Genomic instability in quartz dust exposed rat lungs: Is inflammation responsible?

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Abstract. Exposure to quartz dusts has been associated with lung cancer and fibrosis. Although the responsible mechanisms are not completely understood, progressive inflammation with associated induction of persistent oxidative stress has been discussed as a key event for these diseases. Previously we have evaluated the kinetics of pulmonary inflammation in the rat model following a single intratracheal instillation of 2mg DQ12 quartz, either in its native form or upon its surface modification with polyvinylpyridine-N-oxide or aluminium lactate. This model has been applied now to evaluate the role of inflammation in the kinetics of induction of DNA damage and response at 3, 7, 28, and 90 days after treatment. Bronchoalveolar lavage (BAL) cell counts and differentials as well as BAL fluid myeloperoxidase activity were used as markers of inflammation. Whole lung homogenate was investigated to determine the induction of the oxidative and pre-mutagenic DNA lesion 8-hydroxy-2-deoxy-guanosine (8-OHdG) by HPLC/ECD, while mRNA and protein expression of oxidative stress and DNA damage response genes including hemeoxygenase-1 (HO-1) and apurinic/apyrimidinic endonuclease (APE/Ref-1) were evaluated using Western blotting and real time PCR. Isolated lung epithelial cells from the treated rats were used for DNA strand breakage analysis using the alkaline comet assay as well as for micronucleus scoring in May-Gruenwald-Giemsa stained cytospin preparations. In the rats that were treated with quartz, no increased 8-OHdG levels were observed, despite the presence of a marked and persistent inflammation. However, DNA strand breakage in the lung epithelial cells of the quartz treated rats was significantly enhanced at 3 days, but not at 28 days. Moreover, significantly enhanced micronucleus frequencies were observed for all four time points investigated. In the animals that were treated with the PVNO modified quartz, micronuclei scores did not differ from controls, while in those treated with the aluminium coated quartz intermediate effects were found. These findings were in line with the kinetics of inflammation and epithelial proliferation in the rat lungs for the different treatments. Notably, a highly significant correlation was...
observed between neutrophil numbers and micronucleus frequencies, indicative for a role of inflammation in eliciting genomic instability in target cells of quartz-induced carcinogenesis. Our ongoing investigations focus on the evaluation of the causality between both in relation to quartz exposure.

1. Introduction

Inflammation has been recognised as an important factor in cancer development and progression [1, 2, 3]. Chronic inflammation is also considered to be crucial in driving lung cancer formation after inhalation of quartz as well as poorly-soluble low toxicity dusts. Herein, specifically a mechanism is considered to be involved whereby inflammatory cells release excessive amounts of reactive oxygen species (ROS) which are known to be able to cause oxidative DNA damage and mutagenesis [reviewed in 4]. This mechanism can be referred to a secondary genotoxicity, in contrast to primary genotoxicity, which is considered to involve a direct genotoxic action of a particle [5]. Previous studies have demonstrated the carcinogenicity of quartz as a variably entity [6]. We and others have shown that especially the surface reactivity of the particles contributes to their biological response, since surface modifications by polyvinylpyridine-N-oxide (PVNO) or aluminium lactate (AL) are able to reduce the inflammatory and genotoxic potential of DQ12 quartz particles [7, 8, 9, 10]. The present study was undertaken to evaluate the relation between the kinetics of quartz-induced lung inflammation and genotoxicity in vivo at 3, 7, 28 and 90 days after single intratracheal (i.t.) instillation. Inflammation was characterised by inflammatory cell counts of the bronchoalveolar lavage (BAL) and differentials. For the evaluation of genotoxicity three independent methods were used. In whole lung tissue homogenate we determined the formation of the oxidative and pre-mutagenic lesion 8-hydroxydeoxyguanosine [11]. Two further measurements of genotoxicity were determined specifically in the lung epithelial cells, isolated from the treated animals. These cells were specifically analysed because of their relevance as target cells in pulmonary carcinogenesis [8]. For the determination of DNA strand breakage the alkaline comet assay was used. This assay is a sensitive genotoxicity indicator assay that allows for quantification of DNA-damage in individual cells [12]. The isolated lung epithelial cells were also evaluated for micronuclei (MN), which are small extranuclear bodies, arising in dividing cells from chromosome fragments or a whole lagging chromosome. MN analysis in isolated lung epithelial cells from rats has been successfully applied for the evaluation of in vivo genotoxicity of hard metal particles and carbon nanotubes after i.t. instillation [13, 14].

