the 82 subjects, 31 (38%) had radiographic progression of their disease during the period of follow-up during the study, with an increase of from one to six subcategories of the ILO classification (six cases increased one subcategory, 10 cases increased two subcategories, 10 cases increased three subcategories, two cases increased four subcategories, two cases increased five subcategories, one case increased six subcategories). No subjects were judged to have radiographic regression of their disease over the course of the study.

All the serum samples were analysed for levels of PDGF-AB and TGF-β1 using quantitative ELISAs, according to the manufacturer's instructions. The PDGF-AB assay (R&D Systems, Minneapolis, MN, USA) is based on a mouse monoclonal antibody specific for PDGF-BB as the capture molecule and an enzyme-linked rabbit polyclonal antibody specific for PDGF-AA as the reporter molecule. For the assay, 100 μl of sample (serum diluted 50x with buffer) or standard plus 100 μl of buffer is added to individual microtitre plate wells precoated with 2 μg of mouse monoclonal PDGF-BB antibody and incubated for 3 h at room temperature. After four separate washes with 400 μl of buffer, 200 μl of a PDGF-AA rabbit polyclonal antibody conjugated to horseradish peroxidase is added to each well and incubated at room temperature for 1 h. After another four washes with 400 μl of buffer, 200 μl of tetramethylbenzidine chromogen solution is added to each well and incubated at room temperature for 30 min, followed by addition of 50 μl of 2 N sulfuric acid stop solution. Absorbance of each well is read on a spectrophotometric plate reader within 30 min at a 450 nm test wavelength and 620 nm reference wavelength, and concentrations of PDGF-AB are determined by comparison of the absorbance of the samples to the absorbance of a standard curve generated from

solutions of known concentrations of purified, recombinant human PDGF-AB. By this approach, the assay can be seen to have an average intra-assay variability of 3.2% and an average interassay variability of 7.4%, with a lower limit of detection of 1.7 pg ml–[1]. The assay recognizes recombinant and natural PDGF-AB with a recovery in spiked serum samples of 85–115%. The assay shows no significant cross-reactivity or interference with numerous human cytokines and other growth factors.

Similarly, the TGF-β1 assay (Promega, Madison, WI, USA) is based on a monoclonal antibody specific for soluble TGF-β1 as the capture molecule and a TGF-β1 specific polyclonal antibody as the reporter molecule. For the assay, serum samples are pre-treated to activate latent TGF-β1 to the reactive form. For the pretreatment, 10 μl of serum sample is diluted 1:5 in 40 μl of PBS, then mixed with 1 μl of 1 N HCl and incubated for 15 min at room temperature. The mixture is then neutralized with 1 μl 1 N NaOH and 30-fold with buffer (final serum dilution 1:150). Then, 100 μl of the sample solution or standard is added to individual microtitre plate wells precoated with the capture monoclonal antibody and incubated at room temperature for 90 min. After five separate washes with 400 μl of buffer, 100 μl of a TGF-β1 rabbit polyclonal antibody is added to each well and incubated at room temperature for 2 h. After five washes with buffer again, 100 μl of horseradish peroxidase conjugate is added to each well and incubated at room temperature for 2 h. After another five washes with 400 μl of buffer, 100 μl of tetramethylbenzidine chromogen solution is added to each well and incubated at room temperature for 15 min, followed by the addition of 100 μl of 1 N HCl stop solution. Absorbance of each well is read on a spectrophotometric plate reader within 30 min at 450 nm, and concentrations of TGF-β1 are determined by comparison of the absorbance of the samples to the absorbance of a standard curve generated from solutions of known concentrations of purified, recombinant human TGF-β1. By this approach, the assay can be seen to have an average intra-assay variability of 4.5%, 1.6% and 3.3% and an average interassay variability of 19.1%, 11.6% and 7.6% in low, medium and high concentrations of human TGF-β2, respectively, with a lower limit of detection of 15.6 pg ml^{-1}. The assay shows no significant cross-reactivity or interference with numerous human cytokines and other growth factors.

Average serum levels of PDGF-AB and TGF-β2 for all subjects were evaluated by the various categories of interest, e.g. cancer status, radiographic severity of disease, and radiographic progression of disease. As these values were not normally distributed, the data were logarithmically transformed to achieve normality, and geometric mean levels were compared among the various categories by analysis of variance (ANOVA). The interest category data were analysed by the Mantel–Haenszel χ^2 test. For multivariate analysis, covariance analysis for time-dependent repeated measurements was employed using mixed model procedures of SAS software to control for age, gender, smoking status, and estimated cumulative asbestos exposure in fibre-years ml^{-1}.

