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Rapid Communication

Scavengers of Active Oxygen Species Prevent Cigarette Smoke-Induced Asbestos Fiber Penetration in Rat Tracheal Explants

A. Churg, J. Hobson, K. Berean, and J. Wright
From the Department of Pathology, University Hospital, University of British Columbia, Vancouver, British Columbia, Canada

It was previously shown that rat tracheal explants first exposed to cigarette smoke and then to amosite asbestos take up more asbestos fibers than explants exposed to air and asbestos. To examine the mechanism of this process, the same experimental design was followed but test groups were added in which the asbestos was mixed with catalase or superoxide dismutase, scavengers of active oxygen species, or deferoxamine, an iron chelator that prevents formation of hydroxyl radical. All three agents protected against the cigarette smoke effect. Heat inactivated catalase or superoxide dismutase was not protective. These observations indicate that active oxygen species, probably derived from the cigarette smoke, play a role in smoke-mediated fiber transport into tracheobronchial epithelia. (Am J Pathol 1989, 135:599–603)

Asbestos workers who smoke cigarettes are known to have increased incidences of lung cancer and probably increased incidences of asbestosis (diffuse interstitial fibrosis) compared with asbestos workers who do not smoke.[1-3] Experimentally, cigarette smoke was shown to increase pulmonary retention of many types of mineral particles in humans and animals,[4-6] and, in the whole intact animal, to increase the number of asbestos fibers entering into tissue.[7] Tissue penetration by asbestos fibers also occurs in tracheal explant systems,[8-11] and, using such explants, we recently demonstrated that brief exposure to cigarette smoke, followed by exposure to amosite asbestos and maintenance in culture, greatly increased the number of asbestos fibers penetrating the tracheal epithelium over a period of several days.[12]

The latter observation indicated that cigarette smoke can directly influence particle uptake by the tracheobronchial epithelium, but the mechanism or mechanisms of this process have not been determined. Cigarette smoke is known to contain many types of free radicals,[13,14] and radical-mediated damage to cells may be one mechanism of enhanced fiber uptake. In this study we tested the hypothesis that active oxygen species are involved in the increased asbestos fiber penetration in tracheal explants exposed to cigarette smoke.

Materials and Methods

Tracheal explants were prepared from 200-g male Sprague-Dawley rats by the method of Mossman et al[11]; details are provided in references 11 and 12. The segments were placed serosal side down on Millipore filters. To test the effects of scavengers of active oxygen species, the tracheas were then exposed to the following treatments:

Experiment 1

1) Sham smoke followed by amosite asbestos;
2) Cigarette smoke followed by amosite asbestos;
3) Cigarette smoke followed by amosite asbestos plus catalase; and

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Address reprint requests to Andrew Churg, MD, Department of Pathology, University of British Columbia, 2211 Wesbrook Mall, Vancouver, British Columbia, Canada V6T 2B5.

4) Cigarette smoke followed by amosite asbestos plus inactivated catalase.

Experiment 2

1) Sham smoke followed by amosite asbestos;
2) Cigarette smoke followed by amosite asbestos;
3) Cigarette smoke followed by amosite asbestos plus superoxide dismutase; and
4) Cigarette smoke followed by amosite asbestos plus inactivated superoxide dismutase.

Experiment 3

1) Sham smoke followed by amosite asbestos;
2) Cigarette smoke followed by amosite asbestos; and
3) Cigarette smoke followed by amosite asbestos that had been preincubated with deferoxamine.

Cigarette smoke was generated by drawing 20 ml of air through a burning cigarette once per minute and expelling the whole smoke into a 2-liter humidified chamber housing the tracheal cultures.[12] One commercial nonfilter cigarette was used and the total exposure was 10 minutes (10 puffs of smoke totalling 200 ml in 2 liters of air). Sham smoked tracheas were exposed to air in the same chambers.

After smoking or sham smoking, a solution of 5 mg/ml UICC amosite asbestos in serum-free Dulbecco's medium was added to the Petri dishes to a level sufficient to completely cover the tracheas and left for 1 hour at 37 C. In experiment 1, Catalase (Boehringer-Mannheim) was added to the asbestos solution as indicated above at a final concentration of 1300U/ml; in experiment 2, superoxide dismutase (Sigma Chemical Co., St. Louis, MO) was added at a final concentration of 600 U/ml. In experiment 3, the asbestos was preincubated with 10 mM deferoxamine methylate (Desferal, Ciba-Geigy) for 24 hours at 37 C; the asbestos fibers were then spun down and excess deferoxamine removed by washing before the fibers were added to the tracheas.

