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Potential Late Health Effects of Depleted Uranium and Tungsten Used in Armor-Piercing Munitions: Comparison of Neoplastic Transformation and Genotoxicity with the Known Carcinogen Nickel

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Limited data exist to permit an accurate assessment of risks for carcinogenesis and mutagenesis from embedded fragments or inhaled particulates of depleted uranium (DU). Ongoing studies have been designed to provide information about the carcinogenic potential of DU using in vitro and in vivo assessments of morphological transformation as well as cytogenetic, mutagenic, and oncogenic effects. For comparison, we also examined tungsten alloys used in military projectiles and the known carcinogen nickel. Quantitative and qualitative in vitro transformation studies were done to assess the carcinogenic potential of radiation and chemical hazards. Using a human osteosarcoma cell model, we demonstrated that soluble and insoluble DU compounds can transform cells to the tumorigenic phenotype, as characterized by morphological, biochemical, and oncogenic changes consistent with tumor cell behavior. Tungsten alloys and nickel were also shown to be neoplastic transforming agents, although at a frequency less than that of DU. Sister chromatid exchange, micronuclei, and alkaline filter elution assays showed DU and tungsten alloys were genotoxic. Exposure to a nontoxic, nontransforming dose of DU induced a small but statistically significant increase in the number of dicentrics formed in cells. These results suggest that long-term exposure to DU or tungsten alloys could be critical to the development of neoplastic disease in humans and that additional studies are needed.

Introduction

The first widespread combat use of depleted uranium (DU) munitions occurred in the Gulf War. Several friendly fire incidents resulted in casualties with DU fragment injuries. Following long-established fragment removal policies, the fragments were not removed unless they presented a known immediate or long-term threat to the health of the wounded individual. However, little data exist to assess whether there are long-term health consequences of leaving fragments of unusual metals like DU in place. Our laboratory has developed a human cell culture model that is applicable for transformation, mutagenicity, and genotoxicity studies. These immortalized, nontumorigenic human osteosarcoma (HOS) cells have been widely used to assess the potential carcinogenicity of soluble metal salts (e.g., nickel, chromium, lead)[1,2] and of other compounds.[3,4] Previously, we demonstrated that soluble DU-uranyl chloride could transform HOS cells to a tumorigenic phenotype.[5] Therefore, we chose to use HOS cells to examine the transforming and genotoxic potential of an insoluble DU compound, DU-UO_2. DU transformants were selected, cloned, and analyzed for oncogene and tumor suppressor alterations. Cytogenetic assessments include micronuclei induction, sister chromatid exchange (SCE), DNA strand break analysis, and dicentric formation. Crystalline nickel, a known carcinogen and transforming agent in vitro, was used for comparison.

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Materials and Methods

Cell Lines and Culture

HOS cell lines (TE85, clone F-5) were obtained from the American Type Culture Collection (Manassas, VA). Cell cultures were propagated as previously detailed.[4,5] Cells were tested for *Mycoplasma* using a MycoTect kit (Sigma-Aldrich, St. Louis, MO), and only cells negative for *Mycoplasma* were used.

Metal Powders

DU-UO_2 was obtained from Sigma-Aldrich. The various tungsten alloys used in military applications have the following range of weight percentages: tungsten (W), 91 to 93%; nickel (Ni), 3 to 5%; and cobalt (Co) 2 to 4%.[6] These alloys are not commercially available, so we simulated them with a mixture of commercially available fine powders. The following powders were used to prepare the mixtures: (1) extra-fine Co metal (Alfa Aesar 10455, 99.5% pure), median particle size, d_{50} 1 to 4 μM; (2) extra-fine Ni metal (Alfa Aesar 10256, 99% pure), median particle size, d_{50} 3 to 5 μM; (3) extra-fine W metal (Alfa Aesar 10400, 99.9% pure), median particle size, d_{50} 1 to 3 μM. For cell exposures, we selected a representative mixture consisting of 92% W, 5% Ni, and 3% Co (hereafter referred to as rWNiCo). All stock solutions and metal suspensions were freshly prepared in ultrapure, sterile water. The suspensions were carefully mixed and dispersed before adding to the cells.

