Free Radical Activity of Industrial Fibers: Role of Iron in Oxidative Stress and Activation of Transcription Factors

Peter S. Gilmour,1 David M. Brown,1 Paul H. Beswick,1 William MacNee,2 Irfan Rahman,2 and Kenneth Donaldson1

1Department of Biological Sciences, Napier University, Edinburgh, United Kingdom; 2Rayne Laboratory, Unit of Respiratory Medicine, University of Edinburgh, Edinburgh, United Kingdom

We studied asbestos, vitreous fiber (MMVF10), and refractory ceramic fiber (RCF1) from the Thermal Insulation Manufacturers' Association fiber repository regarding the following: free radical damage to plasmid DNA, iron release, ability to deplete glutathione (GSH), and activate redox-sensitive transcription factors in macrophages. Asbestos had much more free radical activity than any of the man-made vitreous fibers. More Fe3+ was released than Fe2+ and more of both was released at pH 4.5 than at pH 7.2. Release of iron from the different fibers was generally not a good correlate of ability to cause free radical injury to the plasmid DNA. All fiber types caused some degree of oxidative stress, as revealed by depletion of intracellular GSH. Asbestos upregulated nuclear binding of activator protein 1 transcription factor to a greater level than MMVF10 and RCF1; long-fiber asbestos was the only fiber to enhance activation of the transcription factor nuclear factor κB (NFκB). The use of cysteine methyl ester and buthionine sulfoximine to modulate GSH suggested that GSH homeostasis was important in leading to activation of transcription factors. We conclude that the intrinsic free radical activity is the major determinant of transcription factor activation and therefore gene expression in alveolar macrophages. Although this was not related to iron release or ability to deplete macrophage GSH at 4 hr, GSH does play a role in activation of NFκB. — Environ Health Perspect 105(Suppl 5):1313–1317 (1997)

Key words: asbestos, fiber, MMVF, RCF, free radicals, oxidative stress, glutathione, transcription factors

Introduction

Extensive research in laboratory animals and human populations on the effects of asbestos has determined that exposure increases the risk of pulmonary fibrosis, pleural mesotheloma, and bronchial carcinoma (1,2). Much less is known of the potential toxicity of man-made respirable industrial fibers (2). Early research into the mechanisms of fiber pathogenicity implicated fiber geometry, length and dimension (2,3), and surface area (4). The physical properties of respirable fibers appear to persist in the lung milieu and express their pathogenic potential and their ability to cause genetic effects in vitro (5). Man-made vitreous fiber(s) (MMVF) and refractory ceramic mineral fibers (RCF) also have been shown to be pathogenic in animal inhalation studies (6). Surface-associated free radical activity has been suggested as a mechanism of asbestos-mediated toxicity (7), but man-made fibers appear to have little activity in this respect (8). Free radicals may cause damage by causing DNA, lipid, and protein oxidation but more subtle effects in initiating gene transcription may also occur (9). Iron is released readily from selected fibers (8) and can produce free radicals via Fenton chemistry, which ultimately results in the production of the hydroxyl radical.

In the present study we examine the free radical activity of three fibers and relate this to the release of iron and its ability to cause oxidative stress and to activate nuclear transcription factors AP-1 and NFκB in alveolar macrophages.

Materials and Methods

Fibers

For this study asbestos and two types of man-made fibers were used. The amosite asbestos was used as previously described (10). RCF 1, 2, 3, and 4, and MMVF 10, 11, 21, and 22 were obtained from the Thermal Insulation Manufacturers' Association (TIMA) fiber repository and were as used in the Research and Consulting Company (RCC) studies (6). The characteristics of these different samples are as previously described (8). All fibers were used at equal fiber numbers of 9.3 × 10⁴ fibers/DNA assay, 8.24 × 10⁷/ml for iron detection, and 8.24 × 10⁶/ml for reduced glutathione (GSH) and gel mobility shift assays.

Measurement of Iron Release from Fibers

All fiber types were suspended in either pH 4.6 or pH 7.2 citric acid/NaHPO₄ buffer at a fiber concentration of 8.24 × 10⁷ fibers/ml and iron release over 8 hr at 37°C was assessed using desferrioxamine-B (DFSB) for Fe²⁺, or ferrozine 10 mM for Fe³⁺ (8). The amount of iron present in solution after 8 hr of incubation was expressed as μM Fe²⁺ or Fe³⁺/8.24 × 10⁷ fibers.

Measurement of Induction of Fiber-mediated Free Radical Damage to DNA

As described in detail by Gilmour et al. (8), φ X174 RF1 closed-circular supercoiled DNA was used to detect free radical activity.

