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Comparative evaluation of particle properties, formation of reactive oxygen species and genotoxic potential of tungsten carbide based nanoparticles in vitro

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HIGHLIGHTS

- Assessment of toxic potential of tungsten carbide-based nanoparticles.
- Evaluation of ROS and micronuclei induction of three hard metal nanomaterials.
- Dependency of observed toxic effects on the materials physical–chemical properties.
- Differences in several particle properties seem to modulate the biological response.

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ABSTRACT

Tungsten carbide (WC) and cobalt (Co) are constituents of hard metals and are used for the production of extremely hard tools. Previous studies have identified greater cytotoxic potential of WC-based nanoparticles if particles contained Co. The aim of this study was to investigate whether the formation of reactive oxygen species (ROS) and micronuclei would help explain the impact on cultured mammalian cells by three different tungsten-based nanoparticles (WC_S, WC_L, WC_L–Co (S: small; L: large)). The selection of particles allowed us to study the influence of particle properties, e.g. surface area, and the presence of Co on the toxicological results. WC_S and WC_L/WC_L–Co differed in their crystalline structure and surface area, whereas WC_S/WC_L and WC_L–Co differed in their cobalt content. WC_L and WC_L–Co showed neither a genotoxic potential nor ROS induction. Contrary to that, WC_S nanoparticles induced the formation of both ROS and micronuclei. $CoCl_2$ was tested in relevant concentrations and induced no ROS formation, but increased the rate of micronuclei at concentrations exceeding those present in WC_L–Co. In conclusion, ROS and micronuclei formation could not be associated with the presence of Co in the WC-based particles. The contrasting responses elicited by WC_S vs. WC_L appear to be due to large differences in crystalline structure.

1. Introduction

Tungsten carbide (WC) and cobalt (Co) are the main constituents of hard metals used for the production of extremely hard and wear resistant tools, e.g. for milling and drilling. While WC is a ceramic material with high hardness, cobalt metal is added as a binder, improving the toughness and strength of the compound material. Recently, nano-scaled hard metals have become very attractive because strength and hardness can be increased by reducing the WC particle size.

It is expected that exposure occurs in the workplace. In fact, an increased risk of lung cancer has been reported for workers exposed to tungsten carbide–cobalt (WC–Co) dusts ("hard-metal lung") [1].

Abbreviations: BET, Brunauer–Emmet–Teller; BSE, back scattered electron detector; CFDA-AM, 5-carboxyfluorescein diacetate, acetoxymethyl ester; Co, cobalt; DLS, dynamic light scattering; EDS, energy dispersive X-ray spectroscopy; FBS, fetal bovine serum; IARC, International Agency for Research on Cancer; PBS, phosphate buffered saline; ROS, reactive oxygen species; RPMI, Roswell Park Memorial Institute Medium; SEM, scanning electron microscopy; v, volume; WC, tungsten carbide; WC–Co, tungsten carbide cobalt; XDR, X-ray diffraction.

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Previous studies using μm-sized particles have indicated a lack of toxicity for WC particles and a hazardous potential for cobalt metal particles toward macrophages derived from mammals [2]. WC–Co, a combination of both materials, features an enhanced toxicity compared to cobalt alone [3]. WC–Co is classified by the International Agency for Research on Cancer (IARC) as 'probably carcinogenic to humans' [4], cobalt alone as 'possibly carcinogenic to humans' [4]. A similar combinatorial effect has recently been demonstrated for nano-sized WC and WC–Co in different mammalian cells [5] as well as in a rainbow trout gill cell line [6]. Whereas WC nanoparticles were found to exert no cytotoxic effects, WC–Co induced moderate cytotoxicity even though both nanomaterials were internalized by cells. The role of the Co leached from WC–Co particle mixtures was assessed as well. Co^{2+} concentrations equivalent to those contained in the particle mixtures were found not to be cytotoxic [5,6]. At the level of the transcriptome, genes differentially expressed upon exposure to WC–Co and Co^{2+} were nearly indistinguishable and therefore did not help to explain the differential cytotoxicity observed for WC, WC–Co and Co^{2+} [7].

To identify possible mechanisms for the enhanced toxicity of WC–Co, the present study aimed to analyze the potential of WC and WC–Co to induce ROS and micronuclei.

Excess ROS lead to damage of cellular components and the generation of ROS in biological systems is considered a major mechanism of nanoparticle induced toxicity [8,9]. The redox-sensitive probe dichlorofluorescein diacetate (H_2DCFDA) was applied for ROS measurement. For μm-sized hard metal particles, no evidence was found that production of ROS contributes to WC–Co cytotoxicity [10], even though ROS formation by WC–Co was demonstrated in cell-free systems [11,12].

The induction of micro-nucleated cells is an indicator for chromosome aberrations and fixed DNA damage. ROS may induce DNA damage, but other mechanisms of micronuclei formation also exist. WC particles at the μm size showed no DNA damaging potential, whereas for Co and WC–Co particles a dose dependent damage to human leukocyte DNA was observed, with an enhanced potential for WC–Co [13,14].

