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Increased 8-isoprostane, a Marker of Oxidative Stress in Exhaled Breath Condensate in Subjects with Asbestos Exposure

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Abstract: Asbestosis and pleural plaques exhibit unpredictable but progressive development, and there are no markers routinely available to measure their prognosis. Asbestos exposure induces the generation of reactive oxygen species, and 8-isoprostane is involved in experimental asbestos-related lung toxicity. This oxidative stress marker was measured in exhaled breath condensate (EBC) in 92 former asbestos workers with mean age 68.8 ± 1.7 yr and mean duration of asbestos exposure 24.1 ± 2.0 yr. The control group had 46 subjects with mean age 65.2 ± 3.3 yr. The mean level of 8-isoprostane, analyzed by liquid chromatography —electrospray ionization— mass spectrometry, was higher in asbestos-exposed subjects (69.5 ± 6.6 pg/ml, p=0.0001) compared with the control group, where the concentration was 47.0 ± 7.8 pg/ml. The results presented support the hypothesis that oxidative stress due to asbestos is the main cause of increased 8-isoprostane in EBC. Measurement of 8-isoprostane in EBC is a promising non-invasive means for assessing the activity of asbestos-induced diseases.

Key words: Asbestos, Asbestosis, Pleural hyalinosis, 8-isoprostane, Breath condensate, Rheumatoid factor, α1-microglobulin, α1-antitrypsin, Cotinine

Introduction

Asbestos exposure leads to asbestosis and to the development of pleural plaques; it also increases the risk of mesothelioma and lung cancer. It would be of utmost importance to better understand the relations between benign pleural diseases and the risk of mesothelioma and lung cancer. There are no markers routinely available to measure the activity and prognosis of asbestos-induced diseases, and there is no accepted treatment for them.

Current evidence suggests a role for reactive oxygen species (ROS) in the pathogenesis of asbestos-induced diseases. Asbestos can cause oxidative damage to the lungs directly, through hydroxyl radical formation via the Haber-Weiss reaction with fiber surface iron, and indirectly through recruitment and activation of ROS-producing inflammatory cells, such as macrophages[1)]. Mesothelial cells and lung fibroblasts, to a lesser extent, are also able to generate ROS species in response to asbestos[2, 3)].

Oxidative stress can be quantified in a biologic specimen by measuring the products of the effects of oxidative stress. An increase in the exhaled breath condensate (EBC) of 8-isoprostane, a stable prostaglandin-*F2*-like compound, originating from a major pathway of arachidonic acid disposition, indicates that there is an ongoing lipid peroxidation that is incompletely suppressed by antioxidant defenses[4)]. EBC examination appears to be a simple and non-invasive method to study lower respiratory tract events *in vivo*[5)]. EBC contains several bio-

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markers of inflammation and oxidative stress, and collection can be repeated several times without any adverse effects, even in children[6] or severely ill patients. 8-isoprostane has been observed to increase in the EBC of patients with idiopathic pulmonary fibrosis[7] and in our limited study with asbestos exposed subjects[8]; it was recently confirmed in 15 patients with asbestosis, in parallel with the inflammation marker, alveolar nitric oxide concentration[9].

Because the effect of recent smoking, another source of reactive oxygen species on the concentrations of 8-isoprostane on breath condensate has been described[5], cotinine, a metabolite of nicotine in the urine and a tobacco smoke biomarker[10] was also analyzed in our study to eliminate this possible influence. On the other hand, antioxidants and free-radical scavengers, such as vitamin C and E, can act as defenses against free-radical-mediated oxidative damage[4].

Methods

Subjects and sampling

A total of 92 subjects were examined –46 women and 46 men. Data on the asbestos-exposed subjects and controls are given in Table 1.

