Hydroxyl radical mediated DNA base modification by manmade mineral fibres

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ABSTRACT Manmade mineral fibres (MMMFs) were examined for their ability to hydroxylate 2-deoxyguanosine (dG) to 8-hydroxydeoxyguanosine (8-OH-dG), a reaction that is mediated by hydroxyl radicals. It appeared that (1) catalase and the hydroxyl radical scavengers, dimethylsulphoxide and sodium benzoate, inhibited the hydroxylation, whereas Fe^{2+} and H_2O_2 potentiated it; (2) pretreatment of MMMFs with the iron chelator, deferoxamine, or with extensive heat (200–400° C), attenuated the hydroxylation; (3) the hydroxylation obtained by various MMMFs varied considerably; (4) there was no apparent correlation between the hydroxylation and the surface area of different MMMFs, although increasing the surface area of a fibre by crushing it increased its hydroxylating capacity; and (5) there was good correlation between the hydroxylation of dG residues in DNA and the hydroxylation of pure dG in solution for the 16 different MMMFs investigated. These findings indicate that MMMFs cause a hydroxyl radical mediated DNA base modification in vitro and that there is considerable variation in the reactivity of different fibre species. The DNA modifying ability seems to depend on physical or chemical characteristics, or both, of the fibre.

Epidemiological studies have indicated an increased risk of lung cancer associated with occupational exposure to manmade mineral fibres (MMMFs).^{1,2} Animal studies with glass fibre and other MMMFs have indicated that these minerals are carcinogenic³⁻⁵ and in vitro experiments have shown that glass fibres as well as asbestos may cause chromosomal aberrations,⁶ morphological transformations,⁷ and chromosomal abnormalities⁸ in cultured cells. Recently, the International Agency for Research on Cancer classified glasswool, rockwool, slagwool, and ceramic fibres as possibly carcinogenic to man.⁹

The underlying molecular mechanism for MMMF induced carcinogenesis has not been resolved. One possibility is that reactive oxygen species—for example, hydroxyl radicals ($\text{OH}^\cdot$)—are implicated in the carcinogenic process. Hydroxyl radicals are implicated in several harmful processes, including DNA base modifications,¹⁰ lipid peroxidation, single strand breaks in DNA, and carcinogenesis.¹¹ Asbestos fibres catalyse the formation of hydroxyl radicals and superoxide anions ($\text{O}_2^\cdot$) in a cell free system containing H_2O_2 ,^{12,13} and using electron spin resonance; this has also been shown for glass fibres.¹⁴ With the same technique, asbestos¹⁵ and rockwool and glasswool¹⁶

may produce hydroxyl radicals in a cell free system without any addition of hydrogen peroxide.

We have previously reported that certain MMMFs and asbestos fibres may produce hydroxyl radicals that modify hydroxylate 2-deoxyguanosine (dG)¹⁷ and calf thymus DNA¹⁸ in a cell free system. In the present investigation we have extended our studies and examined a variety of MMMFs for their ability to hydroxylate DNA—that is, dG residues in DNA—and pure dG in solution. The results indicate that there is a large variation between different MMMFs in their DNA hydroxylation and that this variation is caused by differences in chemical or physical properties, or both, of the fibre surface.

Materials and methods

CHEMICALS

Calf thymus DNA (type I), nuclease P_i from *Penicillium citrinum*, *Escherichia coli* alkaline phosphatase (type III), and catalase were obtained from Sigma Chemical Co, St Louis, MO, USA; 2'-deoxyguanosine (research grade) from Serva Feinbiochemica GMBH & Co, Heidelberg, FRG; iron(II) chloride (FeCl_2) from Aldrich-Chemie, Steinheim, FRG; dimethylsulphoxide (DMSO, pro analysi) from Riedel-de Haan AG, Seelze, FRG; sodium benzoate and perhydrol

(H₂O₂) (pro analysi) from Merck, Darmstadt, FRG; deferoxamine (Desferal) from CIBA-GEIGY, Basle, Switzerland; and phosphate buffered saline (PBS, Dulbecco's medium, modified) from Flow Laboratories, Irvine, UK. 8-Hydroxydeoxyguanosine (8-OH-dG) was synthesised in our laboratory according to the method described by Kasai and Nishimura.¹⁹

FIBRE SAMPLES

Sixteen different types of MMMFs from European plants were provided by Mr Ingemar Öhberg, Rockwool Inc, Skövde, Sweden. Determination of the surface area of these fibres was carried out on a Flow-Sorb II 2300, from Micromeritics, Norcross, Georgia, USA, and based on gas desorption with single point measurements using a gas mixture containing helium (70%) and nitrogen (30%).²⁰ The coefficient of variation in the determination of the fibre area was $\pm 5\%$.

