Formation and Persistence of 8-Oxoguanine in Rat Lung Cells as an Important Determinant for Tumor Formation following Particle Exposure

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Exposure of rats to quartz (or various other particles) can lead to the development of lung tumors. At the moment, the mechanisms involved in particle-induced tumor formation are not clarified. However, it is suggested that inflammation, in conjunction with the production of reactive oxygen species (ROS) and an enhancement of epithelial cell proliferation, may play a key role in the development of lung tumors. ROS induces 8-oxoguanine (8-oxoGua) and other mutagenic DNA oxidation products, which can be converted to mutations in proliferating cells. Mutation formation in cancer-related genes is a critical event with respect to tumor formation. In this study we investigated the effects of quartz (DQ12) and of the non-tumorogenic dust corundum on the induction of 8-oxoGua in the DNA of rat lung cells, as well as on cell proliferation and pulmonary inflammation. Wistar rats were exposed by intratracheal instillation to quartz (2.5 mg/rat) or corundum (2.5 mg/rat) suspended in physiological saline; control animals exposed to physiological saline or left untreated. Measurements were carried out 7, 21, and 90 days after the exposures. 8-oxoGua levels were determined in lung tissue sections at the single cell level by immunocytochemical assay using a rabbit anti-8-oxoGua antibody. After exposure to quartz, 8-oxoGua levels were significantly increased at all time points of investigation. Additionally, we observed inflammation and an enhanced cell proliferation. Exposure to corundum had no adverse effects on the lung; neither increased 8-oxoGua levels nor enhanced cell proliferation or inflammation were detected. These observations support the suggestion that inflammation associated with increased 8-oxoGua levels in lung cells and increased cell proliferation is an important determinant for particle-induced development of lung tumors in the rat. — Environ Health Perspect 105(Suppl 5):1291–1296 (1997)

Key words: dust, quartz, corundum, rat, inflammation, cell proliferation, 8-oxoguanine, anti-8-oxoguanine antibody, immunocytochemical assay.

Introduction

The pathogenesis of tumors caused by chemical or physical carcinogens proceeds in multiple subsequent steps involving defined genetic alterations (mutations) in cancer-related genes of target cells. These genes comprise two distinct classes: proto-oncogenes, which are involved in the control of cell division, and tumor-suppressor genes, which inhibit neoplastic cell growth. Alterations in the sequence context of these genes lead to activation or overexpression of protooncogene products and inactivation or loss of tumor-suppressor gene products (1–3). Genetic alterations are induced by a variety of DNA-reactive agents, such as chemical carcinogens, ultraviolet light, ionizing radiation, and reactive oxygen species (ROS). A common feature of these agents is that they cause structural modifications in genomic DNA. DNA modifications can be converted into heritable mutations in dividing cells.

Recent in vitro and in vivo studies demonstrated that the mutagenic and tumorigenic effects of particles and fibers in the lung are closely linked to the formation of ROS (4–8). ROS generate different types of DNA modifications, including single strand breaks in the DNA double helix, 8-oxoguanine (8-oxoGua), the most frequently occurring mutagenic base modification, and various other mutagenic DNA oxidation products (9–12). These DNA modifications can induce genetic alterations in cancer-related genes, which implies a major role of ROS in carcinogenesis (13–18).

Most cells possess potent defense mechanisms to protect DNA from oxidative damage. These mechanisms comprise antioxidant systems for the deactivation of ROS molecules and efficient repair proteins for the elimination of oxidative DNA damage (19–23). Formation of large amounts of ROS and/or inefficient cellular defense mechanisms can lead to persistent (unrepaired) DNA modifications. Persistence of mutagenic DNA modifications in the genome of proliferation competent cells (e.g., pneumocytes II, Clara cells, and bronchial epithelial cells in the lung) constitutes a critical event with respect to cancer development. Therefore, quantification of persistent DNA modifications represents a reliable parameter for tumor risk assessment.

We developed a number of highly sensitive immunoanalytical techniques for the detection and quantification of defined DNA modifications in the DNA isolated from cells or tissues and in the DNA of individual cells in suspension or in histological sections (24–27). These techniques can be applied for the determination of any DNA modification for which an appropriate monoclonal or polyclonal antibody is available. Using antibodies aimed at 8-oxoGua in this study, the immunocytochemical assay (ICA) was used for the quantification of 8-oxoGua in the DNA of individual...
cells in rat lungs after the exposure of the animals to moderate amounts of quartz (DQ12), corundum, or physiological saline as a control. Additionally, the proliferation of lung cells was measured and inflammation markers in bronchoalveolar lavage (BAL) fluids were investigated.

