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In vitro expression of hard metal dust (WC–Co) — responsive genes in human peripheral blood mononucleated cells

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Abstract

Hard metals consist of tungsten carbide (WC) and metallic cobalt (Co) particles and are important industrial materials produced for their extreme hardness and high wear resistance properties. While occupational exposure to metallic Co alone is apparently not associated with an increased risk of cancer, the WC–Co particle mixture was shown to be carcinogenic in exposed workers. The *in vitro* mutagenic/apoptogenic potential of WC–Co in human peripheral blood mononucleated cells was previously demonstrated by us. This study aimed at obtaining a broader view of the pathways responsible for WC–Co induced carcinogenicity, and in particular genotoxicity and apoptosis. We analyzed the profile of gene expression induced *in vitro* by WC–Co versus control (24 h treatment) in human PBMC and monocytes using microarrays. The most significantly up-regulated pathways for WC–Co treated PBMC were apoptosis and stress/defense response; the most down-regulated was immune response. For WC–Co treated monocytes the most significantly up- and down-regulated pathways were nucleosome/chromatin assembly and immune response respectively. Quantitative RT-PCR data for a selection of the most strongly modulated genes (*HMOX1*, *HSPA1A*, *HSPA1L*, *BNIP3*, *BNIP3L*, *ADORA2B*, *MT3*, *PLA2G7*, *TNFAIP6*), and some additionally chosen apoptosis related genes (*BCL2*, *BAX*, *FAS*, *FASL*, *TNFα*), confirmed the microarray data after WC–Co exposure and demonstrated limited differences between the Co-containing compounds. Overall, this study provides the first analysis of gene expression induced by the WC–Co mixture showing a large profile of gene modulation and giving a preliminary indication for a hypoxia mimicking environment induced by WC–Co exposure.

Keywords: Cobalt; Tungsten carbide; Hard metal; Gene expression; Apoptosis; Carcinogenesis

Introduction

Occupational exposure to cobalt-containing dust has been associated with pulmonary toxicity including bronchial asthma, fibrosing alveolitis and lung cancer. While bronchial asthma seems to be associated with many industrial applications, irrespective of the chemical or physical form of cobalt, fibrosing alveolitis (hard metal disease) and the development of lung cancer are especially associated with the hard metal industry where exposure is to a mixture of cobalt metal and carbide particles (Lison, 1996; Moulin et al., 1998). Hard metals consist of tungsten carbide and metallic cobalt particles, and are produced for their extreme hardness and wear resistance properties.

The carcinogenic potential of cobalt and cobalt compounds was first evaluated by IARC in 1990 (IARC, 1991). The working group concluded that cobalt and its compounds were *possibly carcinogenic to humans (Group 2B)*. In 2003, cobalt (with or without tungsten carbide) in hard metals and cobalt sulfate were reconsidered. Cobalt metal with tungsten carbide is now classified as *probably carcinogenic to humans (Group 2A)* on the basis of *limited evidence* in humans for an increased risk of lung cancer and *sufficient evidence* in experimental animals.

Abbreviations: ROS, reactive oxygen species; Co, metallic cobalt; $CoCl_2$, cobalt chloride; FCS, foetal calf serum; PBMC, peripheral blood mononucleated cells; PHA, phytohaemagglutinin; WC, tungsten carbide; WC–Co, tungsten carbide–cobalt or hard metal; qRT-PCR, quantitative reverse transcription PCR.

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Cobalt metal without tungsten carbide, cobalt sulfate and other soluble cobalt (II) salts are classified as *possibly carcinogenic to humans (Group 2B)* (IARC, 2006). However, the mechanisms responsible for cancer induction are not fully understood. In 1995, Lison et al. demonstrated, using a spin trapping system, that hard metal particles produce enhanced quantities of reactive oxygen species (ROS) as compared to cobalt metal alone. They proposed that, because of the particular surface characteristics of WC particles, when Co and WC particles are in close contact, electrons provided by cobalt are transferred to the surface of tungsten carbide particles, reduce ambient oxygen and generate ROS; cobalt metal is concomitantly oxidized and passes into solution (Co^{2+}). These ROS are highly reactive and known to alter DNA integrity, and may therefore account in part for the mechanisms of hard metal carcinogenicity. In addition, cobalt 2+ ions may bind to proteins involved in essential cellular processes such as DNA repair, cell cycle control and apoptosis (Hartwig, 2001). The higher *in vitro* mutagenic potential of the WC–Co mixture compared to its individual components was demonstrated by us (Anard et al., 1997; Van Goethem et al., 1997; De Boeck et al., 1998, 2003c); for review (Lison et al., 2001; De Boeck et al., 2003b) in human peripheral blood mononucleated cells (PBMC) suggesting that WC–Co can induce primary genotoxicity in lung epithelial cells. This was confirmed *in vivo* in rat type II pneumocytes after a single intra-tracheal exposure (De Boeck et al., 2003a). Whether the observed *in vivo* mutagenic effect was the result of primary genotoxicity only or also resulted from secondary genotoxicity caused by inflammation is unclear. Overall, simultaneous exposure to cobalt metal and tungsten carbide particles seems to be required to exert a mutagenic effect, which may contribute to its carcinogenicity.

