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Evaluation of immunohistochemical markers to detect the genotoxic mode of action of fine and ultrafine dusts in rat lungs

Susanne Rittinghausen[a,*], Bernd Bellmann[a], Otto Creutzenberg[a], Heinrich Ernst[a], Angelika Kolling[a], Inge Mangelsdorf[a], Rupert Kellner[a], Sascha Beneke[b], Christina Ziemann[a]

[a] Fraunhofer Institute for Toxicology and Experimental Medicine ITEM, Hannover, Germany
[b] Institute of Veterinary Pharmacology and Toxicology, University of Zurich, Zurich, Switzerland

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ABSTRACT

Data on local genotoxicity after particle exposure are crucial to resolve mechanistic aspects such as the impact of chronic inflammation, types of DNA damage, and their role in lung carcinogenesis. We established immunohistochemical methods to quantify the DNA damage markers poly(ADP-ribose) (PAR), phosphorylated H2AX (γ-H2AX), 8-hydroxyguanosine (8-OH-dG), and 8-oxoguanine DNA glycosylase (OGG1) in paraffin-embedded tissue from particle-exposed rats. The study was based on lungs from a subchronic study that was part of an already published carcinogenicity study where rats had been intratracheally instilled with saline, quartz DQ12, amorphous silica (Aerosil® 150), or carbon black (Printex® 90) at monthly intervals for 3 months. Lung sections were stained immunohistochemically and markers were quantified in alveolar lining cells. Local genotoxicity was then correlated with already defined endpoints, *i.e.* mean inflammation score, bronchoalveolar lavage parameters, and carcinogenicity. Genotoxicity was most pronounced in quartz DQ12-treated rats, where all genotoxicity markers gave statistically significant positive results, indicating considerable genotoxic stress such as occurrence of DNA double-strand breaks (DSB), and oxidative damage with subsequent repair activity. Genotoxicity was less pronounced for Printex® 90, but significant increases in γ-H2AX- and 8-OH-dG-positive nuclei and OGG1-positive cytoplasm were nevertheless detected. In contrast, Aerosil® 150 significantly enhanced only 8-OH-dG-positive nuclei and oxidative damage-related repair activity (OGG1) in cytoplasm. In the present study, γ-H2AX was the most sensitive genotoxicity marker, differentiating best between the three types of particles. The mean number of 8-OH-dG-positive nuclei, however, correlated best with the mean inflammation score at the same time point. This methodological approach enables integration of local genotoxicity testing in subchronic inhalation studies and makes immunohistochemical detection, in particular of γ-H2AX and 8-hydroxyguanine, a very promising approach for local genotoxicity testing in lungs, with prognostic value for the long-term outcome of particle exposure.

1. Introduction

Carcinogenicity studies have demonstrated that long-term exposure to various respirable micro- and nanoscale particles (MNP) can induce lung tumors, in particular in the rat model (Saffiotti and Stinson, 1988; Wiessner et al., 1989; Donaldson and Borm, 1998; Muhle et al., 1989; Nikula, 2000; Roller, 2009). Especially the surface characteristics of poorly soluble particles predominantly determine the carcinogenic potential of MNP (Oberdörster et al., 2005; Duffin et al., 2007), as they do not act as single molecules, but more likely in a physico-mechanical or physico-chemical way.

Different genotoxic modes of action could explain the carcinogenic potential of particles in the lung in non-overload and overload situations. Possible genotoxic mechanisms of MNP *in vivo*, as summarized earlier by Knaapen et al. (2004), seem to comprise indirect (secondary) mechanisms that are phagocytosis- and/or inflammation-driven, but also directly particle-related (primary) genotoxic modes of action. Release of reactive oxygen (ROS) and nitrogen (RNS) species either by (i) oxidative burst of phagocytes, (ii) disturbance of the respiratory chain, (iii) activation of

Abbreviations: γ-H2AX, gamma-H2AX; 8-OH-dG, 8-hydroxyguanosine; ANOVA, analysis of variance; BAL, bronchoalveolar lavage; DSB, DNA double-strand breaks; LDH, lactate dehydrogenase; MNP, microscale and nanoscale particles; NO, nitric oxide; OGG1, 8-oxoguanine DNA glycosylase; ONOO—, peroxynitrite anions; PAR, poly(ADP-ribose); PARP, poly(ADP-ribose) polymerase; PMN, polymorphonuclear neutrophils; RNS, reactive nitrogen species; ROS, reactive oxygen species.