2. Materials and methods

2.1. Particle characteristics and coating procedure

Dörentruper quartz (DQ12) was used as native quartz as well as for the preparation of surface-modified quartz samples. The procedure for particle coating with aluminium or PVNO and quality control evaluation (i.e. determination of coating efficiency and size distribution) have been described elsewhere [8, 9, 10].

2.2. Animals and treatment

Female Wistar rats (Janvier, Le Genest St. Isle, France) were maintained in an accredited on-site testing facility, responding to the guidelines of the Society for Laboratory Animals Science (GV-SOLAS). At a age of eight weeks (220±1g) animals were i.t. instilled following anesthetization (Isofluran, Essex Pharma GmbH, Munich, Germany) using 400µl volume containing phosphate buffered saline (PBS) as vehicle control, 2mg DQ12 quartz or DQ12 (2mg) coated with either PVNO or with AL. At days 3, 7, 28 and 90 post-instillation, animals were sacrificed by deep anesthetization with pentobarbital (50 mg/kg body weight) followed by exsanguination via the A. abdominalis.
2.3. Broncho-alveolar lavage and analysis
Lungs of 5 animals per treatment group and time point were lavaged as described previously (Albrecht et al., 2004). The BAL fluid was spun (500g, 10min, 4°C), and cells were collected for cell counting and May-Grunwald-Giemsa (MGG)-stained cytospin preparations. Inflammatory and cytotoxic data are published elsewhere [9].

2.4. DNA isolation and analysis of 8-hydroxy-2’-deoxyguanosine by HPLC/ECD
Lung tissue was removed, chopped, aliquots were snap frozen in liquid nitrogen and stored at –80°C until later measurement of 8-OHdG using high performance liquid chromatography with electrochemical detection (HPLC-ECD) [15]. Values are expressed as the total amount of 8-OHdG per 106 nucleotides.

2.5. Lung epithelial cell isolation
In order to investigate the specific target cell for particle-induced lung tumours, lung epithelial cells were isolated according to the method of Richard and colleagues [16] as modified by Knaapen and co-worker [8]. A part of the obtained epithelial cell fraction was used to prepare MGG-stained cytospin preparations in order investigate the appearance of MN. Another part was used for the evaluation of DNA strand breakage by the alkaline comet assay.

The purity of the isolated cell fraction, as determined by microscopy evaluation of the MGG-stained slides was found to be 63 – 75 % epithelial cells. Other cell types were neutrophils (4 – 18 %) and macrophages (13 – 20 %).

2.6. Comet assay
DNA strand breakage was investigated immediately after cell isolation using the alkaline comet assay as described previously [8] according to the guidelines published by Tice and Colleagues [17]. For each animal three independent slides were prepared, and on each single slide 50 cells were randomly evaluated. Cells were categorised according to presence or absence of a tail. The comet assay analysis was performed for 2 time points, i.e. at 3 days and 28 days.

2.7. Micronucleus evaluation in lung epithelial cells
In order to determine the proportion of the micronucleated cells, in each MGG-stained slide 1000 lung cells were counted at x1000 magnification with immersion oil using a Zeiss Axioscope 2 microscope. The identification and scoring of the MN was done according to Tolbert and colleagues [18]. Characteristics of each evaluated cell are an intact cytoplasm and an intact nucleus with a smooth and distinct nuclear perimeter. A cellular structure was then identified as a MN if its size was less than a third of the diameter of the associated nucleus, but large enough to discern the round shape and a comparable staining intensity to the nucleus.

2.8. Statistical analysis
Data are expressed as mean ± SD. Statistical analysis was performed using SPSS version 15.0 for Windows, post hoc Tukey-HSD. Correlation between MN formation and percentage of PMN was tested using Person’s r-test. Differences were considered as statistically significant when p < 0.05.