The asbestos-exposed subjects came to the Department of Occupational Medicine for regular follow-up due to their past exposure. An estimate of asbestos fiber-years was calculated according to Hagemeyer *et al.*[11], based on workplace measurements and duration of exposure in years. Classification of the estimated cumulative fiber dust dose in three fiber-year classes yielded the following distribution: 58 (nearly 63%) subjects were classified in fiber-year class III (100 and more fiber-years), 18 (20%) in fiber class II (25–99 fiber-years) and 16 (17%) in fiber class I (<25 fiber-years). Median asbestos fiber-years amounted to 147.5 (in the range of 6–1150).

Workers were employed for a mean 24.1 ± 2.0 yr in three asbestos manufacturing plants (using about 95% chrysotile and 5% crocidolite), mostly in the production of asbestos insulation and textile materials, asbestos-cement roofing, and pipe. The median beginning was in 1964, with median ending in 1990. Median latency time since the first year of work was 41.0 yr, median latency since the end of exposure was 15.0 yr.

The control group was represented by 46 subjects (23 men and 23 women), employed as hospital technical workers (gatekeepers, adjuncts and helpers, hospital mailmen, etc.) without occupational exposure to asbestos but with a lifestyle similar to the asbestos-exposed group. They did not differ in age, sex or factors potentially influencing oxidative stress, such as smoking and alcohol consumption, from the subjects in the exposed group (Table 1).

Informed consent was obtained from each participant prior to taking blood samples for erythrocyte sedimentation (ESR), rheumatoid factor, α1-antitrypsin, and α1-microglobulin, which were measured by standard laboratory methods. Clinical and laboratory data (including cholesterol, triglycerides, liver enzymes and bilirubin in blood) were collected to search for factors potentially influencing oxidative stress.

Lung functions were measured using a body plethysmograph (Jaeger, Germany). Chest radiography, conducted in all asbestos-exposed subjects, revealed findings in the range of s0-u3[12]. Asbestosis with pleural plaques was seen in 47 (51.1%) patients. Profusion of irregular opacities in this group was classified as grade 1 in 25 subjects (i.e. 53.2%), as grade 2 in 17 subjects (i.e. 36.2%), and as grade 3 in 5 subjects (i.e. 10.6%). Asbestosis only was found in 9 (9.8%) subjects, among them 7 subjects with profusion of irregular opacities grade 1 (77.8%) and 2 subjects (22.2%) with profusion grade 2. Pleural changes only were found in 23 (25.0%) subjects. Normal findings were observed in 13, i.e. 14.1% subjects. HRCT was performed in selected patients (53 subjects, i.e. about 58%) when a more exact picture was needed for the classification and compensation of the

Table 1. Characteristics of subjects exposed to asbestos and controls

	Asbestos-exposed, n=92		Controls, n=46		
	Mean ± CI	Range	Mean ± CI	Range	*p* exact
Age	68.8 ± 1.7	48–91	65.2 ± 3.3	51–85	0.133
Cigarette Pack-years	14.7 ± 4.5	0–96	10.5 ± 4.8	0–50	0.115
Alcohol ml/wk	99.1 ± 28.8	0–700	111 ± 63	0–1,050	0.693

Notes: CI — confidence interval of the arithmetic mean is calculated as follows:

$$CI = t_{0.05;n-1}\frac{s}{\sqrt{n}}$$

where s is calculated SD, $t_{0.05;n-1}$ *t*-coefficient on the level of significance 0.05 and n is number of results involved in the computation.

occupational disease. The study was carried out according to the Helsinki Declaration and approved by the university ethics committee.

The smokers in our study were restricted from smoking at least 2 h before collection of breath condensate. In addition, nicotine metabolite cotinine in the urine was measured to verify the non-smoking category.

EBC samples were collected using the EcoScreen (Jaeger, Germany). Each subject was asked to breathe through the collection kit for 15 min, with more than 2 ml of EBC collected. Samples were immediately frozen after collection (–80˚C) and stored for a period not exceeding 1 month. Contamination of saliva in the EBC was excluded by the colorimetric detection of α-amylase (α-Amylase-Liquid BIO-LA-TEST kit, Pliva-Lachema, Czech Republic).