* Corresponding author at: Fraunhofer Institute for Toxicology and Experimental Medicine ITEM, Department of Pathology, Nikolai-Fuchs-Str. 1, 30625 Hannover, Germany. Tel.: +49 5115350310; fax: +49 5115350155.
E-mail address: susanne.rittinghausen@item.fraunhofer.de (S. Rittinghausen).

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ROS-/RNS-producing enzyme systems, or (iv) reactive particle surfaces with subsequent oxidative DNA damage is thought to be of principal importance. However, translocation into the nucleus and physical interaction with genomic DNA is also being discussed as putative genotoxic insult, in particular for nanoparticles.

Various genotoxicity endpoints have been used to evaluate the diverse hypotheses on the mechanistic principles of particle-induced tumor development, as reviewed in several recent publications (Gonzalez et al., 2008; Landsiedel et al., 2009; Schins and Knaapen, 2007; Singh et al., 2009). Nevertheless, knowledge about the *in vivo* situation is still insufficient. To enlarge the body of knowledge, new experimental approaches are highly needed. In the present study, we therefore investigated whether local DNA damage in particle-exposed lung tissue can be detected and quantified *in situ* with immunohistochemical methods. One advantage of this approach is the possibility to use paraffin-embedded lung tissue from previous studies. In the present study, we used lung tissue from 3-month satellite groups of an existing carcinogenicity study, where animals had been exposed to particles by intratracheal instillation of high doses of crystalline silica (quartz DQ12), carbon black (Printex® 90), or amorphous silica (Aerosil® 150). A variety of parallel data on histopathology, inflammation, toxicity, and tumor incidences enabled assessment of the feasibility and informative value of the approach.

A panel of genotoxicity markers with different degrees of informative value was chosen to enable identification of the genotoxic modes of action in alveolar lining cells predominantly consisting of epithelial cells, as target cells of lung tumor development. The well-established genotoxicity markers poly(ADP-ribose) (PAR), phosphorylated H2AX (γ-H2AX), 8-hydroxy-2′-deoxy-guanosine (8-OH-dG), and 8-oxoguanine DNA glycosylase (OGG1) were selected for immunohistochemical detection and quantification in the available lung tissue samples. PAR is a posttranslational protein modification that has been used as a general, overall marker of genotoxic stress (Bürkle, 2001). Its synthesis reflects an early cellular reaction to DNA single- (SSB) or double-strand breaks (DSB). Additionally, PAR is involved in the regulation of cell division and cell cycle progression (for review, see Hakmé et al., 2008) and plays a role in inflammatory processes in asthma and other lung diseases (Virág, 2005). Gamma-H2AX is a phosphorylated core histone variant phosphorylated after DSB induction (Rogakou et al., 1998) and γ-H2AX-containing foci seem to correlate directly with the number of DSB (Sedelnikova et al., 2002). In addition, γ-H2AX formation also occurs during apoptosis (Sluss and Davis, 2006), but nevertheless can be used as a sensitive genotoxicity marker (Watters et al., 2009). 8-OH-dG, a well-characterized oxidative DNA base lesion, is an important and well-established marker of oxidative stress (Kasai, 1997). It is probably the most mutagenic oxidative DNA base modification (Shibutani et al., 1991) and is commonly found in lung tumors (Husgafvel-Pusiainen et al., 2000). Because of the hypothesized importance of oxidative stress for the genotoxic potential of particles, the base excision repair protein OGG1 was chosen as a second marker of oxidative DNA damage. OGG1 is involved in recognition and excision of 8-OH-dG in both nuclear and mitochondrial DNA if mispairing with cytosine occurs (Dianov et al., 1998; Aburatani et al., 1997). OGG1 thus indicates oxidative stress-related DNA repair capacity. Interestingly, mutations or polymorphisms of the *OGG1* gene (Chevillard et al., 1998; Mambo et al., 2005) as well as low OGG1 activity (Paz-Elizur et al., 2003) seem to be strongly associated with an increased risk of lung cancer. Besides PAR and γ-H2AX, our study thus aimed at quantitative detection of these oxidative stress markers to evaluate one hypothesized principal mechanism for the genotoxic potential of MNP.

Improving the immunohistochemical methods for reliable *in situ* detection and quantification of different types of DNA damage in paraffin-embedded lung tissue would enable re-evaluation of existing inhalation and instillation studies with MNP and also integration of local genotoxicity testing in new *in vivo*, in particular subchronic toxicity studies and also carcinogenicity studies. It is of special interest in this context whether such a methodological approach would be of prognostic value for the long-term outcome of particle exposure.