**Methods**

**Exposures**

Wistar rats (10 animals/exposure and per time point) were exposed by intratracheal instillation to quartz (DQ12; 2.5 mg/rat) or corundum (2.5 mg/rat) suspended in physiological saline (0.5 ml); control animals were exposed to physiological saline or left untreated. Animals were sacrificed 7, 21, and 90 days after treatment. Lung tissue sections were used for the quantitative determination of 8-oxoGua in individual cells by ICA (26).

**Antibodies against 7,8-Dihydro-8-oxo-2'-deoxyguanosine**

Synthesis of 7,8-dihydro-8-oxo-2'-deoxyguanosine (8-oxodGuo) and 7,8-dihydro-8-oxoguanosine (8-oxoGuo) was carried out as described (28). 3H-8-oxoGuo (tracer) was prepared from 2'-deoxy-(1',2'-3H)-guanosine (3H-dGuo) as above. 3H-dGuo was obtained by dephosphorylation of the corresponding commercially available triphosphate. Thyroglobulin (TG) was conjugated to 8-oxoGuo as described (29). The hapten–protein conjugates used for immunization contained 400 and 1000 8-oxoGuo molecules/TG molecule. White New Zealand rabbits were injected sc and im with an emulsion containing 600 μg of the conjugate and ABM-ZK adjuvant (Linaris GmbH, Bettingen, Germany). Injections were repeated 4, 10, and 22 weeks after the initial immunization. Serum was collected 12 days after the last injection. Antibodies were precipitated with solid ammonium sulphate (50% saturation), dialyzed, and stored at −80°C. The affinity constants \( K_a \) of the antibodies for 8-oxoGuo, 8-oxoGuo, deoxyguanosine, guanosine, and other unmodified DNA constituents were calculated from the data obtained by competitive radioimmunoassay (30). The \( K_a \) values of the rabbit antibody used for the ICA are listed in Table 1.

**Immunocytological Assay**

The ICA was performed essentially as described for the quantification of DNA alkylation products (26). Frozen lung sections were fixed for 15 min in methanol and rehydrated in 2× SSC (300 mM sodium chloride, 30 mM sodium citrate, pH 7.2). After treatment with RNase A (200 μg/ml 2× SSC) and RNase T1 (50 U/ml 2× SSC) for 1 h at 37°C, sections were washed in 0.14 M NaCl. Cellular DNA was denatured by incubation in a solution containing 70 mM NaOH, 40% EtOH, and 0.14 M NaCl for 5 min at 4°C, followed by incubation with proteinase K (2 μg/ml) for 10 min at 37°C. To avoid nonspecific antibody binding, first incubated sections with phosphate-buffered saline (PBS) containing 20% bovine serum albumin (BSA) for 20 min at room temperature. The sections were then incubated with a rabbit anti-8-oxoGua antibody (1 μg antibody/ml PBS containing 1% BSA) for 16 h at 4°C. Unbound antibodies were removed by extensive washing with PBS containing 0.1% BSA. Goat anti-rabbit-IgG F(ab)2 fragments conjugated to rhodamine isothiocyanate (2 μg/ml PBS containing 1% BSA; Dianova, Hamburg, Germany) were added for 45 min at 37°C. Nuclear DNA was washed with PBS, then stained with 0.3 μM 4,6-diamidino-2-phenylindole in PBS. Samples were then mounted in an antifading medium (PBS, pH 8.2, containing 20% glycerol, 10% elvanol, and 0.03 M dithiothreitol).

**Proliferation**

The fraction of proliferating lung cells was determined by treating rats (3 animals/exposure and per time point) with bromodeoxyuridine (BrdUrd) (100 mg/kg body weight, ip) 2 h prior to sacrifice. Frozen sections of the lungs were fixed in ethanol and rehydrated in PBS. The sections were then treated with 2 M HCl for 15 min, neutralized with 0.1 M sodium tetraborate, pH 8.5, and preincubated with a solution containing PBS, 1% BSA, and 0.1% Triton X100 for 15 min. Mouse anti-BrdUrd antibody (Becton Dickinson, Mountain View, CA) were added to the samples for 45 min at 37°C, then washed with 0.1% BSA in PBS. Finally, goat anti-mouse IgG conjugated to Texas Red (Sigma, Deisenhofen, Germany, diluted 1:50 in PBS/0.1% BSA) was added for 45 min at 37°C.