Analytical methods

8-isoprostane in EBC was analyzed after immunoaffinity separation using LC-ESI-MS (liquid chromatography —electrospray ionization— mass spectrometry), where the multiple reaction monitoring (MRM) mode was used for its extremely high degree of selectivity, and stable-isotope-dilution assay for its high precision of quantification[13]. The limit of detection (LOD) for 8-isoprostane was determined to be 1 pg/ml, and the limit of quantification (LOQ) 5 pg/ml; recovery was 84% and precision was statistically evaluated as 8%.

Cotinine in the urine was determined by gas chromatography/mass spectrometry (SIM mode).

Statistical analysis

Student's *t*-test (for equal variances and for equal means), *F*-test, ANOVA, χ^2 and linear regression (correlation coefficient) methods were used for statistical comparison of the groups.

Results

8-isoprostane, ESR 1.H, ESR 2.H, α1-microglobulin and α1-antitrypsin were higher in the asbestos-exposed than in the control group (Table 2). Moreover, α1-antitrypsin was positively correlated with 8-isoprostane (p=0.04). Positivity of rheumatoid factor (above 25 IU/ml) was more frequent in exposed than controls (20.7% and 4.4%, respectively, p=0.0119).

Several lung function parameters were lower in exposed subjects than in the controls, as can be seen in Table 2. There was not a significant difference between exposed men and women in the 8-isoprostane level (70.3 ± 9.8 vs. 68.7 ± 9.3 pg/ml, p=0.813). No correlation for 8-isoprostane was found with asbestos fiber-years and latency since first or last exposure until this study.

In both asbestos-exposed and control subjects, 8-iso-

Table 2. Comparison of laboratory findings and lung functions in asbestos-exposed and controls

Group		Asbestos-exposed	Control	
Parameter	Units	Mean ± CI	Mean ± CI	*p* Exact
8-isoprostane	pg/ml	69.5 ± 6.6	47.0 ± 7.8	0.0001
ESR 1.H	mm	24.8 ± 4.0	12.2 ± 2.6	0.000011
ESR 2.H	mm	39.6 ± 4.9	24.7 ± 4.5	0.000011
α1-antitrypsin	g/l	1.580 ± 0.06	1.42 ± 0.10	0.001
α1-microglobulin	mg/l	10.8 ± 2.2	6.9 ± 2.2	0.014
PEF	% pred	102.6 ± 5.7	110.0 ± 7.6	0.134
FVC	% pred	100.4 ± 4.7	110.0 ± 5.4	0.014
FEV1	% pred	99.7 ± 5.0	109.7 ± 6.1	0.018
MEF 25–75	% pred	73.8 ± 6.7	91.4 ± 14.2	0.012
TLC	% pred	96.5 ± 3.3	103.6 ± 4.2	0.013
RV/TLC	% pred	43.2 ± 2.4	38.3 ± 2.8	0.016
TLCO	% pred	73.4 ± 4.1	82.7 ± 4.8	0.006

Notes: CI — confidence interval of the arithmetic mean is calculated as follows:

$$CI = t_{0.05;n-1}\frac{s}{\sqrt{n}}$$

where s is calculated SD, $t_{0.05;n-1}$ *t*-coefficient on the level of significance 0.05 and n is number of results involved in the computation, pred - predicted, PEF - peak expiratory flow, FVC - forced vital capacity, FEV1 - forced expiratory volume in 1 s, MEF 25–75 maximal expiratory flow rate at 25–75% of the vital capacity, TLC - total lung capacity, RV - residual volume, TLCO - diffuse lung capacity for carbon monoxide, ESR - erythrocytes sedimentation.