2. Materials and methods

2.1. Lung tissue for immunohistochemical detection of genotoxicity markers

For immunohistochemical detection and quantification of DNA damage in lung tissue, we used existing paraffin-embedded lung tissue samples from the German Federal Environment Agency (Umweltbundesamt, UBA)-funded project entitled: "Pathogenetische und immunbiologische Untersuchungen zur Frage: Ist die Extrapolation der Staubkanzerogenität von der Ratte auf den Menschen gerechtfertigt?" (FKZ 203 61 215). Samples of the 3-month study part (satellite groups) were selected, as a period of 3 months seemed long enough to guarantee particle-driven perpetual chronic inflammation in the lungs.

These lung tissue samples offered the unique possibility to correlate the data on local genotoxicity of repeatedly intratracheally instilled particles (Tables 1 and 2) in the lungs of female Wistar WU rats [strain: Crl:WI(WU)] three months after the first and one month after the last instillation with parameters such as tissue inflammation (at the same time point), tumor incidence (in a lifetime study), and specific pathological findings (Ernst et al., 2002, 2005; Kolling et al., 2008). The samples had originally been embedded for histology and immunohistochemistry and had a fixation time of 24 h. The corresponding histopathology data of the 3-month samples were published by Ernst et al. (2002).

2.2. Particle characteristics and particle administration

In the original carcinogenicity study, the biological effects of inflammatory doses of crystalline silica (quartz DQ12), carbon black (Printex® 90), and amorphous silica (Aerosil® 150) had been compared. The origin, preparation, and properties of the particles used in the 3-month study part and in the carcinogenicity lifetime study are given in Table 1, besides details on preparation of the particle suspensions. At the time of the original study (end of last century), the physico-chemical characterization of particles, in this case nanoscale particles in an aqueous suspension, was generally poor. Data on hydrodynamic particle diameters or ζ potential are thus missing. Nevertheless, the approach already aimed to achieve an effective dispersion of particles in saline by stirring. Being aware of the agglomeration problem with nanoscale particles an ultrasonic treatment of 10–30 s was included. Based on today's knowledge and the dispersion characterization, the dispersions will have had mean agglomerate sizes of about 300–500 nm.

For details on treatment groups, numbers of investigated animals, and dosing regimes, see Table 2. Animals were exposed to the particle suspensions by intratracheal instillation. Due to the completely different focus of the original study, however, aimed at inducing comparable grades of chronic inflammation for all three granular dusts, mass doses of the three particle types in the subacute, subchronic and chronic study parts were not identical (see Table 2). The administered mass doses thus depended on known particle characteristics. Quartz DQ12 (highly reactive crystalline silica, triggering progressive lung injury) and Printex® 90 (carbon black) are poorly soluble dusts, whereas amorphous silica (Aerosil® 150) is a non-biopersistent dust that is eliminated relatively fast (half-life in rats approx. 1 day; rat study by Fraunhofer ITEM, 1999) and triggers acute toxicity but only temporary inflammation in the lung. Printex® 90-treated animals received three times higher particle mass doses in the 3-month study part than silica-treated animals (quartz DQ12 and Aerosil® 150). Consequently, correlations regarding expression of the genotoxicity markers between Printex® 90-treated animals and animals treated with the other particle materials were limited. However, quartz DQ12 and Aerosil® 150 were instilled at the same doses and intervals, thus enabling material-based direct comparison of the data. As the ratios of doses of the different dusts also varied between the 3-month and lifetime study parts, correlations of genotoxicity marker expression and tumor data could be evaluated only with certain restrictions.

2.3. Immunohistochemical detection of genotoxicity markers

For immunohistochemical detection of the chosen genotoxicity markers in lung tissue, 3-μm paraffin sections were cut from the lung material, using one block of the left lung lobe for each animal, and were mounted on glass slides. Paraffin sections were then dewaxed and subject to DNA hydrolysis with 4 N HCl and the corresponding antigen retrieval methods, which had been validated for each of the primary antibodies.

The primary antibodies used comprised protein A column-purified mouse monoclonal antibody 10 H (generous gift from Prof. A. Bürkle, University of Konstanz, Germany) for detection of PAR, a rabbit polyclonal antibody directed against γ-H2AX (phospho S139) as DSB marker (Abcam, Cambridge, UK, ab2893), mouse monoclonal antibody N45.1 against 8-OH-dG (Abcam, Cambridge, UK, ab48508), and rabbit polyclonal anti-Ogg1 antibody (Novus Biologicals, Littleton, USA, NB100-106).