To inactivate the enzymes, the catalase was boiled for 10 minutes and the superoxide dismutase for 30 minutes. A few tracheal segments were removed from each group before asbestos exposure; these served as controls to check the morphologic effects of culture and smoking.

After the 1 hour exposure, segments were rinsed with fresh culture medium and transferred to new Millipore filters. They were then incubated mucosal side up using just enough culture medium to cover the filters in an atmosphere of 95% air, 5% CO_2 for 1 day (24 hours), 3 days, or 7 days at 37 C. At each period, six to eight segments were removed from each test group and routinely processed for histologic examination. Five-micron sections were cut and stained with Nuclear Fast Red to permit easy visualization of fibers. The direction of histologic sectioning was from serosa to mucosa to prevent artificial movement of any fibers adherent to the mucosa into the tissue.

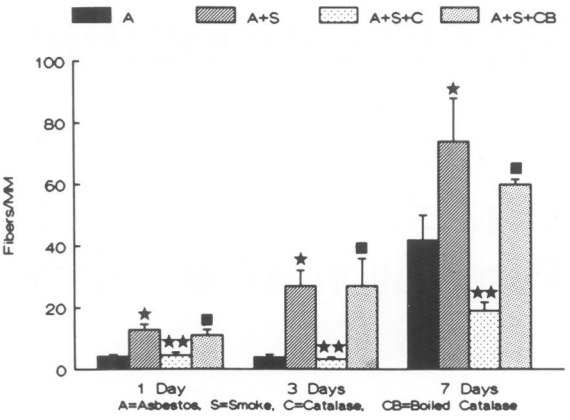

Figure 1. *Effects of catalase on fiber penetration (fibers/mm epithelium). Heat-inactivating the catalase completely abolishes the protective effect. Values as mean (SE). *, significantly greater than treatment with asbestos alone; **, significantly lower than treatment with asbestos plus smoke; ■, significantly greater than treatment with asbestos plus smoke plus scavenger.*

The number of asbestos fibers in or under the tracheal epithelium was determined by counting fibers longer than 4 μm using a light microscope fitted with an eyepiece graticule for measurement. The 4 μm length restriction was imposed to insure that only real fibers were counted. Epithelial length was measured as length of basement membrane, using a computer-assisted digitizer, and a value of fibers/mm of epithelial length generated for each explant. Hyperplastic lesions, heaped up masses of proliferating epithelial cells (see reference 12, Figure 1b), were not included in the counts because of the huge numbers of fibers in such lesions. However, a separate evaluation of length of hyperplastic lesions per total length of epithelium (expressed as the percentage of epithelial length occupied by hyperplastic lesions) was measured for each segment, again using the computer-assisted digitizer.

Data were evaluated using the SYSTAT system.[15] Inspection showed that the counts of fibers per mm or hyperplastic lesions could be normalized using a log transformation. Analysis was then performed on the transformed data by analysis of variance followed by contrasts with Bonferroni correction for multiple comparisons. Values of $P < 0.05$ or less after correction were considered significant. Because the means both of fibers per mm and of the percentage of hyperplastic lesions expressed the trend of the data, these values were used for constructing

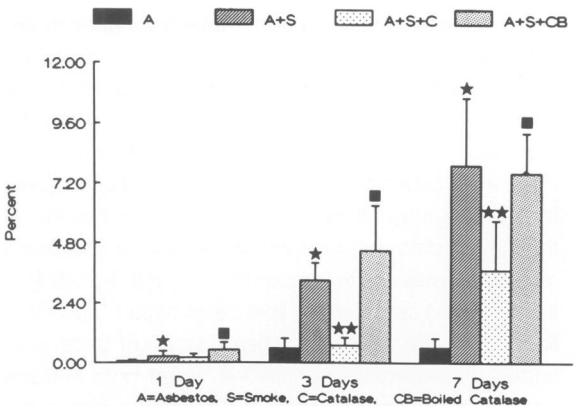

Figure 2. *Effects of catalase on development of hyperplastic lesions (% of epithelium). Heat-inactivating the catalase completely abolishes the protective effect. Values as mean (SE). *, significantly greater than treatment with asbestos alone; **, significantly lower than treatment with asbestos plus smoke; ■, significantly greater than treatment with asbestos plus smoke plus scavenger.*