Microdosimetry

The term "specific energy" is used rather than "dose" because dose refers to macroscopic averages, whereas specific energy is a microdosimetric equivalent or a dose to a single cell. Microdosimetric methods using Monte Carlo computer simulations were applied as previously detailed.[5]

Colony Formation, Transformation, Soft Agar Clonability, and Saturation Density Assay Studies

Exponentially growing cells were incubated with the metal powder for 24 hours and then processed and incubated as

previously described for each type of assay.[4,5] For all assays, data are representative of three independent experiments.

Cytogenetic Assays

A detailed description of the micronuclei induction, SCE, and DNA single-strand break elution assays have been previously published by our laboratory.[4,5] Dicentrics were analyzed according to the procedures of Martin et al.[7]

Results and Discussion

Transformation of HOS Cells by DU-UO_2 or rWiNiCo: Comparison to Crystalline Ni

To assess morphological cell transformation, the standard focus formation assay[3-5] was used. HOS cells were exposed to insoluble DU-UO_2, rWNiCo, or Ni for 24 hours. Microdosimetric calculations showed that a single cell traversed by one alpha particle received a radiation dose of 27 cGy and that 8.5% of the cells received a single traversal (none received more than one). Following exposure to DU or rWNiCo, a morphological change in HOS cells was observed that was consistent with transformed morphology previously observed when these cells were exposed to the known carcinogen *N'*-methyl-*N'*-nitro-*N*-nitrosoguanidine or to soluble DU.[3] Untreated HOS cells exhibit a flat, epithelial-like morphology and appear to grow in a monolayer. In contrast, after treatment with DU or rWNiCo and weekly changes of nutrient medium for 5 weeks, diffused type II foci appeared.[3-5]

Table I shows transformation frequencies (normalized per surviving cell) for HOS cells treated with DU-UO_2 (10 mg/mL) or rWNiCo (10 mg/mL). The data demonstrate that treatment with DU-UO_2 resulted in a 25.5 (±2.8)-fold increase in transformation frequency compared with untreated HOS cells. Exposure to rWNiCo induced a 9.5 (±0.9)-fold increase in transformation. The positive control, crystalline Ni, which has been shown previously to be a transforming agent,[1] induced a 7.1 (±2.1)-fold increase in transformation frequency. Several foci were chosen by cloning cylinders and expanded by mass culture to establish transformed clonal lines, which were used to further characterize the transformation induced by these heavy metals. Alteration in growth control is critical to neoplastic transformation; so metal-transformed clones were further characterized by assessing differences in growth properties associated with the neoplastic phenotype, such as saturation density and soft colony-forming efficiency. Additionally, to determine whether cells transformed in vitro by these heavy metals were capable of producing tumors in immunosuppressed mice, the nude mouse assay was used to test the tumorigenicity of the heavy metal-transformed cells.

Table I shows that the saturation densities and anchorage-independent growth-plating efficiencies of DU- and rWNiCo-transformed cells were significantly higher than those of the parental HOS cells. Inoculation of athymic nude mice with DU or rWNiCo transformants resulted in the development of animal tumors within 4 weeks. Similarly, nude mice inoculated with Ni transformants also formed tumors (Table I). In contrast, parental HOS cells injected into nude mice did not form any tumors over a period of 6 months after cell inoculation.

Histological analysis indicated that the tumors formed by DU-transformed cells resemble a carcinoma characterized by undifferentiated sheet-like growth. Tumors were reestablished in tissue culture and confirmed as human; their resemblance to the cells of origin was determined by karyological analysis (data not shown). These data demonstrate (1) that cellular exposure to insoluble DU or rWNiCo can transform human cells into the tumorigenic phenotype and (2) that this transformation is similar to that observed for nickel, a known carcinogen.

Cytogenetic Assessment

Several in vitro cytogenetic analyses were performed on HOS cells exposed to these heavy metals to determine whether these cellular exposures are genotoxic. Table II shows data obtained from studies examining micronuclei induction, SCE levels, DNA single-strand breaks, and dicentric formation following exposure to DU-UO_2 (5 mg/mL per 24 hours), rWNiCo (5 mg/mL per 24 hours), or crystalline Ni (5 mg/mL per 24 hours). Data are reported as a percentage of the levels found in untreated HOS cells. The induction of micronuclei in binucleate cells, SCE/cell, and DNA single-strand breaks was significantly elevated (158–810%) in DU- and rWNiCo-treated cells. The difference between metal-treated and control cells was highly significant in all cases ($p < 0.001$). Similarly, Ni exposure resulted in a significant increase in these three cytogenetic markers (150–559%).