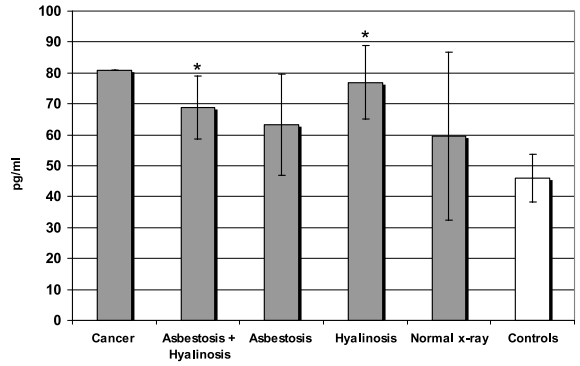

Fig. 1. 8-isoprostane levels in EBC according to radiological findings in asbestos-exposed (grey) groups and control (white) group.
Note: Error bars represent ± CI (CI — confidence interval of the arithmetic mean, $p \leq 0.01$); *denotes columns which differ from the control group ($p \leq 0.01$). Error bars were not evaluated for the "Cancer" column, because this group comprised only two patients.

prostane did not correlate with daily alcohol consumption or cigarettes/day. In the controls, no effect of daily intake of vitamins with antioxidant properties, such as vitamin C and E on 8-isoprostane was observed. Among the subjects with asbestos exposure, only 19 subjects used vitamins daily, compared with 73 subjects who did not use them daily. Interestingly, in the group of subjects with vitamin intake, the exposure to asbestos as expressed by fiber-years was 1.5-fold higher. However, the level of 8-isoprostane was not higher in more-exposed subjects, on the contrary, 53 ± 19 and 71.1 ± 7.9 pg/ml (p=0.088) was measured in subjects with and without daily vitamin intake, respectively.

More detailed analysis of 8-isoprostane levels according to the findings on chest radiographs is shown in Fig. 1. As can be seen, only the differences between patients with both asbestosis and hyalinosis (47 subjects) and controls and between subjects with hyalinosis (23 subjects) and controls were significant. In addition, two patients with lung cancer had a high level of 8-isoprostane.

The significant correlation of fiber-years was seen for crepitation (p=0.000039), chronic cough (p=0.00066), dyspnoea (p=0.0001) and history of tuberculosis of the lungs (p=0.042).

Cigarette pack-years in asbestos-exposed subjects were associated with a decrease of FEV1%, FEV1 and TLCO (p=0.0012, 0.0009, and 0.0009, respectively), and in the controls of FEV1%, FEV1/FVC, and MEF25-75 (p=0.036, 0.036, and 0.049, respectively).

Cotinine in the urine was found in smokers in both groups; the highest level in asbestos-exposed subjects was 794 ng/ml, in the controls 430 ng/ml. No effect of smoking status and cotinine level on 8-isoprostane level in the EBC was found in either asbestos-exposed or controls.

Discussion

Several experimental studies have established the importance of oxidative stress in the pathophysiology of asbestos damage. Our finding of an increase of 8-isoprostane in EBC in human asbestos-induced respiratory disorders supports these data. In this study we found an important elevation of 8-isoprostane in a group of subjects with benign asbestos-induced diseases and in two subjects with asbestos exposure and lung cancer. However correlation with asbestos fiber-years was not found.

The mean level of 8-isoprostane was increased in the whole group of asbestos-exposed subjects as compared with the controls. It was not significantly increased in patients with asbestosis. This group was relatively small; it comprised 9 patients only and profusion of their irregular opacities was relatively low, which could have influenced the result.

Inflammatory response, expressed by ESR, rheumatoid factor, α1-microglobulin and α1-antitrypsin, was more frequent in the whole asbestos-exposed group. α1-antitrypsin is an acute phase reactant which is elevated in acute and chronic inflammatory conditions, infections and with some cancers. It is synthesized in the liver and secreted into the plasma. From the plasma, α1-antitrypsin diffuses into various body compartments, including the lungs, where it provides the antiprotease protection[14]. Elevation of α1-antitrypsin has already been described in a study where asbestos exposure was also associated with ANCA positivity[15]. α1-microglobulin is synthesized in the liver and present in plasma, but was found in the extracellular interstitial matrix of many tissues, including lung tissue. Moreover, the specific presence of this protein in lung airways and alveolar septa resembles that of extracellular superoxide dismutase, a scavenger of ROS[16].