**Image Analyses**

Fluorescence images were recorded and quantitated by a Hamamatsu cooled CCD video camera (Hamamatsu City, Japan) and a multiparameter image analysis program (AHRENS ACAS cytometry analysis system, Bargeheide, Germany) as described in Seiler et al. (26). Each value represents the average fluorescence intensity of about 100 individual cell nuclei.

**Inflammation Markers**

Bronchoalveolar lavages were performed 7, 21, and 90 days after exposures. Cells contained in BAL fluids were sedimented (400 g x 10 min, 4°C). After removal of the supernatant, cells were suspended in Hank’s solution and counted. Differential cell counts were made from cytocentrifuge smears stained with Pappenheim (Merck, Darmstadt, Germany). Protein concentrations of the BAL supernatants were measured by an assay based on the method of Lowry et al. (31). The activity of tumor necrosis factor alpha (TNF-α) in BAL fluids was determined by a cell lytic assay, as described by Aggarwal et al. (32). Analyses of surfactant phospholipids (phosphatidylglycerol, phosphatidylinositol, phosphatidylethanolamine, phosphatidylcholine, and sphingomyelin) in BAL fluids were performed as described by Folch et al. (33) and Bruch et al. (34).

**Results**

**Quantiﬁcation of 8-oxoGuanine in Single Cells of Lung Tissue Sections**

The ICA makes it possible to visualize and quantify defined DNA lesions in single cells. This technique requires an antibody that binds to a defined DNA lesion in the cellular genome. In this work a partially purified rabbit anti-8-oxoGua antibody was used. As shown in Figure 1A, binding of the antibody molecules (Figure 1Aii, red fluorescence) is restricted to the nuclear DNA of the alveolar cells (Figure 1Aii, blue fluorescence). Other components of the lung tissue (Figure 1Ai, phase contrast) are not labeled, which indicates that binding of the antibody is specific.

The exposure of rats to quartz (DQ12; 2.5 mg/rat; Figure 1Aiii) resulted in signal...
Figure 1. Visualization of 8-oxoGua in individual cells of the rat lung by ICA at day 90 after exposures. Exposure to (A) quartz (DQ12; 2.5 mg/rat), (B) corundum (2.5 mg/rat), and (C) physiological saline. i) phase contrast; ii) nuclear fluorescence (blue) after staining of DNA with 4,6-diamidino-2-phenylindole; iii) 8-oxoGua specific fluorescence (red).
At lower than corundum, 8-oxoGua levels in lung cells were markedly elevated after exposure of rats to quartz.

The results obtained by quantitative evaluation of the fluorescence signals with an image analysis system are summarized in Figure 2. It is evident that the exposure to quartz induced significantly increased 8-oxoGua levels in lung cells at all time points of investigation. The exposure to corundum, however, resulted in 8-oxoGua levels lower than those determined in control animals exposed to physiological saline. At day 21, all data were elevated in comparison to the control (untreated animals). At present, we have no satisfactory explanation for this effect; however, it cannot be excluded that the animals of this exposure group developed a low-level inflammation.

A more detailed analysis of samples from animals exposed to quartz revealed that no cells were detected that significantly differed from the average 8-oxoGua levels calculated for alveolar cells.

Analyses of Bronchoalveolar Lavage Fluids

Exposure of Wistar rats to quartz resulted in significant changes in BAL fluid parameters. Total cell numbers were elevated 5-fold at day 7, 7- to 8-fold at day 21, and more than 30-fold at day 90 (Figure 3A). The percentage of neutrophils was about 44% at day 7 and increased slightly to 56% at day 90 (not shown). The protein concentration in BAL fluids steadily increased from day 7 (2.5 times above control) to day 90 (8.5 times above control) (Figure 3B). Also, the amount of the surfactant component phosphatidylcholine was significantly elevated in BAL fluids at days 7, 21, and 90 (3-, 4.3-, and 5.7-fold, respectively) (Figure 3C). Finally, we observed a significantly increased activity of TNF-α in BAL fluids at day 90 after instillation of quartz (Figure 3D).

After exposure of rats to corundum, no statistically significant changes in the corresponding BAL fluid parameters were detected.

Cell Proliferation

As shown in Figure 4, no significant differences in the percentage of proliferating lung cells were detected between animals exposed to corundum and control animals exposed to physiological saline. Exposure to quartz resulted in a 3- to 4-fold increased cell proliferation at all time points of investigation (Figure 4).