3. Results
Investigation of whole lung tissue homogenate showed no enhanced 8-OHdG/dG ratios in the animals after treatment with native quartz at all investigated time points (Figure 1). Surprisingly, at all time points 8-OHdG levels tended to be higher in the lungs from rats that were exposed to the coated quartz preparations than from those that were treated with the original quartz sample. At the 7 day time point, 8-OHdG levels were significantly enhanced in animals that were treated with the PVNO-coated quartz sample (p<0.05, Fig. 1).
Figure 1: 8-OHdG analysis by HPLC/ECD in lung tissue, obtained from rats exposed to a single dose of 2 mg non-coated DQ12 or DQ12 coated with PVNO or AL at 3, 7, 28 and 90 days after i.t. instillation. Data are shown as mean ± SD (n=5). *p<0.05 vs. PBS

The comet assay was used for the determination of DNA strand breakage in freshly isolated epithelial cells. The measurements were performed at the 3 day and the 28 day time points (Table 1).

Table 1: Percent of isolated lung epithelial cells showing DNA damage as determined by the alkaline comet assay. Cells were isolated from the lungs of rats that were exposed to a single dose of 2 mg non-coated DQ12 or DQ12 coated with PVNO or AL (* p<0.05).

| Treatment       | 3 days (% of cells with tail) | 28 days (% of cells with tail) |
|-----------------|-------------------------------|--------------------------------|
| PBS             | 32.4 ± 6.6                    | 56.4 ± 13.8                    |
| DQ12            | 41.1 ± 11.3 *                 | 51.2 ± 14.6                    |
| DQ12 – PVNO     | 28.6 ± 12.9                   | 62.6 ± 14.7                    |
| DQ12 – AL       | 31.3 ± 10.2                   | 60.0 ± 10.4                    |

Three days after quartz instillation a significant higher number of cells with DNA damage were observed, when compared to the control animals. Particle surface modification with PVNO and AL inhibited the ability of quartz to induce these DNA damage. After 28 days, these differences were not present. In fact, the DQ12 treated animals tended to show the lowest DNA damage at this time point.

As a further marker for the genotoxic potential of the quartz particles, the appearance of MN were evaluated at MGG-stained cyto spin preparations of lung epithelial cells. In quartz-treated animals significantly higher MN % were observed at all times points (Figure 2). Interestingly, for animals that had received AL-coated quartz also enhanced MN frequencies were found at the 28 and 90 day time points. In contrast, animals that were treated with the PVNO-coated quartz MN % in the lung epithelial cells isolates were not increased.
Figure 2: Data of MN formation calculated as absolute number per 1000 cells evaluated from MGG stained cytospin preparations. Cells were obtained from rats exposed to a single dose of 2 mg non-coated DQ12 or DQ12 coated with PVNO or AL at 3, 7, 28 and 90 days after single i.t. instillation. Data are shown as mean ± SD (n=5). *p < 0.05 vs. PBS; **p < 0.01.

In order to evaluate the relation between the inflammation as induced by the different quartz preparations and genotoxicity, correlation analyses were performed between inflammatory cell counts from bronchoalveolar lavage and the MN frequencies as determined in the lung epithelial cells. For each of the four investigated time points there was a significant linear correlation between the % of neutrophils and the MN frequencies when data were considered on the single animal level (3, 7, 28 and 90 days, all: p<0.01). The relation between neutrophilic inflammation and epithelial lung cell genotoxicity was also observed on the group level for the entire study (Figure 3). This effect was also clearly exhibited on the single animal level (n = 76; R² = 0.46; p < 0.0001).
4. Discussion and conclusion