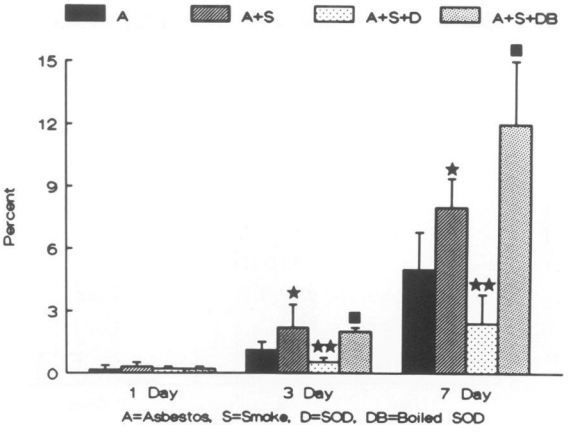

Figure 4. *Effects of superoxide dismutase (SOD) on development of hyperplastic lesion (% of epithelium). Heat-inactivating the superoxide dismutase abolishes the protective effect. Values as mean (SE). *, significantly greater than treatment with asbestos alone; **, significantly lower than treatment with asbestos plus smoke; ■, significantly greater than treatment with asbestos plus smoke plus scavenger.*

Figures 1 through 6, but the statistical values indicated in the figures were calculated as just described.

Results

Figures 1 and 2 show the effects of catalase on the number of fibers penetrating the epithelium and the extent of hyperplastic lesions; Figures 3 and 4 show the same data for superoxide dismutase, and Figures 5 and 6 show the data for deferoxamine. By itself, cigarette smoke greatly increased the number of fibers penetrating the tracheal epithelium and also increased the extent of hyperplastic lesions, findings essentially identical to our previous observations.[12] When catalase or superoxide dismutase was added to the asbestos solution, or when the asbestos was preincubated with deferoxamine, the smoke-mediated increases in fiber penetration were variably, but usually completely, abolished. The same was true for hyperplastic lesions, except that, as was previously observed, there was considerably more variation in the extent of hyperplastic lesions than in numbers of fibers per mm at any given time. Heat-inactivated catalase or superoxide dismutase failed to protect the epithelium.

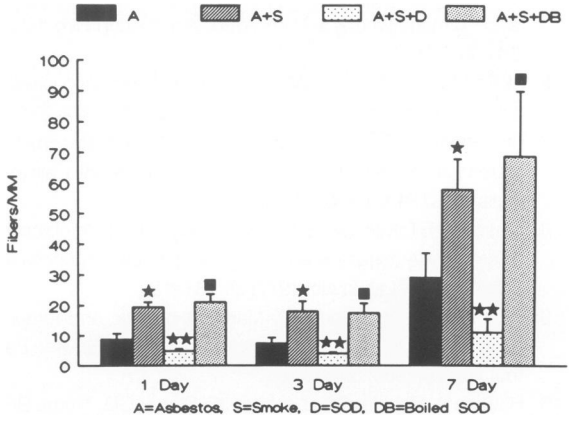

Figure 3. *Effects of superoxide dismutase (SOD) on fiber penetration (fibers/mm epithelium). Heat-inactivating the superoxide dismutase abolishes the protective effect. Values as mean (SE). *, significantly greater than treatment with asbestos alone; **, significantly lower than treatment with asbestos plus smoke; ■, significantly greater than treatment with asbestos plus smoke plus scavenger.*

Discussion

The mechanism of asbestos fiber penetration into pulmonary epithelial cells is not known, and there is no detailed

