Rat Pleural Mesothelial Cells Show Damage after Exposure to External but Not Internal Cigarette Smoke

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The combination of cigarette smoke and high-level occupational asbestos exposure produces a synergistic increase in the incidence of lung cancer; however, smoking does not affect the incidence of mesothelioma. Here we present the results of tests of two theories that have been proposed to explain this phenomenon; namely, that pleural mesothelial cells are resistant to cigarette smoke-induced damage and that the pleural connective tissue acts as a barrier that prevents smoke from reaching the mesothelial cells. To test these hypotheses, excised whole rat lung preparations were exposed to either internal (intratracheal) or external (pleural surface) smoke. For comparison, additional excised lung preparations were exposed to solutions of hydrogen peroxide either externally or intratracheally. Mesothelial cells exposed to external smoke showed widespread, dose-dependent uptake of Trypan blue. Mesothelial cells did not take up Trypan blue after exposure to internal smoke. Bronchial epithelial cells exposed to internal smoke did show uptake, but to a lesser degree than externally exposed mesothelial cells. Examination by scanning and transmission electron microscopy showed that internal smoke did not affect mesothelial cell ultrastructure, whereas external smoke produced obvious mesothelial cell damage and mesothelial cell detachment. Catalase and deferoxamine, scavengers of active oxygen species, provided protection against smoke-induced mesothelial cell injury, but inactivated catalase did not. External hydrogen peroxide produced a very similar, dose-dependent pattern of Trypan blue uptake and ultrastructural changes. Intratracheal hydrogen peroxide also damaged mesothelial cells, but the extent of damage was always less than with comparable concentrations of external hydrogen peroxide. We conclude that 1) pleural mesothelial cells are sensitive to damage by smoke-derived active oxygen species; 2) the pattern of injury is similar to that produced by hydrogen peroxide; 3) bronchial epithelial cells appear to be less sensitive to smoke-induced oxidant damage than mesothelial cells; and 4) at least acutely, the pleura appears to act as a barrier to smoke and penetration of active oxygen species. Key words: active oxygen species, catalase, cigarette smoke, deferoxamine, mesothelial cells.

Many asbestos workers are or have been cigarette smokers, and numerous epidemiological reports have shown that the combination of high-level occupational asbestos exposure and cigarette smoking produces a synergistic increase in the incidence of lung cancer; i.e., the lung cancer rate is greater than that expected from simply adding the rates for asbestos exposure and smoking alone (1). In contrast, although amphibole asbestos is a powerful mesothelial carcinogen in humans, it has been repeatedly observed that cigarette smoking has no effect on mesothelioma incidence (2).

The reason(s) for this difference between the effects of smoke on mesothelioma and lung cancer rates is not known, but a variety of hypotheses have been proposed. One hypothesis is that the actual mechanism of mesothelial carcinogenesis is completely different from bronchial carcinogenesis. It has been suggested that asbestos acts as a complete carcinogen in the pleura but as a promoter, with cigarette smoke as the initiator, in the bronchial tree (3). Another possibility is that, although smoke is believed to increase retention of asbestos fibers and thus increase the effective dose in the bronchial tree (4), smoke might not augment the uptake of asbestos fibers by mesothelial cells. A third theory relates to the sensitivity or lack of sensitivity of the pleura to oxidant injury. Asbestos fibers have been shown to function as catalysts for the formation of hydroxyl radical, and it has been suggested that active oxygen species may be important mediators of asbestos-induced carcinogenesis (5, 6).

Cigarette smoke is also a source of active oxygen species (6-8), and the combination of asbestos and smoke has been shown to lead to synergistic increases in the incidence of DNA strand breaks in cultured cells (9) and to increases in the uptake of asbestos fibers by tracheal epithelial cells in organ culture (10). Both these effects can be prevented by scavengers of active oxygen species (9-11), suggesting that one effect of smoke may be to augment oxidant injury to bronchial epithelial cells. In contrast, bronchial epithelial cells, the sensitivity of mesothelial cells to oxidant damage has been disputed (6, 12-14; see Discussion).