EXPERIMENTAL SYSTEMS

The fibres were investigated with regard to their ability to hydroxylate dG residues in calf thymus DNA and pure dG in solution. The reaction mixtures contained 10 mg fibre and 1.0 ml PBS with 0.5 mg DNA or 0.5 mM dG. Where indicated, the reaction mixtures were supplemented with additives known to influence the generation of hydroxyl radicals: FeCl₂ (3 and 30 μ M), H₂O₂ (100 μ M and 1 mM), sodium benzoate (10 and 100 mM), DMSO (10 and 100 mM), or catalase (500 Sigma units/ml). To compare the hydroxylating properties of the different fibres the fibre DNA or fibre dG mixtures were incubated (in the dark) for five hours in a shaking waterbath at 37° C. In other experiments fibres were crushed in a glass mortar and fibre DNA mixtures were incubated for one hour at 37° C in small polypropylene tubes on a rotator at slow speed. In one series of experiments the fibres were preheated (200° C or 400° C, 1 h) in air or oxygen atmosphere (fibres were crushed and placed on a watch glass at the bottom of an oven) or pretreated with deferoxamine as described by Weitzman and Weitberg.²¹

After incubation, the amount of 8-OH-dG in the medium was determined, either directly (fibre dG system) or after digestion of DNA (fibre DNA system). In the latter case the ratio between 8-OH-dG and dG was used to measure the DNA damage. After incubation, the fibres were pelleted and supernatants were extracted with 3.0 ml chloroform: isoamylalcohol (24:1), and centrifuged (2000 g, 6 min). The upper aqueous phase containing DNA was collected and organic solvents in the aqueous layer were evaporated under a stream of nitrogen. After the addition of 100 μ l 3 M sodium acetate pH 5.2, the DNA was precipitated with 2 vol 70% ice cold ethanol, centrifuged, washed once with 70% ethanol, dried, and redissolved in 50 μ l

0.10 sodium acetate buffer, pH 4.8. The DNA was digested at 37° C in a water bath with nuclease P₁ for 30 minutes and alkaline phosphatase for one hour (the pH was raised from 4.8 to 7.5 by addition of 1.0 M Tris-HCl, pH 8.0, before treatment with alkaline phosphatase). The amounts of 8-OH-dG and dG were then determined.

ANALYSIS

8-OH-dG and dG were analysed with the HPLC technique originally described by Floyd *et al.*²² The analysis was performed with a Jasco 880-PU HPLC pump and a Jasco 875 ultraviolet spectrophotometric detector from Japan Spectroscopic Co, Tokyo, Japan. An electrochemical detector, Zäta 4 C from Zäta Electronic, Lund, Sweden, was coupled in line with the ultraviolet detector. This detector worked at 254 nm and the EC detector was run in the oxidative mode, +0.600 V. Twenty microlitres of the reaction mixture was injected into a Rheodyne 7125 syringe loading sample injector (Berkeley, CA, USA) and separated on an Apex Octadecyl C₁₈ column (3 μ m, 150 $\times$ 4.8 mm Jones Chromatography, Mid Glam, UK). The mobile phase consisted of 15% aqueous methanol containing 12.5 mM citric acid, 25 mM sodium acetate, 30 mM sodium hydroxide, and 10 mM acetic acid. The flow rate was 1.0 ml/min.

Results

Table 1 illustrates the hydroxylation of dG residues in DNA caused by a MMMF and the influence of various agents known to affect the generation of reactive oxygen metabolites on this hydroxylation. Catalase inhibited the hydroxylation (heat treated enzyme did not) and so did the hydroxyl radical scavengers, sodium benzoate and dimethyl sulphoxide.