Results

In ANOVA using all serum sample values before the time of cancer diagnosis, levels of TGF-β and PDGF did not vary meaningfully by cancer status. For example, geometric mean serum levels of TGF-β were 12.9 ng ml^{-1} in those without cancer and 14.2 ng ml^{-1} in those with cancer, and geometric mean serum levels of PDGF were 16.1 ng ml^{-1} in those without cancer and 16.2 ng ml^{-1} in those with cancer. Similarly, as shown in Tables 1 and 2, in ANOVA using all serum sample values up to and including the time of the last available chest radiograph, levels of PDGF did not vary meaningfully by severity or progression of asbestosis by radiographic status. For example, geometric mean serum PDGF levels ranged from 18.6 ng ml^{-1} in major category 0 to 16.0 ng ml^{-1} in major category 1 to 16.9 ng ml^{-1} in major category 2 to 19.0 ng ml^{-1} in major category 3 in terms of radiographic severity, and geometric mean serum PDGF levels in those without radiographic progression of disease were 16.9 ng ml^{-1} compared with 16.4 ng ml^{-1} in those with progression. None of these differences were statistically significant (p >0.05).

In addition in this cohort, by Mantel–Haenszel χ^2 testing radiographic severity and/or progression of disease tended to be greater in male subjects than female subjects and to increase with estimated cumulative

Table 1. Association of serum growth factor levels with radiographic severity of asbestosis.

ILO classification	Mean serum PDGF-AB* (ng ml^{-1})	Mean serum TGF-β1** (ng ml^{-1})
Category 0 (n = 9)	18.6	9.8
Category 1 (n = 43)	16.0	13.3
Category 2 (n = 25)	16.9	15.0
Category 3 (n = 5)	19.0	23.4

PDGF, platelet-derived growth factor; TGF, transforming growth factor.
* p = 0.59; ** p = 0.04.

Table 2. Association of serum growth factor levels with radiographic progression of asbestosis.

Progression	Mean serum PDGF-AB* (ng ml^{-1})	Mean serum TGF-β1** (ng ml^{-1})
No (n = 51)	16.9	13.0
Yes (n = 31)	16.4	15.3

PDGF, platelet-derived growth factor; TGF, transforming growth factor.
* p = 0.73; ** p = 0.21.

asbestos exposure, although these trends were not statistically significant. For example, the odds ratio for severe disease (major categories 2 and 3) was 4.5 (95% confidence interval (CI) 0.6–33.1) in males compared with females, and compared with the lowest tertile of exposure (<237 fibre-years ml^{-1}), the odds ratio for severe disease increased to 2.6 (95% CI 0.8–8.5) in the middle tertile of exposure (237–690 fibre-years ml^{-1}) to 2.8 (95% CI 0.9–9.1) in the highest tertile of exposure (>690 fibre-years ml^{-1}) (p for trend = 0.13). Similarly, compared with the lowest tertile of exposure, the odds ratio for disease progression increased to 3.0 (95% CI 1.0–9.7) in the middle tertile of exposure and to 2.8 (95% CI 0.9–9.1) in the highest tertile of exposure (p for trend = 0.15).

On the other hand, as shown in Tables 1 and 2, variations in serum TGF-β levels with severity or progression of disease were more suggestive. For example, geometric mean serum TGF-β levels ranged from 9.8 ng ml^{-1} in major category 0 to 13.3 ng ml^{-1} in major category 1 to 15.0 ng ml^{-1} in major category 2 to 23.4 ng ml^{-1} in major category 3. Geometric mean serum TGF-β levels in those without radiographic progression of disease were 13.0 ng ml^{-1} compared with 15.3 ng ml^{-1} in those with progression; compared with serum levels in those without disease progression (13.0 ng ml^{-1}), levels increased to 13.9 ng ml^{-1} in those whose disease progressed one to two radiographic subcategories, to 15.0 ng ml^{-1} in those whose disease progressed three to four radiographic subcategories, and to 28.0 ng ml^{-1} in those whose disease progressed five to six radiographic subcategories. Although the differences by progression did not reach statistical significance (p >0.05), the differences by severity were statistically significant (p = 0.04), so the relationship of serum TGF-β levels to radiographic status was explored further. In the multivariate regression model controlling for age, gender, smoking status, and estimated cumulative asbestos exposure in fibre-years ml^{-1}, serum TGF-β levels still remained statistically significantly associated with radiographic severity of disease whether designated by major category (p = 0.01) or by subcategory (p = 0.03), and the association with radiographic progression of disease now approached statistical significance (p = 0.07).

Discussion

The results of this study suggest that serum levels of TGF-β are correlated with the state of pneumoconiosis in individuals with asbestosis. This finding is consistent with prior results on the role of TGF-β in pulmonary fibrosis and its expression in response to exposure to asbestos and other fibrogenic agents.

