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Molecular basis of carcinogenicity of tungsten alloy particles

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ABSTRACT

The tungsten alloy of 91% tungsten, 6% nickel and 3% cobalt (WNC 91–6–3) induces rhabdomyosarcoma when implanted into a rat thigh muscle. To investigate whether this effect is species-specific human HSkMc primary muscle cells were exposed to WNC 91–6–3 particles and responses were compared with those from a rat skeletal muscle cell line (L6-C11). Toxicity was assessed by the adenylate kinase assay and microscopy, DNA damage by the Comet assay. Caspase 3 enzyme activity was measured and oligonucleotide microarrays were used for transcriptional profiling. WNC 91–6–3 particles caused toxicity in cells adjacent to the particles and also increased DNA strand breaks. Inhibition of caspase 3 by WNC 91–6–3 occurred in rat but not in human cells. In both rat and human cells, the transcriptional response to WNC 91–6–3 showed repression of transcripts encoding muscle-specific proteins with induction of glycolysis, hypoxia, stress responses and transcripts associated with DNA damage and cell death. In human cells, genes encoding metallothioneins were also induced, together with genes related to angiogenesis, dysregulation of apoptosis and proliferation consistent with pre-neoplastic changes. An alloy containing iron, WNF 97–2–1, which is non-carcinogenic *in vivo* in rats, did not show these transcriptional changes *in vitro* in either species while the corresponding cobalt-containing alloy, WNC 97–2–1 elicited similar responses to WNC 91–6–3. Tungsten alloys containing both nickel and cobalt therefore have the potential to be carcinogenic in man and *in vitro* assays coupled with transcriptomics can be used to identify alloys, which may lead to tumour formation, by dysregulation of biochemical processes.

Background

Alloys of tungsten have been developed as potentially safer alternatives to depleted uranium in the manufacture of armour-piercing munitions. These materials have many attractive characteristics for this purpose including the fact that they are both dense and tough and their physical properties can be tuned by modifying their elemental composition (Gero et al., 2001). Due to the high melting point of tungsten, these alloys are manufactured by mixing a fine tungsten powder with small quantities (typically less than 10% w/w) of other metal powders, such as cobalt, copper, iron and nickel. The mixture is heated so that the other metals melt and some of the tungsten powder dissolves in the liquid alloy so that when the mixture cools it forms a composite of the residues of the tungsten particles bound together by an alloy enriched in the non-tungsten constituents (Gero et al., 2001). Fig. 1 shows the structure and composition of the alloy WNC-91–6–3 which is comprised of 91% W, 6% Ni and 3% Co by mass. The composition of the binding alloy is actually 49% W; 34% Ni; and 17% Co when expressed by weight (w/w) and 23.5% W; 51% Ni; and 25.5% Co when expressed by molarity. Because of this, there are safety concerns about these materials since it has long been established that metallic Ni and Co, in both powder and pellet form, are carcinogenic when implanted into rat muscles (Heath and Webb, 1967; Kalinich et al., 2005). Furthermore, WNC 91–6–3 itself has also been investigated and, when implanted into the thigh muscles of F344 rats all animals developed aggressive rhabdomyosarcomas that metastasized to the lung within 4 to 5 months (Kalinich et al., 2005). It is believed that the mechanism of toxicity of these materials is related to preferential dissolution of the binding alloy into biological fluids resulting in the release of high concentrations of nickel and cobalt ions, which are detectable in urine of rats following implantation with pellets of alloy WNC 91–6–3 (Kalinich et al., 2008; Schuster et al., 2012). WNF 97–2–1 pellets in contrast do not contain cobalt and also display far less corrosion in rat muscles, leading to lower concentrations of metals detectable in urine and no elevated cancer rate (Schuster et al., 2012).

There is a clear need to develop robust *in vitro* screening tests to identify potentially carcinogenic alloys thereby reducing the need for expensive *in vivo* testing. We have previously described the development of a rapid *in vitro* exposure model capable of differentiating between carcinogenic and non-carcinogenic alloys using a cultured rat skeletal muscle cell line (L6-C11). On dissolution, particles of WNC-91–6–3 were found to generate reactive oxygen species, killing cells in their immediate vicinity, and induce DNA damage in the bulk population of cells in culture. Further mechanistic studies also showed that WNC 91–6–3 inhibited caspase 3 activity and altered gene expression in a manner consistent with carcinogenesis (Harris et al., 2011). The aim of the current study was to further assess the likely hazard to