Table 1 Influence of various additives on MMMF mediated hydroxylation of dG residues in DNA. Mixtures were incubated for one hour and the amount of 8-OH-dG in DNA determined. The hydroxylation in control incubations (without any fibres) amounted to 5.1 8-OH-dG residues/10⁵ dG and was subtracted throughout. (Means $\pm$ SD of four experiments)

Experimental system	Hydroxylation (8-OH-dG/10 ⁵ dG)	
Fibre alone	4.2 $\pm$ 0.2	100 (%)
+ Catalase	0.2 $\pm$ 0.3	4
+ Catalase, heated*	4.2 $\pm$ 0.4	100
+ Na-benzoate 10 mM	2.8 $\pm$ 0.9	68
+ Na-benzoate 100 mM	2.3 $\pm$ 0.8	54
+ DMSO 10 mM	2.0 $\pm$ 0.5	48
+ DMSO 100 mM	0.9 $\pm$ 0.1	22
+ FeCl ₂ 3 μ M	7.3 $\pm$ 0.8	175
+ FeCl ₂ 30 μ M	17.2 $\pm$ 0.3	410
+ H ₂ O ₂ 100 μ M	11.3 $\pm$ 1.3	270
+ H ₂ O ₂ 1 mM	26.5 $\pm$ 1.1	630

*Boiled for five minutes.

Table 2 Hydroxylation of dG residues in DNA (DNA hydroxylation) and of dG in solution (dG hydroxylation) by 16 different MMMFs (g = glasswool, c = ceramic fibre, s = slagwool, and r = rockwool). The DNA hydroxylation was assessed as number of 8-OH-dG residues/10⁵ dG, whereas the dG hydroxylation was determined and normalised to that in control incubations (the 8-OH-dG formation formed in incubations without fibres was set to 1). (Means $\pm$ SD of three experiments)

Fibre (type)	Surface area (m ² /g)	DNA hydroxylation	dG Hydroxylation
1 (c)	0.95	0.6 $\pm$ 0.1	2.1 $\pm$ 0.1
2 (g)	0.91	0.1 $\pm$ 0.1	2.9 $\pm$ 1.6
3 (c)	1.10	0.6 $\pm$ 0.2	3.0 $\pm$ 0.9
4 (r)	1.30	0.7 $\pm$ 0.1	6.4 $\pm$ 2.2
5 (r)	0.36	0.3 $\pm$ 0.1	6.4 $\pm$ 2.8
6 (r)	0.60	1.5 $\pm$ 0.6	7.8 $\pm$ 0.9
7 (r)	0.73	4.5 $\pm$ 0.5	8.1 $\pm$ 2.1
8 (r)	1.01	1.7 $\pm$ 0.3	8.4 $\pm$ 0.9
9 (r)	1.28	3.8 $\pm$ 2.6	9.7 $\pm$ 0.6
10 (r)	1.16	4.3 $\pm$ 0.3	9.8 $\pm$ 1.2
11 (r)	1.18	3.3 $\pm$ 0.5	14.5 $\pm$ 1.5
12 (s)	1.14	2.5 $\pm$ 0.9	17.1 $\pm$ 1.9
13 (r)	1.30	5.0 $\pm$ 1.0	18.4 $\pm$ 3.1
14 (r)	1.06	3.9 $\pm$ 0.5	28.2 $\pm$ 6.5
15 (s)	0.90	9.0 $\pm$ 1.1	29.3 $\pm$ 1.1
16 (r)	0.62	7.4 $\pm$ 1.6	39.2 $\pm$ 2.0

ide. On the other hand, FeCl₃ and H₂O₂ potentiated the hydroxylation. Heat treatment of the fibres, particularly in an oxygen rich atmosphere, reduced their ability to hydroxylate DNA: fibres heated at 200° C and 400° C in air for one hour caused only 76% and 19%, respectively, of the hydroxylation by untreated fibres, whereas corresponding value for fibres treated at 400° C in oxygen was 7% (mean of six experiments). Furthermore, pretreatment of the fibres with deferoxamine reduced the hydroxylation by 29% (mean of six experiments).

Table 2 shows the hydroxylation of dG residues in calf thymus DNA and pure dG in solution by 16 different MMMFs. All the fibres caused significant hydroxylation, although the extent of hydroxylation varied considerably. Fibres containing little or no iron—for example, glasswool and ceramic fibres—were poor in their hydroxylating capacity.