Discussion

Exposure of rats to silica, diesel exhaust, carbon black, or other defined particles results in the development of lung tumors in these animals (35–39). The detailed route by which these materials can exert their tumorigenic potential still remains to be clarified. It is, however, evident that tumor development is preceded by a permanent inflammatory response in the lung evoked by the deposition of low solubility particles. Though inflammation does not necessarily lead to tumors, e.g., as in rheumatoid inflammation of the joints,
persistent pulmonary inflammation seems to be a risk factor for carcinogenesis. Pulmonary inflammation is associated with a series of processes including a) cytotoxic effects leading to cell leakage and apoptotic cell death, b) an enhanced synthesis of surfactant lipids in pneumocytes II, c) an increase in epithelial cell proliferation, and d) the release of large amounts of genotoxic ROS (nitric oxide and other oxidants) from activated macrophages and neutrophils. (8,14,18,40–45).

In particular, formation of ROS and cell proliferation may play a key role in the formation of particle-induced lung tumors: ROS can produce 8-oxoGua and various other mutagenic lesions in genomic DNA that can activate protooncogenes or inactivate tumor-suppressor genes with cell replication (13–18); an enhanced cell proliferation increases the number of target cells for mutagenesis and carcinogenesis and, as a consequence, the probability of tumor formation. Recently, Driscoll et al. (8) detected a significant increase in the hprt mutation frequency in alveolar epithelial cells of rats after exposure to carbon black. The mutational effects have been observed only after carbon black exposures that resulted in significant inflammation and epithelial hyperplasia. This finding strongly supports the hypothesis that inflammation coupled with ROS production and enhanced cell proliferation plays a major role in the development of lung tumors (at least in rats exposed to carbon black).

In general, the cellular content of DNA lesions depends on two counteracting processes: a) the amount and ability of an ultimate carcinogen to react with the constituents of genomic DNA that are packed in chromatin in situ (25,46), and b) the ability of a cell to recognize and eliminate the lesions from DNA by repair proteins. Formation of large numbers of DNA lesions and/or an inefficient cellular repair activity lead to increased levels (persistence) of DNA lesions. In studies performed mainly with alkylating carcinogens, the cellular phenotype can vary considerably from species to species, cell type to cell type, and individual to individual (47). As the repair activity of a proliferation-competent target cell has great influence on the persistence of DNA lesions, it constitutes an important determinant for tumor formation (48). Quantitative determination of persistent DNA lesions, therefore, represents a reliable parameter for tumor risk assessment. Through the ICA, persistence of distinct DNA lesions, such as the oxidation product 8-oxoGua, can be determined at the single cell level.

In this study we analyzed the effects of quartz (DQ12) and the nonmutagenic dust corundum on the alveolar cells of the rat lung. We found that exposure to quartz results in significantly increased cellular 8-oxoGua levels at all time points of the investigation (7, 21, and 90 days after a single intratracheal instillation). This indicates that formation of 8-oxoGua in the DNA of lung cells clearly exceeds the repair activity of these cells. As a consequence, this mutagenic DNA oxidation product persists in the genome of lung cells at least for the time period of investigation (90 days). Analyses at the single cell level showed that 8-oxoGua contents were similarly high in all cell types and areas of the lung. Therefore, it seems that genomic DNA of all alveolar lung cells is oxidatively damaged to an equal extent, at least during the first 3 months.

After exposure of rats to corundum, we found no differences between control animals and exposed animals; i.e., no increase in cellular 8-oxoGua levels or cell proliferation or changes in BAL fluid parameters were observed. On the other hand, exposure to quartz resulted in a 3- to 4-fold enhancement of cell proliferation and significant changes in all BAL fluid parameters so far examined. Thus, the concentrations of protein and surfactant, as well as the activity of TNF-α and the number of neutrophils in BAL fluids, were markedly elevated 7 days after exposure and increased steadily during the first 3 months.

In summary, exposure of rats to quartz produced inflammation, an increase in cell proliferation, and persistence of the mutagenic DNA oxidation product 8-oxoGua in lung cells. Corundum, a noncarcinogenic particle, was not harmful to the rat lung. These observations support the suggestion that inflammation, in conjunction with the persistence of mutagenic DNA oxidation products (e.g., 8-oxoGua) in target cells and an increased proliferation of target cells, plays an important role in the particle-induced pathogenesis of rat lung tumors. It is not known how long 8-oxoGua levels in target cells must be elevated until the multistep process of carcinogenesis is initiated and completed. In addition, we do not know why the rat but not, for example, the hamster is easily susceptible to tumor formation after exposure to quartz and other distinct particles. Further experiments involving measurements of ROS-induced oxidation products in proliferating cells of rats and other species at early and later stages after particle exposure may answer these questions.

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