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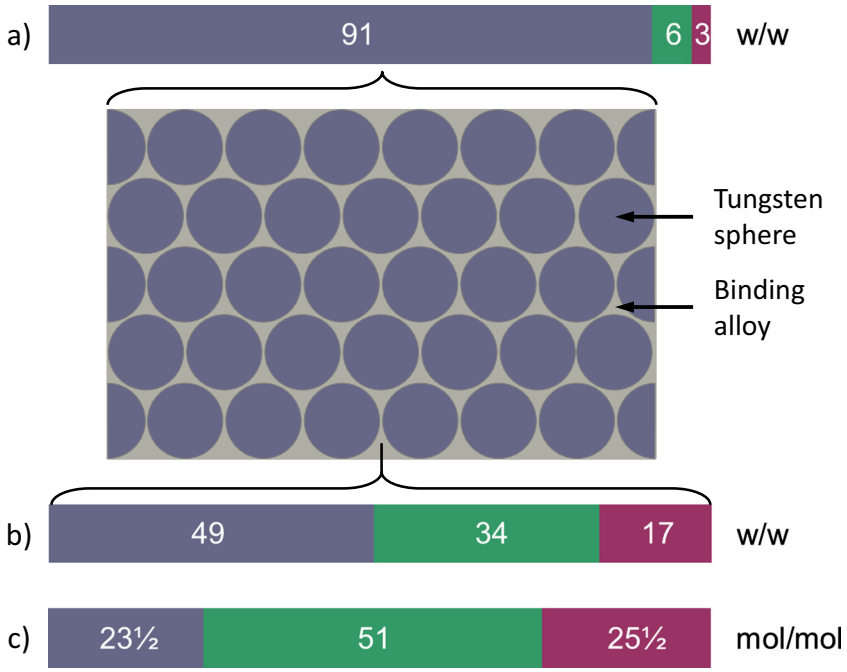

Fig. 1. Structure and composition of tungsten–nickel–cobalt alloy 91–6–3. Percentage composition of (a) entire alloy by weight, (b) and (c) binding alloy by weight and molarity respectively. Key: Tungsten, mid grey; nickel, white; cobalt, black; and binding alloy, light grey.

human health of these materials by investigating whether human primary skeletal muscle cells (HSkMC) respond similarly to the previously studied rat cell line, and whether the properties of particles change upon long term storage, thereby altering their toxicity in a cell culture system.

Results

Behaviour of particles in cell culture

The overall viability of HSkMC and L6-C11 cultures treated with alloy particles was assessed using the Toxilight® adenylate kinase assay (Lonza), revealing no significant increase in adenylate kinase release into the media. Visual analysis by microscopy, however, showed that treatment with all types and ages of WNC particles resulted in the formation of regions of dead and apoptotic cells around the particles (Fig. 2). In contrast, no damage was apparent in response to tantalum or WNF 97–2–1 particles. Unlike the L6-C11 cells (Harris et al., 2011), it was not possible to maintain the differentiated HSkMC cells in culture for long enough to follow the process until particle disintegration occurred.

Induction of DNA strand breaks by WNC particles

DNA strand breaks were determined by the Comet assay. Treatment of HSkMC cells with WNC 97–2–1 statistically significantly elevated DNA strand breaks compared with untreated controls (Fig. 3A). Similar results were obtained for WNC 91–6–3 particles that had been stored for 7 or 67 months (Fig. 3B) but tantalum or WNF 97–2–1 exposures resulted in no significant changes. However, treatment with WNC 91–6–3 particles that had been stored for 39 months did not significantly alter DNA strand breaks (Fig. 3A). Similarly, when rat L6-C11 cells were exposed to the 39-month old particles no significant induction of DNA strand breaks was observed (data not shown).

Inhibition of caspase 3 by WNC particles

Caspase 3 enzyme activity was not significantly altered in human HSkMC cells following treatment with any of the particles investigated (Figs. 4 A, B). In rat L6-C11 cells however, caspase 3 activity was significantly reduced following treatment with WNC 91–6–3 particles. Furthermore, the newest particle preparation WNC 91–6–3 (7 months) elicited a significantly lower caspase 3 activity in the rat L6-C11 cells than the oldest particle preparation WNC 91–6–3 (67 months) (Fig. 4).

Transcriptional changes following treatment with WNC particles

Two independent transcriptomic experiments were carried out using microarrays and analysed separately. Lists of differentially expressed transcripts were generated by finding those statistically significantly (FDR < 0.05) altered in expression *versus* the relevant untreated control group, and restricting these lists to only those transcripts displaying a 1.3-fold or higher fold change *versus* the controls. The fold change cut-off was performed in order to focus on those transcriptional changes that were more likely to be biologically significant. The numbers of differentially expressed transcripts discovered under these criteria are shown in Table 1. The tantalum control treatment resulted in differential expression of 776 transcripts in the first experiment, but this was not confirmed in the second experiment, where no significant changes were detected. WNF 97–2–1 led to minimal transcriptional changes. WNC 97–2–1 led to 827 transcriptional changes, 91% of which were also detected in one or more WNC 91–6–3 treated groups (Fig. 5A). WNC 91–6–3 exposures led to very extensive transcriptional changes (Table 1), 87% of the transcripts identified as altered after WNC 91–6–3 (39) exposure were also altered by WNC 91–6–3 (7) or WNC 91–6–3 (67) exposure (Fig. 5B). The second transcriptomic experiment appeared more sensitive, detecting additional changes in response to WNC 91–6–3. There were no significant differences in transcript expression between WNC 91–6–3 (7) and WNC 91–6–3 (67) but there was a general trend towards a greater magnitude of