The major goal of our study was to evaluate the relation between inflammation and genotoxicity, considered to be a crucial mechanism of particle-induced carcinogenesis. Therefore, we evaluated the kinetics of lung inflammation after single i.t. instillation of different preparations of quartz in rat lungs in relation to various genotoxicity readouts. The application of two different surface-modifications of DQ12 quartz (i.e. PVNO and aluminium) along with the original DQ12 sample resulted in levels of inflammation which largely differed in extent as well as over time [9]. In this study we have now examined over the time different markers of particle-induced genotoxicity. Being the most-well investigated oxidative DNA lesion, the induction of 8-OHdG was measured in whole lung tissue homogenates by HPLC/ECD [11, 15]. Increased 8-OHdG immunoreactivity in lung alveolar cells has been previously shown by Seiler and colleagues [19] three weeks after a single i.t. instillation of 1.5 mg quartz in rats, while 3 days after instillation such an increase was absent. Surprisingly, no significant 8-OHdG induction was observed by the original quartz sample in the current study despite a persistent inflammation, neither with HPLC/ECD nor with supportive investigations by immunohistochemistry (data not shown). The divergence between the different in vivo quartz studies may have resulted from use of different doses, exposure times or quartz samples, i.e. as an effect of a “batch to batch variation” [6]. Also the possibility of an artificial induction of 8-OHdG during DNA extraction and processing as required for HPLC/ECD analysis, leading to high background levels would neither explain positive findings by others [e.g. 11] as well as our unexpected effects in the animals which received PVNO-coated quartz samples.

In contrast to the lack of detectable increases in 8-OHdG upon DQ12 treatment, a significant increase in DNA strand breakage of lung epithelial cells from quartz-treated animals could be
measured three days after quartz instillation. This difference might be explained by the specific isolation of the lung epithelial cells and the high sensitivity of the comet assay method. Importantly, DNA-damage was not observed in the animals that were treated with the surface modified quartz samples. As these data were related to the extent of neutrophilic inflammation as induced by the different sample treatments for this time point, they are in support of the causal role of inflammation in particle-induced genotoxicity [8]. However, 28 days after the instillation, no difference in DNA strand breakage could be detected in the lung epithelial cells from the different treatment groups. These findings may be explained by an induction of DNA repair, as indicated from our previous investigations were we observed enhanced expression of the oxidative DNA damage repair protein apurinic/apyrimidinic endonuclease/redox factor 1 (APE/Ref1) [10]. However, the role of epithelial cell proliferation and differentiation known to occur after quartz treatment should also be considered. In this regard, the third genotoxicity assay evaluated in our current study, i.e. MN in the lung epithelial cells, are considered to be the most relevant: While the comet assay is a measure of the bare presence of a potentially broad spectrum of DNA lesions which may - or may not - lead to a mutation, a MN represents the product of a clastogenic or aneugenic effect and cell proliferation. The relevance of MN test has been further supported by recent observations that demonstrated the predictive value of their increases in peripheral blood lymphocytes for lung cancer risk in humans [20].

In the present study, MN frequencies were found to be enhanced in lung epithelial cells up to the period of 90 days after quartz instillation. In the knowledge that the quartz treatment in our study has been shown to lead to a persistent increasing inflammation over this time period [9], it may be proposed that the observed in vivo genotoxicity results from (a) yet to be identified mechanism(s) operating in inflamed lung tissues. This is further supported by observed gradients in inflammation and MN in the lungs of the rats that received AL- and PVNO-modified DQ12 respectively, visualised by a highly significant correlation between the percentages of neutrophils and MN in the over all study. Although previous in vitro studies by us and others have demonstrated that both neutrophils and quartz particles can cause genotoxic insult to epithelial cells [reviewed in 5] our present data are in further support of the secondary mechanism of genotoxicity. Noteworthy in this regard, the MN frequency in epithelial cells of the control rats as well as those that were treated with PVNO-coated DQ12 was about 0.5 – 1 % indicative of the absence of an increased genotoxicity up to an approximate 30 % of neutrophils in the lung as observed with the DQ12- PVNO group.

In conclusion, our study demonstrates that modification of the reactive surface of quartz particles can have significant impact on pulmonary inflammation and associated genotoxicity and therefore provides further clues for the observed variable cancer risk of quartz exposure [6]. Moreover, our data are the first to show a marked in vivo association between neutrophilic inflammation in the lung and the formation of MN in lung target cells for (particle-induced) lung carcinogenesis. Additional experiments are needed to establish the causality of this relation.

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