Alternatively, it is possible that mesothelial cells are indeed damaged by smoke but that smoke cannot penetrate the pleura. Even in small laboratory animals that have a relatively thin connective tissue layer in the visceral pleura, physical agents such as inhaled asbestos fibers appear to be largely prevented from crossing into the pleural cavity (15). The pleural connective tissue in humans is much thicker than it is in laboratory animals. Nothing is known about smoke penetration through the pleura, but it is entirely possible that the pleural connective tissue acts as a barrier that prevents smoke from reaching the mesothelial cells. In this paper we examine the effects of active oxygen species in cigarette smoke on mesothelial cells and also evaluate the question of whether smoke penetrates the pleura.

Materials and Methods

We divided female Sprague-Dawley rats weighing 250 g (Charles River Laboratories, Quebec) into groups as shown in Table 1. Each group contained three or four animals. The rats were anesthetized and exsanguinated, the tracheas cannulated, and en block preparations of heart and lungs excised. We immediately dipped the heart and lung preparation into Dulbecco’s modified Eagle medium (DMEM) to prevent drying of the pleural surface and to wash off any blood. The lung preparation was then transferred to a humidified chamber at 37°C, and the cannulated trachea was connected to a pressure-controlled lung inflation device.

We generated whole cigarette smoke by drawing 20 cc puffs of air through a burning, commercial, nonfilter cigarette once per minute and injecting the smoke into a 2-l humidified chamber. Each puff was added sequentially to the chamber (10). For external smoke exposures, we suspended the excised lung preparation in the smoke chamber and inflated the lung with air. For internal smoke exposures, we suspended the lung in humidified air and first deflated (see below) and then inflated it with smoke drawn from the smoke chamber. This procedure ensured that the concentration of external or internal smoke delivered to the lung parenchyma or pleura was the same. Air controls received internal and external air. All procedures were carried out at 37°C.

During exposures, we inflated lungs from the various groups with air or smoke as appropriate via the trachea to a pressure...
of 20 cm of water, held them at that pressure for 45 sec and then deflated the lungs by drawing the air or smoke out at a pressure of -20 cm of water. Each inflation and deflation cycle lasted about 1 min. This procedure ensured that fresh air or smoke reached the periphery of the lungs when administered via the trachea and that any structural changes induced in the mesothelial cells by inflating and deflating the lungs would be the same in all treatment groups. For groups receiving 6 puffs of air or smoke, total exposure time was 10 min, and for groups receiving 15 puffs of air or smoke, total exposure time was 15 min.

To test the protective effect of scavengers of active oxygen species, we made additional lung preparations and dipped them before exposure to smoke or air in culture medium (DMEM) containing a final concentration of 1300 U/ml catalase (Boehringer-Mannheim, Laval, Quebec) or inactivated catalase (1300 U/ml, boiled for 10 min), or 10 mM deferoxamine (Desferral, Ciba-Geigy, Toronto). Because internal smoke exposure produced no mesothelial cell damage, these experiments were only carried out with external smoke exposure.

We carried out hydrogen peroxide exposures using various concentrations of hydrogen peroxide (Table 1) in culture medium. Because hydrogen peroxide exposure required solutions rather than air or smoke, for these protocols the lung preparations with external hydrogen peroxide exposure were initially deflated as described above and then inflated via the trachea with culture medium without hydrogen peroxide. For internal hydrogen peroxide exposure, the solutions were reversed. Exposure time was 10 min at 37°C. After 10 min we rinsed lungs exposed to external hydrogen peroxide in several changes of culture medium. For lungs exposed to internal hydrogen peroxide, the hydrogen peroxide solution was lavaged out with culture medium.