Based on these previous data for μm sized particles, nano sized WC–Co was expected to induce the formation of micronuclei, but not of ROS, whereas WC_{L+S} were expected to induce neither ROS nor micronuclei.

Further, we aimed to identify whether ROS and micronuclei formation are influenced by additional nanoparticle properties, specifically surface area and crystalline structure. Thus, in addition to the previously studied WC and WC–Co nanoparticles, we added a third WC-based particle type [15]. Based on the smaller diameter of the latter particle type, it was designated WC_S. For clarity and consistency, the other two particle types formerly referred to as WC and WC–Co were now designated WC_L and WC_L–Co.

As cobalt is known to exert cytotoxic and genotoxic effects in vitro, both as ions and as metal [13,14,16], and in order to be able to differentiate between the effects of WC_L–Co and soluble cobalt, $CoCl_2$ was included in all in vitro assays as a control substance.

2. Materials and methods

2.1. Preparation and characterization of properties of particle suspensions

From a WC powder and a WC–Co mixture, two particle suspensions were prepared: WC_L and WC_L–Co. Both suspensions were characterized by size, agglomeration behavior and behavior in cell culture media, as described previously [5,6]. To distinguish these particles from smaller size WC particles included in this study, WC and WC–Co of the former studies were here renamed WC_L and WC_L–Co. WC_L–Co contained 10% (w/w) cobalt, amounting to ~50 μM cobalt in 33 μg/ml WC_L–Co, the highest concentration tested in in vitro assays. WC_L and WC_L–Co particle suspensions were found to be stabilized by fetal bovine serum (FBS) containing cell culture media [5]. From a second WC powder, named WC_S, suspensions were prepared and characterized in a similar way to ensure comparable experimental conditions. Agglomeration behavior of WC_S particles was analyzed at a concentration of 30 μg/ml by dynamic light scattering (DLS) in RPMI medium with and without 5% FBS. Particle and particle suspension characteristics are summarized in Table 1.

2.2. Dissolution behavior and crystalline structure of WC_L, WC_L–Co and WC_S

Dissolution of metal ions from particle suspensions (assessed one week after preparation) was 6% of tungsten for WC_L and 15% of tungsten and 76% of cobalt for WC_L–Co [5]. For WC_S, 24% of tungsten was found to be in solution after one week [15]. Regarding the chemical structure, the two initial powders differed in crystalline phase. The WC_L and WC_L–Co are WC phase, whereas the WC_S consist of W_2C crystallites. Additionally, due to the preparation process, WC_S contains about 5% carbon black (Table 2).

2.3. Preparation of particle suspensions for cell culture experiments

For cell culture experiments, 300 (WC_L, WC_S) and 330 (WC_L–Co) μg/ml particle stock solutions with equal amounts of tungsten were prepared. WC_L was suspended in water, WC_S and WC_L–Co in sodium polyphosphate solution (Graham's salt; Merck, Darmstadt, Germany). Stock suspensions were sterilized by autoclaving. Prior to exposure of cells, suspensions were redispersed by treatment in an ultrasonic bath (RK 255 H, Bandelin, Berlin, Germany) for 10 min.

2.4. Preparation of cobalt chloride solutions

Cobalt chloride (Fluka/Sigma–Aldrich, Seelze, Germany) stock solution was prepared in distilled water at a concentration of 20 mM. The stock solution was sterilized by autoclaving. For measurement of ROS production, working solutions of 250, 500 and 1000 μM were prepared in cell culture grade water (PAA Laboratories GmbH, Pasching, Austria) under sterile conditions. For exposure of cells in the micronucleus assay, a 2 mM working solution was prepared by diluting the stock solution with cell culture grade water under sterile conditions.

2.5. Cell culture

Human keratinocyte cells, HaCaT (CLS – Cell Lines Service, Eppelheim, Germany) [18], were maintained in RPMI medium (Biochrom, Berlin, Germany) supplemented with 5% (v/v) FBS (Sigma Chemicals, St. Louis, USA) and 1% (v/v) penicillin/streptomycin (PAA, Pasching, Austria) in 75 cm^2 flasks (TPP, Trasadingen, Switzerland). HepG2, a human hepatocellular liver carcinoma cell line, was provided by the German Federal Environmental Agency (UBA, FG II, Tamara Grummt) and were maintained in RPMI medium supplemented with 2 mM glutamine (PAA, Pasching, Austria), 1% (v/v) penicillin/streptomycin and 10% (v/v) FBS in 75 cm^2 flasks. Both cell lines were cultured in monolayer at 37 °C in a humidified, 5% (v/v) CO_2 atmosphere with sub-cultivation twice a week. For sub-cultivation, cells were washed three times with Versene (Invitrogen, Karlsruhe, Germany) and detached by trypsin (0.25% (v/v) in phosphate-buffered saline (PBS) (Biowest,