Alveolar Macrophage Preparation

Alveolar macrophages were obtained by lavage from in-house-bred, adult Wistar rats, as described previously. Macrophages

This paper is based on a presentation at The Sixth International Meeting on the Toxicology of Natural and Man-Made Fibrous and Non-Fibrous Particles held 15–18 September 1996 in Lake Placid, New York. Manuscript received at EHP 27 March 1997; accepted 11 July 1997.

We acknowledge the help of W. Nicholson in this study and the Health and Safety Executive for their support of DMB.

Address correspondence to Dr. P.S. Gilmour, Applied Chemical and Physical Sciences, Napier University, 10 Colinton Road, Edinburgh EH10 5DT, UK. Telephone: 0131 455 2505. Fax: 0131 455 2290. E-mail: p.gilmour@napier.ac.uk

Abbreviations used: AP-1, activator protein 1; BSO, buthionine sulfoximine; CME, cysteine methyl ester; DFSB, desferrioxamine-B; GSH, reduced glutathione; GSSG, oxidized glutathione; LFA, long-fiber amosite; LPS, lipopolysaccharide; Man, mannitol; MMVF, man-made vitreous fibers; NFκB, nuclear factor κB; RCC, Research and Consulting Company; RCF, refractory ceramic fiber(s); TIMA, Thermal Insulation Manufacturers' Association.
were seeded into 24-well plates at a concentration of 1 × 10⁶ cells/ml and allowed to attach for 1 hr, after which non-adherent cells were removed by washing with medium and cell treatments were performed in the culture medium for 4 hr. For gel mobility shift assays 3 × 10⁵ rat alveolar macrophages were seeded and incubated with treatment for 16 hr.

**GSH and GSSG Assays**

The alveolar macrophages were assessed for GSH and oxidized glutathione (GSSG) by spectrophotometry according to the modified method of Tietze (11, 12). The protein content of the cell lysates was measured by the BioRad Bradford protein reagent (BioRad Laboratories, Hemel, Hempstead, UK) to allow the GSH and GSSG to be described as nanomolar per milligram of protein. To alter the levels of GSH, cysteine methyl ester (CME) was incubated with macrophages for 30 min before fiber addition at a concentration of 300 µM to increase the GSH levels. Buthionine sulfoximine (BSO) was used similarly to decrease the GSH at a concentration of 100 µM.

**Gel Mobility Shift Assays**

Nuclear proteins were extracted from treated, washed macrophages, and all gel mobility shift assays were carried out according to the method modified by Nicholson et al. (13). Briefly, alveolar macrophages were treated with fibers for 4 hr, after which nuclear proteins were extracted as previously described (14). For each mobility shift reaction, 7 µg of nuclear protein was incubated with 1.75 pmol of freshly 32P-labeled AP-1 or NFκB oligonucleotide. This was carried out at room temperature in the presence of 0.25 mg/ml poly(dI-dC)·poly(dI-dC) competitor oligonucleotides to prevent nonspecific nuclear protein-DNA binding. Samples were separated in 6% polyacrylamide gel for 18 hr at 20 V. Bands were quantified using scanning laser densitometry.

**Statistical Analysis**

One- or two-way analysis of variance was used to determine the significance of treatment effects. The Tukey correction was used when comparisons between multiple treatment groups were required.

**Results**

**Fiber-mediated Free Radical Activity**

Amosite asbestos produced considerable free radical damage to DNA, as shown by the significant 85% depletion compared to 10% for untreated controls (p < 0.001). This asbestos free radical activity was partially attributable to hydroxyl radicals, as shown by mannitol intervention and chelation of fiber-associated iron (Figure 1). RCF1 and MMVF10 fibers were significantly less able to produce free radicals in this assay (p < 0.001), and the slight reduction in supercoiled DNA attributed to these fibers could not be accounted for by iron or hydroxyl radical (Figure 1).

**Release of Iron from Fibers**

To assess a possible connection between free radical activity and fiber-associated iron and the effect of physiologically important pHs, the release of iron from a range of fibers was determined. More Fe³⁺ than Fe²⁺ was released at both pH 4.6 and 7.2; there clearly was more iron release at pH 4.6 than at 7.2 (Figure 2). There did not appear to be any correlation between iron release and free radical activity because both RCF1 and MMVF10 released substantial amounts of iron, with RCF1 releasing significantly more iron of both forms than long-fiber amosite (LFA) (Figure 2).

**Rat Alveolar Glutathione Concentration**

All fibers significantly lowered intracellular GSH (p < 0.01), with MMVF10 causing...
the greatest depletion (Figure 3). Chelation of fiber-associated iron did not significantly ameliorate the fiber-mediated decrease in GSH levels, except in the case of LFA treatment, indicating that iron was not responsible for the intracellular redox status (Figure 4).