To demonstrate the extent of pleural mesothelial cell damage caused by each protocol, we used the Trypan blue (Gibco Laboratories Inc, Grand Island, New York) exclusion technique. Immediately after exposure, the right lung and heart were clamped, ligated, and removed. The right lung with cannulated trachea was inflated with air, submersed in 0.15% Trypan blue for 1 min, and then rinsed with normal saline for 5 min to remove excess dye. We then photographed the lateral surface of the caudal lobe by reflected light and prepared a digitized image of the surface using a Leitz TAS-plus Image Analysis System (Wetelar, Germany). We used the pleural surface of a lung from an untreated rat to establish the reference gray level, and the extent of darker areas (i.e., foci of Trypan blue uptake) was then determined and expressed as a percentage of the total measured surface for each treatment group. We compared the percentage of Trypan blue staining areas among the different groups by analysis of variance. In initial experiments, we prepared frozen sections of the peripheral Trypan blue stained lung to ensure that the dye did not penetrate through the pleura and stain underlying parenchyma, which might show up on the digitized image. No staining of underlying parenchyma was seen on the frozen sections.

To compare the sensitivity of the bronchial epithelium to that of the mesothelium, we exposed additional excised lung preparations to internal smoke (six puffs over 10 min) or air, and dissected the trachea and mainstem bronchi from the lung, opened flat it by longitudinal dissection, and exposed it to Trypan blue in a similar fashion. Photographs of the treated airways were then taken by reflected light, and the images were digitized as above.

After air or smoke exposure, the left lung was removed and fixed overnight in 2.5% glutaraldehyde in 0.1 M cacodylate buffer. Samples from the same area of the lateral pleural surface of each lung were then excised, postfixed in osmium tetroxide, dehydrated with graded ethanol, embedded in epoxy resin, and sectioned for electron microscopic examination.

We used blocks taken immediately adjacent to the transmission electron microscopy blocks for scanning electron microscopy. The fixed blocks were rinsed with 0.1 M cacodylate buffer, dehydrated, critical point dried, and coated with gold.

**Results**

Mesothelial cells from the lungs exposed only to air demonstrated no visually apparent uptake of Trypan blue along the mesothelial surfaces, except for occasional minor staining at the edges of the lobes. This phenomenon was seen sporadically in all treatment groups and appears to represent a drying effect in a region of large surface area. (For this reason care was taken that the electron microscopy samples were obtained from the centers of the lateral surface of the lung, and the edges of the lobes were excluded from the image analysis.) Scanning electron microscopy revealed that the pleural mesothelial cells in the air control group were dome-shaped and had abundant surface microvilli (Fig. 1A). Transmission electron microscopy showed well-preserved mesothelial cells with numerous elongated microvilli (Fig. 1B). No Trypan blue uptake was seen in the large airways in this group.

![Figure 1](image-url)
The mesothelial cells from lungs in the group exposed to either 6 or 15 puffs of internal smoke showed no significant Trypan blue uptake compared to air controls (Table 1). Scanning and transmission electron microscopic findings were essentially identical to those seen in the air control groups (Fig. 2). Trypan blue uptake was seen in the airways (Table 2).

Trypan blue uptake was widely distributed over the pleural surfaces of lungs exposed to external smoke (Table 1). External smoke caused swelling of intracellular organelles, loss of microvilli and separation of intercellular junctions, as well as lifting of cells off the underlying pleural connective tissue and sometimes complete detachment of cells (Fig. 3). Changes were always focal and were more severe with 15 than with 6 puffs of external smoke; in particular, 15 puffs of smoke tended to produce large denuded areas (Fig. 3C). The differences between 6 and 15 puffs are greater than might be apparent from Table 1 because of extensive denudation of the mesothelium after 15 puffs of smoke.

In lungs with external smoke exposure, pretreatment with catalase abolished Trypan blue uptake (Table 1) and completely prevented the types of damage seen by electron microscopy (Fig. 4A). Deterofexamine had a similar effect in this group, although protection was not as complete (Table 1). Heat-inactivated catalase did not prevent smoke-induced cell damage (Fig. 4B).