TGF-β is thought to play an important role in the development of fibroproliferative lung disease, because it contributes to the control of cell proliferation and migration and is a potent stimulator of extracellular matrix deposition (Massague et al. 1992, Border & Noble 1994, Polyak 1996). TGF-β is a cytokine family of multiple isoforms with similar but non-overlapping activities. TGF-β1 is the most abundant and possibly most important for lung fibrosis. TGF-β is secreted in a latent form as a high molecular weight complex that can be converted to the mature, active, 12-kD form by proteolytic cleavage. TGF-β isoforms signal by binding dimerized transmembrane receptors of two subtypes with serine/threonine kinase activity. TGF-β-induced growth signal transduction appears to be subsequently mediated via phosphorylation and nuclear localization of the Mad family of transcription factors. Several studies demonstrate the association between high levels of TGF-β expression, deposition of extracellular matrix and fibrotic lung disease. TGF-β stimulates the synthesis and deposition of collagen and various types of fibronectin in several tissues, including the lung, and may also stimulate matrix generation by inhibiting the production of proteases (Ignotz & Massague 1986, Edwards et al. 1987). Exposure of type II alveolar cells in culture to TGF-β1 increases fibronectin, laminin and proteoglycan synthesis and modulates integrin and surfactant protein C expression (Maniscalo & Campbell 1994). Exposure of fetal rat lung epithelial lines to TGF-β is also known to induce synthesis and secretion of types I and III collagen (DiMari et al. 1991), and elevation of 1α (I) procollagen mRNA levels in isolated rat lung fibroblasts treated with monocyte chemotactic protein appears to be mediated via activation of endogenous TGF-β1 expression (Gharaee-Kermani et al. 1996). In rat models, introduction via the trachea of a recombinant adenovirus expressing TGF-β1 causes histopathological changes consistent with pulmonary fibrosis (Yoshida et al. 1995).

The murine model of asbestos-induced fibroproliferative lung disease is also instructive. Inhalation exposure of rats and mice to asbestos produces enhanced TGF-β expression at sites of fibre deposition which correlates with elevated levels of extracellular matrix (Perdue & Brody 1994, Liu & Brody 2001). During the early stages of lung injury and repair after asbestos exposure, TGF-β is found primarily in alveolar macrophages and pulmonary epithelial cells (Lee et al. 1997). This pattern is also seen in the later stages of a fibrogenic response produced by bleomycin (Khalil et al. 1991). Similarly, intratracheal instillation of silica in rats induces TGF-β expression in fibroblasts, macrophages and hyperplastic type II cells that is associated with silicotic granulomas and co-localizes with type I procollagen synthesis (Mariani et al. 1996).

These findings agree with similar results in humans. Bronchoalveolar lavage (BAL) fluid and alveolar

macrophages from individuals with pneumoconiosis possess increased amounts of various cytokines, including TGF-β (Vanhee et al. 1994). Immunohistochemical analysis of asbestotic lungs show increased TGF-β localized to the fibrotic lesions (Jagirdar et al. 1997). TGF-β also co-localizes with procollagen 1 and fibronectin synthesis in patients with pulmonary fibrosis and appears to be associated with silicotic nodules and the development of progressive massive fibrosis (Broekelmann et al. 1991, Jagirdar et al. 1996). In related clinical studies of idiopathic pulmonary fibrosis, higher levels of TGF-β from BAL correlate with a more aggressive disease and a poorer prognosis (DiGiovine et al. 1996). TGF-β has also been analysed in blood, and levels related to the development of fibrotic disease. For example, as noted above, serum levels of TGF-β were found to be elevated in 42% of firefighters with a history of asbestos exposure and/or radiographic evidence of fibrotic lung disease compared with 0% of matched, healthy controls (Ford et al. 1992). Serum levels of TGF-β have also been found to be statistically significantly elevated in patients with silicosis or coal worker's pneumoconiosis compared to healthy controls (Yao et al. 2006). Furthermore, in patients receiving intensive chemotherapy for cancer, elevated circulating levels of TGF-β were found to be predictive of the subsequent development of hepatic and pulmonary fibrosis, with a positive predictive value > 0.90 (Auscher et al. 1993).

All these data tend to indicate that TGF-β plays an important role in fibroproliferative lung disease, including asbestosis, and that increased expression may be associated with severe and progressive disease. These data, together with the results of the present study, suggest that measuring serum levels of TGF-β may be a useful adjunct for monitoring the state of pneumoconiosis in individuals exposed to asbestos and other fibrogenic agents and thus for identifying high-risk individuals to be targeted for more aggressive intervention.

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Declaration of interest: The authors report no conflicts of interest.

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