**Transcription Factors**

Compared to that in the untreated control, MMVF10 and RCF1 fiber samples upregulated the nuclear translocation of AP-1 transcription factor by 11.9 and 9.1% respectively. LFA, however, produced a far greater increase in AP-1, 37.4% more than in the untreated control. This was similar to the levels found with the lipopolysaccharide (LPS) positive control sample (Figure 5). Only LFA was able to upregulate the NFkB transcription factor; MMVF10 and RCF1 produced no effect (Figure 5).

To test the effect of lowering intracellular GSH on the fiber-mediated NFkB activation, fibers were incubated with GSH-depleted cells and NFkB activation was measured. Depletion of GSH increased the upregulation attributed to LFA treatment and also produced upregulation of RCF1 almost double that of the BSO alone control (Figure 6).

To further study the role of GSH in transcriptional activation, we supplemented the intracellular GSH with CME. In cells pretreated this way LFA-mediated NFkB nuclear translocation was almost eliminated.

**Discussion**

The substantial free radical activity associated with LFA, attributable largely to hydroxyl radicals, agrees with the results of our own and other previous studies (7,8,15). This reflects the pathogenicity of this form of asbestos; Johnston and Maples (16) have demonstrated that this free radical activity can induce p53 protein through iron via Fenton chemistry. LFA

**Figure 3.** Levels of intracellular GSH and GSSG after treatment with different fibers.

**Figure 4.** Levels of GSH and GSSG after treatment with normal fibers and fibers treated with the iron chelator DSF. Note no significant effect of chelation of fiber-associated iron.

**Figure 5.** Nuclear translocation of (A) AP-1 and (B) NFkB transcription factors with fiber treatments and control. Graph shows quantification of bands; each bar represents the mean and ± SEM values from three separate experiments.
has been shown to have a large oxygen radical-generating potential compared to that of other fibers (17), and indeed this appeared to be the case in this study where MMVF10 and RCF1 displayed only minimal free radical activity. The minimal free radical generation displayed by MMVF10 and RCF1 could not be accounted for by iron or hydroxyl radicals. The lack of surface activity appears to agree with the relative lack of pathogenicity of MMVF10, but RCF1 fibers have been found to be carcinogenic in animal studies (6).

The extent of release of the two forms of iron from the fibers at both pHs did not relate to the extent of fiber free radical activity. Although iron was responsible for the activity of asbestos, as demonstrated by chelation studies, both MMVF10 and RCF1 released comparable amounts of, if not more, iron. Therefore, the ability of fibers to generate free radicals cannot be accounted for by released iron alone. The importance of iron in free radical activity, however, may lie in the behavior of surface-bound iron; this is under further investigation. The presence in the lung milieu of reductants initiating Fenton chemistry could be a factor in the pathogenicity of man-made fibers that release iron, but obviously this is unimportant in the DNA assay. Phagocytosis by macrophages produces reductants that have been shown to enhance the production of dust-related free radicals (18) and all three fibers used in the present study caused significant depletions of macrophage GSH. The ability to cause GSH depletion is not, therefore, related to the free radical activities of the fibers. Thus, the products of phagocytosis are likely to mediate some of the oxidative stress effects of fibers. Iron chelation at the fiber surface did not affect the ability of fibers to change GSH redox status, which indicates that GS depletion depends on the presence of iron. The decrease in GSH is likely to be a result of the exportation of GSH as a stress response instead of a direct fiber/free radical oxidation of GSH, which would have resulted in an increase in GSSG. However, the low pH of macrophage phagolysosomes may cause the release of chelated iron and therefore re-expose uncathed iron to the macrophage.

It is suggested that the pathogenicity of asbestos involves fiber-mediated initiation of gene expression (19). The upregulation of NFκB and AP-1 transcription factors by LFA, but not significantly by MMVF10 and RCF1, indicates a relationship between fiber free radical activity and gene expression. However, although GSH depletion may play a role in the activation of these transcription factors in other situations (20), all three fibers caused depletion of GSH. Pharmacological depletion of GSH with BSO alone did increase upregulation of NFκB and enhanced RCF1-mediated upregulation, so redox status is important in the activation of transcription, as previously shown (19). Supplementing intracellular GSH with CME inhibited the LFA-mediated NFκB activation, again confirming that GSH status is important. The fact that all fibers lowered GSH but only LFA caused transcriptional activation, conflicts with the evidence that pharmacologic modulation of GSH did influence transcriptional activation; this may be explained at several levels. The time course of GSH depletion may be central to the activating effect and the 4-hr time point used here may not reflect the most important GSH change. The effect of direct fiber reactivity with cellular thiols may be important in this system in which the thiol redox status of the cell is important in thiol-dependent transcription factor activation. In addition to the effect of fibers on anti-oxidant thiols, there is the possibility that transcription factor-related thiols may be directly influenced by the fibers, masking the relative activation potentials of the fiber samples. These possibilities require further study to determine the mechanism of the activation cascade and to differentiate how the different properties of these fibers result in differing cell responses. Also involved may be other oxidative stress-sensitive molecules such as carbon-based free radical and other intracellular signaling pathways. These are under further investigation.