Among the asbestos-exposed subjects and among the controls, no correlation of 8-isoprostane with alcohol intake or cigarette pack-years was found, which confirms that these lifestyle factors did not play an important role. No influence of recent smoking was seen either, which might be explained by the fact that smokers were restricted from smoking 2 h at least, and mostly they abstained from smoking for several hours, as EBC collection was performed in the early morning.

Several systemic diseases, such as atherosclerosis, hypertension and others were described to increase serum and urine 8-isoprostane level[17]. Analysis of serum and urine point to these systemic disorders associated with oxidative stress; on the other side markers in the EBC appear specific for respiratory disorders. In this study, 8-isoprostane did not correlate with blood lipids, bilirubin, or liver enzymes (data not shown).

Limitation and advantage of the study

A limitation of the study is the fact that HRCT has only been performed in patients where needed for diagnosis for compensation, in order to avoid unnecessary exposure to ionizing radiation. Among 13 subjects from the group without radiological changes, HRCT was not performed in 5 subjects. The implication is that minor parenchymal and pleural asbestos-induced changes could be overlooked. This may explain why 8-isoprostane was increased in the whole group of asbestos-exposed workers, including subjects without typical radiological changes.

An advantage of the study is using a more sensitive and precise analytical method for 8-isoprostane in EBC than the more commonly used method of immunoassay detection. A higher sensitivity of the analytical method might also have plaid the role in a higher 8-isoprostane level in our both control and exposed subjects. Another plausible explanation is the older age of subjects with naturally higher level of oxidative stress[18]. It should be mentioned anyway, that comparisons between absolute concentrations of EBC markers that have been reported by different studies are currently difficult due to differences in the EBC collection procedures, condensers, and sample storage and handling, differences in the analytical techniques used, and inter-individual biological variability[19, 20]. Therefore, EBC analysis is not ready yet for screening of more endangered asbestos exposed subjects, and follow-up data are needed for the evaluation of its utility for activity assessment in early stages of asbestos-induced diseases and/or compensation.

Conclusion

The level of 8-isoprostane in EBC was increased in subjects who were exposed to asbestos in the past; a significantly higher level was seen in patients with pleural hyalinosis or pleural hyalinosis combined with asbestosis and in two patients with lung cancer. In this study, asbestos exposure appeared to be the main factor, as no other parameter was significant among several factors studied. EBC analysis is a promising method and there are initial studies in which it has also been performed in asbestos-exposed subjects using analysis of leukotrienes[7, 21].