There was good correlation between the hydroxyla-

Table 3 Relation between a mineral fibre's surface area and its ability to hydroxylate dG residues in DNA. Fibres (rockwool, No 13 of table 2) were crushed in a mortar for different periods (0, 30, and 60 sec), incubated with calf thymus DNA for one hour, and the amount of 8-OH-dG determined. The hydroxylation in control incubations (without any fibres) was subtracted. (Means $\pm$ SD of four experiments)

Treatment	Surface area (m ² /g)	Hydroxylation (8-OH-dG/10 ⁵ dG)
Uncrushed	0.6 $\pm$ 0.1	1.4 $\pm$ 0.2
Crushed for 30 seconds	0.9 $\pm$ 0.1	3.2 $\pm$ 0.4
Crushed for 60 seconds	1.3 $\pm$ 0.1	4.6 $\pm$ 0.3

tion of dG residues in DNA and the hydroxylation of dG in solution ($r = 0.83$, $p < 0.01$, Students t test, $B = 0$). By contrast, there was no apparent correlation between the hydroxylation and the surface area of the different fibres ($r = 0.02$). If the surface area of a fibre was increased by crushing, however, the hydroxylating capacity of that specific fibre was increased (table 3).

Discussion

Our results show that MMMFs can catalyse the formation of hydroxyl radicals. Hydroxyl radical scavengers added to the incubation mixture decreased the hydroxylation, whereas compounds known to increase the formation of hydroxyl radicals had the opposite effect. The findings also show that MMMFs can hydroxylate DNA in vitro. Such a hydroxylation has been shown for asbestos fibres,²³ and Zalma *et al* have suggested that asbestos fibres may cause the formation of reactive hydroxyl radicals by reducing oxygen¹⁵ according to:

The amounts of O₂⁻ and OH[·] seemed dependent on the presence of electron donor sites of the minerals: some of the fibres were active in forming hydroxyl radicals whereas others were poor in this respect. Asbestos fibres have been shown to decompose hydrogen peroxide with subsequent formation of hydroxyl radicals.^{12,13} We did not add any hydrogen peroxide to the reaction mixture but this component could have been formed according to reactions (1) and (2) above. When catalase was added, the hydroxylation vanished. This goes in line with the results of Zalma *et al* who found a decreased hydroxyl radical formation when catalase was added to an asbestos solution.¹⁵

To investigate the mechanisms behind the hydroxyl radical generation further, we examined different types of pretreated fibres. When the fibres were heated before incubation with DNA, the hydroxylation decreased. This could have been due to oxidation of the fibre surface, a possibility that is consistent with the observation that the hydroxylating capacity almost disappeared when the fibres were placed in an oxygen gas flow during the heat treatment. A similar phenomenon has been shown for chrysotile asbestos, whose cytotoxicity towards cultured human fibroblasts and bovine alveolar macrophages was reduced after heat treatment.²⁴ Fibres pretreated with the iron chelator, deferoxamine, showed a reduced ability to hydroxylate DNA. Deferoxamine has been shown to decrease asbestos mediated lipid peroxidation²¹ and glassfibre mediated formation of hydroxyl radicals.¹⁴

The iron content of the fibre examined in our study was approximately 5%, which thus seems to have contributed significantly to the formation of hydroxyl radicals.

When a larger number of different fibres was investigated, there were great differences in the hydroxylation, both of dG residues in DNA and of dG in solution. There was, however, a good correlation between the hydroxylation of dG residues in DNA and of dG in solution. This points to the possibility of using the more simple dG hydroxylation test for estimating the DNA damaging capacity of different MMMFs.

Our results thus indicate that MMMFs are able to reduce oxygen with the subsequent formation of hydroxyl radicals. These radicals may then cause hydroxylation of DNA and formation of oxidative DNA adducts including 8-OH-dG. It is tempting to speculate that this might play a part in the carcinogenicity of MMMFs. If 8-OH-dG is incorporated in a synthetic oligonucleotide this results in misreading during replication, not only at the 8-OH-dG residue itself but also at bases next to the 8-OH-dG.²⁵ When an oxygen atom is inserted at the C-8 position of a guanine base, this entirely changes the electrostatic potential of the molecule, a factor that could affect the action of DNA polymerase.²⁶ Moreover, although in vivo experiments with irradiated mice have indicated the presence of a repair mechanism for removing 8-OH-dG from DNA,¹⁰ this removal was not complete. It may be hypothesised, therefore, that this type of DNA base modification may underlie MMF induced mutation and carcinogenesis.

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