Besides its mutagenic potential, we demonstrated in PBMC that WC–Co induces apoptosis, in a preferentially caspase-9 dependent pathway and in an additive manner when compared to its individual components. Early signs of apoptosis arise between 15′ and 6 h after WC–Co exposure (annexin-V positive staining) while massive apoptosis occurs at 24 h (DNA fragmentation assessed by Cell death ELISA). Apoptosis induced by cobalt metal and WC was shown to be triggered by caspase-8 and -9, respectively. WC-induced apoptosis was linked to its preferential uptake in monocytes as detected by transmission electron microscopy (Lombaert et al., 2004).

Vengellur and LaPres (2004), showed that HIF-1α is playing a major role in mediating cobalt chloride-induced apoptosis in mouse embryonic fibroblasts; this effect of cobalt chloride is part of its capacity to promote a hypoxia-like response. The authors suggested that this may offer a possible mechanism for lung pathologies resulting from occupational exposure to cobalt.

The present study aimed at obtaining a broader view of the pathways responsible for WC–Co induced carcinogenicity, and in particular genotoxicity and apoptosis.

Therefore, we used high density arrays to analyze the profile of gene expression induced *in vitro* by WC–Co versus control (24 h treatment) in human PBMC and monocytes using particle or cobalt doses that were previously shown to induce genotoxicity (Van Goethem et al., 1997; De Boeck et al., 1998) and apoptosis (Lombaert et al., 2004) in human PBMC *in vitro*. Selection of 24 h exposure was based on the *in vitro* data obtained by us on induction of genotoxicity and apoptosis in human PBMC after WC–Co exposure. It was found that the DNA damage observed with the Comet assay (maximum between 15′ and 6 h; at 15′ at a higher extent with WC–Co compared to Co and with a fold increase of 3 and 1.2 respectively) was completely restored after 24 h. $CoCl_2$ induced approximately the same extent of DNA damage as Co and WC had only a marginal effect on DNA migration (De Boeck et al., 1998). Induced chromosome breakage (MN assay), studied for a short exposure period (15′), lead to a fold increase of chromosome/genome mutation frequencies in the micronucleus test (% micronucleated binucleates (MNBN)) of 6.5; 4.49 and 2.47 after WC–Co, Co or WC treatment, respectively (De Boeck et al., 2003c). Moreover, as far as apoptosis (24 h exposure) is concerned, treatment with $CoCl_2$, Co, WC or WC–Co caused a fold increase of 2.1; 2.8; 1.9 and 3.7, respectively, indicating that the highest level of apoptosis was reached upon treatment with WC–Co and the lowest after WC (Lombaert et al., 2004). Therefore, investigation at 24h after WC–Co exposure would concentrate on cells which repaired the major part of the DNA breakage, have some transmissible chromosomal changes and survived the early apoptotic wave. This would allow to identify which genes are contributing to the massive apoptosis as well as the genes that are induced as part of the cellular response against the damage induced by WC–Co treatment.

The most significantly up-regulated pathways for WC–Co treated PBMC are apoptosis regulation and stress/defense response; the most down-regulated is immune response. In WC–Co treated monocytes, nucleosome/chromatin assembly and immune response are the most significantly up- and down-regulated pathways, respectively. We next studied in a time course by quantitative RT-PCR (qRT-PCR) the expression of a selection of the most strongly modulated genes, and some additionally chosen apoptosis-related genes, after *in vitro* exposure of PBMC to WC–Co and compared the responses with that induced by its individual particulate components (Co and WC) and the soluble cobalt cation ($CoCl_2$). These results confirm the microarray data on WC–Co exposure and demonstrate limited differences between the Co-containing compounds.

This study provides the first analysis of gene expression induced by the WC–Co particle mixture showing several affected genes, some of them indicative for a hypoxia mimicking environment.

Methods

Particles and chemicals. Extra fine cobalt metal (Co) was obtained from a cobalt refinery, 99.87% purity, median particle size (d_{50}) 4 μm. Tungsten carbide (WC) from Johnson Matthey (Royston, UK) 625655, 99.5% purity, $d_{50} < 1$ μm. A mixture of Co (6%) and WC (94%) particles was prepared in the laboratory (WC–Co). $CoCl_2$ was obtained from Sigma (St. Louis, MO). Ham's F10 medium and foetal calf serum (FCS) were purchased from Life Technologies (Paisley, UK). Phytohemagglutinin A 16 (PHA) was obtained from Murex Biotech Ltd. (Dartford, UK).

To compare the different cobalt containing preparations ($CoCl_2$, Co, WC–Co) and WC, the doses used always contained equivalent concentrations of cobalt (6.0 μg/ml Co-equivalent). 6 μg of Co alone and 24 μg of $CoCl_2$ is thus equivalent to 100 μg WC–Co mixture and 100 μg WC was considered approximately as the