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References

1) Shukla A, Gulumian M, Hei TK, Kamp D, Rahman Q, Mossman BT (2003) Multiple roles of oxidants in the pathogenesis of asbestos-induced diseases. Free Rad Biol Med **34**, 117–1129.
2) Kopnin PB, Kravchenko IV, Furalyov VA, Pylev LN, Kopnin BP (2004) Cell type-specific effects of asbestos on intracellular ROS levels, DNA oxidation and G1 cell cycle checkpoint. Oncogene **23**, 8834–40.
3) Dopp E, Yadav S, Ansari FA, Bhattacharya K, von Recklinghausen U, Rauen U, Rödelsperger K, Shokouhi B, Geh S, Rahman Q (2005) ROS-mediated genotoxicity of asbestos-cement in mammalian lung cells in vitro. Part Fibre Toxicol **2**, 2–9.
4) Morrow JD (2006) The isoprostanes —unique products of arachidonate peroxidation: their role as mediators of oxidant stress. Curr Pharm Des **12**, 895–902.
5) Barnes PJ (2005) Exhaled breath condensate: a new approach to monitoring lung inflammation. In: New perspectives in monitoring lung inflammation. Analysis of exhaled breath condensate. Montuschi P (Ed.), 1–9, CRC Press, Boca Raton.
6) Baraldi E, Carraro S, Alinovi R, Pesci A, Ghiro L, Bodini D, Piacentini G, Zacchellon F, Zanconnato S (2003) Cysteinyl leukotrienes and 8-isoprostane in exhaled breath condensate of children with asthma exacerbations. Thorax **58**, 505–9.
7) Psathakis K, Mermigkis D, Papatheodorou G, Loukides S, Panagou P, Polychronopoulos V, Siafakas NM, Bouros D (2006) Exhaled markers of oxidative stress in idiopathic pulmonary fibrosis. Eur J Clin Invest **36**, 362–7.
8) Pelclová D, Fenclová Z, Kačer P, Kuzma M, Lebedová J, Klusáčková P, Balíková M, Navrátil T (2005) Exhaled breath condensate in asbestos exposure. CHEST **128** (Suppl), 345–6.
9) Lehtonen H, Oksa P, Lehtimäki L, Sepponen A, Nieminen R, Kankaanranta H, Saarelainen S, Järvenpää R, Uitti J, Moilanen E (2007) Increased alveolar nitric oxide concentration and high levels of leukotriene B(4) and 8-isoprostane in exhaled breath condensate in patients with asbestosis. Thorax **62**, 602–7.
10) Heinrich-Ramm R, Wegner R, Garde AH, Baur X (2002) Cotinine excretion (tobacco smoke biomarker) of smokers and non-smokers: comparison of GC/MS and RIA results. Int J Hyg Environ Health **205**, 493–9.
11) Hagemeyer O, Otten H, Kraus T (2006) Asbestos consumption, asbestos exposure and asbestos-related occupational diseases in Germany. Int Arch Occup Environ Health **79**, 613–20.
12) Guidelines for the use of ILO international classification of radiographs of pneumoconiosis 22, 1980. Revised edition. Geneva, ILO 1980.
13) Syslová K, Kačer P, Kuzma M, Lebedová J, Klusáčková P, Fenclová Z, Pelclová D (2008) Determination of 8-iso-prostaglandin F (2alpha) in exhaled breath conden-

sate using combination of immunoseparation with LC-ESI-MS. J Chromatogr B Analyt Technol Biomed Life Sci **867**, 8–14.
14) Mulgrew AT, Taggart CC, Lawless MW (2004) Z alpha1-antitrypsin polymerizes in the lung and acts as a neutrophil chemoattractant. Chest **125**, 1952–7.
15) Pelclová D, Bartůňková J, Fenclová Z, Lebedová J, Hladíková M, Benáková H (2003) Asbestos exposure and antineutrophil cytoplasmic Antibody (ANCA) positivity. Arch Environ Health **58**, 662–8.
16) Berggård T, Oury TD, Thøgersen, Åkerström B, Enghild JJ (1998) α_1-Microglobulin is found both in blood and in most tissues. J Histochem Cytochem **46**, 887–94.
17) Patrignani P, Tacconelli S (2005) Isoprostanes and other markers of peroxidation in atherosclerosis. Biomarkers **10** (Suppl 1), 24–9.
18) Horváth, I, Hunt J, Barnes PJ. ATS/ERS Task Force on Exhaled Breath Condensate (2005) Exhaled breath condensate: methodological recommendations and unresolved questions. Eur Respir J **26**, 523–48.
19) Rosias PP, Robroeks CMN, Niemarkt HJ, Kester AD, Vernooy JH, Suykerbuyk J, Teunissen J, Heynens J, Hendriks HJ, Höbsis Q, Dompeling E (2006) Breath condenser coatings affect measurement of biomarkers in exhaled breath condensate. Eur Respir J **28**, 1036–41.
20) Montuschi P (2007) Analysis of exhaled breath condensate in respiratory medicine: methodological aspects and potential clinical applications. Therap Adv Respir Dis **1**, 5–23.
21) Pelclová D, Fenclová Z, Kačer P, Kuzma M, Navrátil T, Lebedová J, Klusáčková P (2007) Arachidonic acid derivatives in the exhaled breath condensate in pneumoconioses and their correlation with individual factors. Chem Listy **101**, s144–6.