In conclusion, the hydroxyl radical activity of asbestos exceeds that of MMVF10 and RCF1 fibers and iron does not appear to be a determining factor in this free radical-generating potential or in their relative pathogenicity. The greater free radical activity of LFA was mirrored in the activation of NFκB and AP-1 transcription factors. All three fibers depleted GSH at the 4-hr time point used here. These results indicate that the free radical activity of asbestos and other fibers may be a key factor in gene transcription in macrophages, but the exact role of GSH in these events remains to be elucidated.

REFERENCES

1. Mossman BT, Gee JBL. Medical progress: asbestos-related diseases. N Engl J Med 320:1721–1730 (1989).
2. Donaldson K, Brown RC, Brown GM. Respirable industrial fibers: mechanisms of pathogenicity. Thorax 48:390–395 (1993).
3. Hesterberg TW, Barrett JC. Dependence of asbestos and mineral dust-induced transformation of mammalian cells in culture on fiber dimension. Cancer Res 44:2170–2180 (1984).
4. Palekar LD, Spooner CM, Coffin DL. Influence of crystalline habit of minerals on in vitro cytotoxicity. Ann N Y Acad Sci 330:673–678 (1979).
5. Hesterberg TW, Barrett JC. Induction by asbestos fibers of anaphase abnormalities: mechanism for aneuploidy induction and possibly carcinogenesis. Carcinogenesis 6:473–475 (1985).
6. Bunn WB, Bender JR, Hesterberg TW, Chase GR, Konzen JL. Recent studies of man-made vitreous fibers. J Occup Med 35:101–113 (1994).
7. Kamp DW, Graceffa P, Pryor AW, Weitzman SA. The role of free radicals in asbestos-induced lung disease. Free Radic Biol Med 12:293–315 (1992).
8. Gilmour PS, Beswick PH, Donaldson K. Detection of surface free radical activity of respirable industrial fibers using jX174 RF1 Plasmid DNA. Carcinogenesis 16:2973–2979 (1995).
9. Donaldson K, Beswick PH, Gilmour PS. Free radical activity with the surface of particles: a unifying factor in determining biological activity? Toxicol Lett 88:293–298 (1996).
10. Davis JMG, Addison J, Bolton RE, Donaldson K, Jones AD, Smith T. The pathogenicity of long and short fiber samples of amosite asbestos administered to rats by inhalation and intra-peritoneal injection. Br J Exp Path 67:415–430 (1986).
11. Tietze F. Enzymatic method for quantitative determination of nanogram amounts of total and oxidised glutathione: applications to mammalian blood and other tissue. Anal Biochem 27:502–522 (1969).
12. Roberts JC, Francetic DJ. The importance of sample preparation and storage in glutathione analysis. Anal Biochem 183:16–20 (1989).
13. Nicholson WJ, Slight J, Donaldson K. Inhibition of the transcription factors NF-κB and AP-1 underlies loss of cytokine gene expression in rat alveolar macrophages treated with a diffusible product from the spores of Aspergillus fumigatus. Am J Respir Cell Mol Biol 15:88–96 (1996).
14. Hassanain H, Dei W, Gupta SL. Enhanced gel mobility shift assay for DNA-binding factors. Anal Biochem 213:162–167 (1993).
15. Lund LG, Williams MG, Dodson RF, Aust AE. Iron associated with asbestos bodies is responsible for the formation of single strand breaks in jX174 RF1 DNA. Occup Environ Med 51:200–204 (1994).
16. Johnston NF, Maples KR. Fiber-induced hydroxyl radical formation and DNA damage. In: Cellular and Molecular Effects of Mineral and Synthetic Dusts and Fibers (Davis JMG, Jaurand MC, eds). Berlin:Springer-Verlag, 1994:23–37.
17. Vallyathan V. Oxygen radical generation by asbestos and its correlation to toxicity. In: Davis JMG and Jaurand MC (eds.), Cellular and Molecular Effects of Mineral and Synthetic Dusts and Fibers (Davis JMG, Jaurand MC, eds). Berlin:Springer-Verlag; 1994:3–21.
18. Vallyathan V, Mega JF, Shi X, Dalal NS. Enhanced generation of free radicals from phagocytes induced by mineral dusts. Am J Respir Cell Mol Biol 6:404–413 (1992).
19. Jansen YMW, Heintz NH, Mossman BT. Induction of c-fos and c-jun proto-oncogene expression by asbestos is ameliorated by N-acetyl-L-cysteine in mesothelial cells. Cancer Res 55:2085–2089 (1995).
20. Sen CK, Packer L. Antioxidant and redox regulation of gene transcription. FASEB J 10:709–720 (1996).