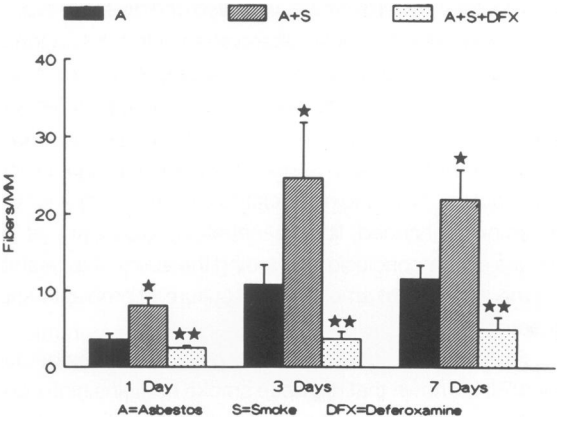

Figure 5. *Effects of deferoxamine on fiber penetration (fibers/mm epithelium). Values as mean (SE). *, significantly greater than treatment with asbestos alone; **, significantly lower than treatment with asbestos plus smoke.*

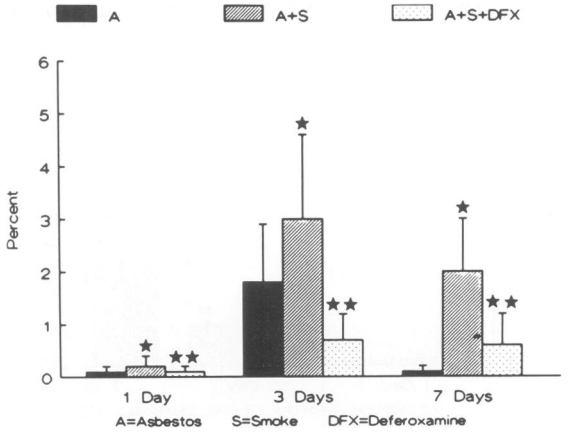

Figure 6. *Effects of deferoxamine on the development of hyperplastic lesions (% of epithelium). Values as mean (SE). *, significantly greater than treatment with asbestos alone; **, significantly lower than treatment with asbestos plus smoke.*

information available on the way cigarette smoke potentiates asbestos effects. Tracheal explants provide a convenient model system in which the undoubtedly important, but often confusing, effects of smoke- and mineral dust-evoked inflammatory cells seen in the intact animal can be removed, and direct effects of smoke and fibers on the tracheal epithelium examined.

As noted earlier, we previously showed, using a tracheal explant system, that cigarette smoke directly increases asbestos fiber uptake.[12] In this study we demonstrated that both catalase and superoxide dismutase, enzymatic scavengers of active oxygen species, abolish the enhanced fiber penetration effects caused by cigarette smoke. The effect is not a nonspecific protection by protein, because heat-inactivated catalase or superoxide dismutase provides no protection against fiber penetration. As well, preincubation of the asbestos with the nonprotein iron chelator, deferoxamine, was also effective in preventing smoke effects. These observations strongly suggest that active oxygen species are responsible for the cigarette smoke effects; more specifically, because deferoxamine chelates iron and prevents formation of OH· from hydrogen peroxide and superoxide anion through a Fenton reaction,[16] our findings point to OH· as a major factor in smoke-enhanced fiber penetration. Mossman et al drew a similar conclusion regarding the ability of asbestos (in the absence of smoke) to kill cultured fibroblasts and macrophages.[16]

From where might such active oxygen species be derived? It is known that cigarette smoke contains high concentrations of free radicals and that bubbling smoke through water generates both superoxide anion and hydrogen peroxide[13,14]; thus, the smoke itself is presumably the source of these molecules, although it should be noted that asbestos fibers by themselves apparently provoke intracellular production of active forms of oxygen.[16]

How might active oxygen species increase fiber penetration? We previously noted from examination of histologic sections of tracheal cultures that smoke appears to make more asbestos stick to the tracheal cells,[12] implying that smoke may affect the apical cell membranes. Although the data are controversial, there is a reasonable amount of evidence to indicate that oxygen radicals (particularly OH·) can produce lipid peroxidation[13]; asbestos fibers by themselves cause peroxidation of lipids when added to phospholipid emulsions,[17] and both Petruska and Mossman[18] and Goodlick et al[19] recently showed that crocidolite asbestos produces measurable increases in malondialdehyde, a product of lipid peroxidation, in lavage fluid or in macrophage cultures exposed to the fiber. We suggest that additional damage to cell membranes in the form of lipid peroxidation produced by active oxygen species either in or derived from the smoke may be the cause of smoke-induced enhanced fiber uptake.

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