The effects of hydrogen peroxide exposure were similar to those seen with external cigarette smoke; namely, extensive Trypan blue uptake (Table 1), ultrastructure evidence of cell swelling and loss of microvilli, and detachment of mesothelial cells from the underlying stroma (Fig. 5). These effects were dose dependent (Table 1). Doses of hydrogen peroxide higher than those shown in Table 1 (for example, 0.1%) completely removed the mesothelial cells. Damage to the mesothelium caused by internal hydrogen peroxide exposure was always of a lesser degree, for any given hydrogen peroxide exposure, than was seen with external exposure (Table 1).

Table 2. Percentage of airway surface showing Trypan blue uptake (means ± SD)

| Treatment       | Percentage of surface |
|-----------------|-----------------------|
| Air control     |                       |
| 10 min          | 0.5 ± 0.3             |
| Internal smoke  |                       |
| 6 puffs          | 4.3 ± 0.6*            |

*Significantly greater than air control (p<0.001); significantly less than uptake by pleura exposed externally to six puffs of smoke (see Table 1) (p<0.001).

Discussion

In this study we designed a simple system of internal or external smoke exposures to evaluate two questions: Are mesothelial cells sensitive to damage by smoke, specifically damage caused by the active oxygen species in smoke, and does the pleura act as a barrier to cigarette smoke and active oxygen species?

In setting up these experiments, we were forced to make some arbitrary choices about exposure methods because mesothelial cells are not normally exposed to smoke or air, but the internal milieu of the lung is. We considered exposing the tissue to solutions through which smoke had been bubbled, but this procedure would not be related to any real inhalation exposure, and is just as arbitrary a method of exposing the pleura to smoke contents as is direct smoke or air exposure. We also wanted to ensure that smoke penetrated deep into the lung and that the smoke concentration internally was as close as possible to that externally; thus we drew the smoke from the same chamber for external and internal exposures and manually deflated the lung before injecting smoke into the trachea. This approach ensured that there was minimal dilution of the smoke with air. If we intentionally punctured the pleura of these lungs exposed internally to smoke, smoke issued from the tear, and we did occasionally note black pigment under the pleura in this group by light microscopy; both of these observations indicate that smoke reached the periphery of the lung.

One additional advantage to this approach is that, because there is little residual air to dilute the smoke and because there are no nasal passages to filter out the smoke, the internal smoke concentration obtained with our method is much higher than could ever be achieved in an intact animal breathing smoke in an exposure chamber. In addition, the lack of blood to the excised lung means that the internal antioxidant defenses of the lung will be rapidly depleted by the smoke and cannot be metabolically replenished. Thus, we purposely manipulated the system to allow artificially high concentrations of smoke to reach the pleura in order to detect damage, if any, from internal smoke exposure.

The use of this type of in vivo lung preparation offers the advantage that one can specifically test the sensitivity of mesothelial cells in situ to internal or external smoke and also test the barrier effects of the pleura, procedures that are more realistic than monolayer cell cultures, even cultures growing on a collagen substrate. At the same time, it must be emphasized that this system is artificial and has a number of limitations. Most notably, although the preparations are satisfactory for 15 min, when we tried to extend the exposure times to 30 min, there was extensive uptake of Trypan blue and obvious ultrastructural damage with air exposure alone. By definition, therefore, our results in regard to the lack of smoke penetration through the pleura only apply to short-term exposures, and we cannot rule out the possibility that with either long or repeated in vivo smoke exposures there would be evidence of smoke penetration.

These problems do not affect our basic conclusion that rat pleural mesothelial cells are sensitive to smoke-induced damage and that this damage appears to be produced by active oxygen species. Cigarette smoke is a rich source of free radicals of many types (6–8), and active oxygen species in the form of superoxide anion and hydrogen peroxide are present in gas-phase smoke and are generated for long periods from quinone radicals in the tar phase (6,8,16).