These data demonstrate for the first time that a DU compound is genotoxic in vitro. Similarly, our data show that rWNiCo is

TABLE I

METAL-INDUCED TRANSFORMATION: BIOLOGICAL PROPERTIES OF TRANSFORMED HUMAN OSTEOSARCOMA CELLS

	Untreated	Depleted Uranium[a]	rWNiCo[a] (Insoluble)	Nickel[a]
Transformation[b] frequency (per survivor × 10^{-4} ± SD)	4.4 ± 1.1	110.2 ± 10.1	41.6 ± 5.1	39.5 ± 3.1
Morphology	Flat	Transformed	Transformed	Transformed
Saturation density (×10^5 cells)	2.6	6.6	6.1	7.1
Soft-agar colony formation (plating efficiency %)	2	61	29	28
Tumorigenicity[c] (mice with tumors/mice inoculated)	0/82	14/20	8/20	6/20

[a]Metal concentrations were all 10 μg/mL, and the exposure time was 24 hours. This exposure resulted in a range of survival levels from 99.5 ± 4.2 to 91.6 ± 5.9 for all metals tested and for untreated controls.

[b]The transformation data illustrated are from three independent experiments. The average number of transformed foci per dish was computed from the proportion of dishes free of transformed colonies, f, by $\lambda = -\ln f$. Transformation frequency = λ/number of surviving cells per dish. The number of surviving cells per dish was corrected for plating efficiency of untreated cells.

[c]Animals were injected with 5×10^{-6} cells in sterile phosphate-buffered saline. Animals were examined three times a week for tumors. After tumor palpation, animals were examined five times a week until the animals were euthanized (tumor volume approximately 90 mm^3). Tumors were histologically identified as adenocarcinomas.

TABLE II

HEAVY METAL-INDUCED GENOTOXICITY: GENOTOXIC EFFECTS IN HOS CELLS EXPOSED TO HEAVY METALS

	Percent of Untreated Levels		
	Depleted Uranium (5 mg/mL)	rWNiCo (5 mg/mL)	Nickel (5 mg/mL)
Micronuclei Induction[a]	210 ± 15	158 ± 8.5	153 ± 8.0
SCE	242 ± 18.9	172 ± 11.2	150 ± 10.4
Alkaline filter elution (DNA strand break)	810 ± 40.1	723 ± 39.5	559 ± 19.4
Dicentric formation	338 ± 14.2	0	0

[a]Three to five replicates were scored for all assays.

also genotoxic. Based on these data, we can speculate that the DNA damage caused by exposure to DU or rWNiCo may be involved in the mechanism of the transformation induced by these metals in vitro. The contribution of other mechanisms such as metal-induced reactive oxygen species and epigenetic factors (DNA repair) may also be essential to DU- or rWNiCo-induced transformation. Further studies are necessary to fully characterize the mechanisms of DU- and rWNiCo-induced transformation.

In an attempt to characterize the contribution of the radiation vs. that of the chemical components in the transformation induced by DU, dicentric chromosomal aberrations were also measured in heavy metal-exposed cells. The data demonstrate that DU exposure resulted in a small but significant increase in dicentric frequency compared with that of untreated parental HOS cells. Human cells exposed to nickel did not exhibit any increase in dicentric formation above control levels. Current studies in our laboratory are examining the frequency of dicentrics in HOS cells following alpha-particle exposure. These data are the first to demonstrate that cellular DU exposure can result in the formation of dicentrics. A previously published report detailed findings that low-dose (~25 cGy) high linear energy transfer radiation can induce a fivefold increase in dicentrics.[8] Therefore, induction of this type of chromosomal aberration suggests that the radiological component of DU exposure may play a role in its ability to induce both DNA damage and neoplastic transformation.

Conclusion

The results of this study show that insoluble DU and rWNiCo can transform human cells to the tumorigenic phenotype. The mechanism of this transformation involves induction of DNA and chromosomal damage. The chromosomal aberrations seen with DU exposure are distinctly characteristic of radiation exposure, suggesting that the alpha-particle component may play a role in the transformation and genotoxic processes. Further studies are warranted to fully understand the potential late health effects caused by exposure to DU or tungsten alloys used in armor-piercing munitions.

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