Because the smoke was directly injected into the trachea and the Trypan blue uptake measurements were made only a few millimeters away from the injection site, it is reasonable to conclude that the tracheal epithelium, after internal smoke exposure, and the pleura, after external smoke exposure, are subjected to similar smoke concentrations. Nonetheless, Trypan blue uptake was seen on average in
34% of the mesothelial cells after external smoke exposure but in only in 4.9% of the tracheobronchial epithelial cells after internal smoke exposure. Thus, a second conclusion is that mesothelial cells are considerably more sensitive to smoke-induced oxidant injury than bronchial epithelial cells. These observations parallel those made by Lechner et al. (17), who showed that in monolayer culture systems, mesothelial cells are about 10 times (roughly what we found) more sensitive than bronchial epithelial cells to the cytotoxic effects of asbestos. This conclusion is not surprising, given that many facets of asbestos cytotoxicity also appear to be mediated by active oxygen species (5,6).

The fact that smoke damage in the current experiments was morphologically similar to that produced by hydrogen peroxide and that it can be prevented by catalase suggests an important role for hydrogen peroxide as a mediator of injury. However, as emphasized by Kamp et al. (6) in a recent review, prevention of oxidant damage by deferoxamine, which we also observed, usually indicates the involvement of iron in producing damage. Iron is present in smoke tar as well as in cells and is released from cellular iron-containing proteins by cigarette smoke (18). In this setting iron most likely acts to catalyze the formation of hydroxyl radical from hydrogen peroxide and superoxide anion [see Kamp et al. (6) for further discussion of this issue].

Our findings also support the idea that mesothelial cells in general are sensitive to oxidant attack. This question has been in dispute (6). Gabrielson et al. (12) were unable to find evidence of asbestos-induced mesothelial damage by active oxygen species using spin trapping, examination of cellular thiol levels, or free radical scavengers. However, Goodglick and Kane (14) found evidence of NBT reduction, believed to reflect the generation of superoxide anion, and mesothelial cell damage as visualized by Trypan blue uptake in the cells around asbestos fibers injected into the peritoneal cavity. More recently, Kinnula et al. (13) demonstrated that cultured rat mesothelial cells were protected against hydrogen peroxide by both the glutathione redox cycle and intracellular catalase, but that sufficiently high concentrations of hydrogen peroxide did produce cell injury. Our findings clearly support the idea that mesothelial cells are sensitive to oxidant, and specifically to hydrogen peroxide, injury.

The third major conclusion in this study is that smoke, or smoke components, either do not penetrate through the pleura or penetrate in relatively small amounts. Because internally administered hydrogen peroxide solutions produce less damage than externally administered hydrogen peroxide solutions at the same concentration, it is clear that even the relatively thin pleural connective tissue of the rat acts as a physical barrier, and undoubtedly the thicker pleura of humans is an even more effective barrier (15). The fact that intratracheal solutions of hydrogen peroxide can produce mesothelial cell damage in a dose-dependent fashion implies that the failure of intratracheal smoke to cause damage is in large part a concentration effect. The amount of hydrogen peroxide that can be detected even in solutions prepared from concentrated smoke tar is several orders of magnitude smaller than the concentrations of hydrogen peroxide that we were using (6,16), and, as noted, the internal milieu of the lung possesses considerable antioxidant defenses that would further decrease the amount of oxidants available to penetrate the pleura in vivo. In addition, the hydroxyl radical is extremely reactive (7) and would react with tissue components long before it could diffuse across the relatively thick pleural barrier.

We cannot rule out the possibility that low concentrations of hydrogen peroxide or other active oxygen species from smoke cross the pleura and are either detoxified...
by antioxidant defenses of the mesothelial cells or damage these cells (13). Our system does not allow evaluation of subtle types of mesothelial damage (e.g., oxidant-induced DNA single strand breaks). This is a limitation inherent in using this type of excised lung preparation. We chose to examine oxidant damage, but other forms of smoke-mediated mesothelial injury might also occur, for example, from aromatic hydrocarbon carcinogens in smoke that may diffuse across the pleura. Nonetheless, our observations do lend support to the idea that the pleura acts as a barrier to the penetration of cigarette smoke oxidants and that this barrier effect might help explain the lack of synergism between smoke and asbestos exposure in producing pleural mesotheliomas.

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