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Effects of ascorbic acid on carcinogenicity and acute toxicity of nickel subsulfide, and on tumor transplants growth in gulonolactone oxidase knock-out mice and wild-type C57BL mice

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ABSTRACT

The aim of this study was to test a hypothesis that ascorbate depletion could enhance carcinogenicity and acute toxicity of nickel. Homozygous *L-gulono-<gamma>-lactone oxidase gene* knock-out mice (Gulo−/− mice) unable to produce ascorbate and wild-type C57BL mice (WT mice) were injected intramuscularly with carcinogenic nickel subsulfide (Ni_3S_2), and observed for the development of injection site tumors for 57 weeks. Small pieces of one of the induced tumors were transplanted subcutaneously into separate groups of Gulo−/− and WT mice and the growth of these tumors was measured for up to 3 months. The two strains of mice differed significantly with regard to (1) Ni_3S_2 carcinogenesis: Gulo−/− mice were 40% *more* susceptible than WT mice; and (2) transplanted tumors development: Gulo−/− mice were more receptive to tumor growth than WT mice, but only in terms of a much shorter tumor latency; later in the exponential phase of growth, the growth rates were the same. And, with adequate ascorbate supplementation, the two strains were equally susceptible to acute toxicity of Ni_3S_2. Statistically significant effects of dietary ascorbate dosing levels were the following: (1) reduction in ascorbate supplementation increased acute toxicity of Ni_3S_2 in Gulo−/− mice; (2) ascorbate supplementation extended the latency of transplanted tumors in WT mice. In conclusion, the lack of endogenous ascorbate synthesis makes Gulo−/− mice more susceptible to Ni_3S_2 carcinogenesis. Dietary ascorbate tends to attenuate acute toxicity of Ni_3S_2 and to extend the latency of transplanted tumors. The latter effects may be of practical importance to humans and thus deserve further studies.

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Introduction

The idea that ascorbic acid confers resistance to neoplasia was introduced by Linus Pauling in 1970-s following the findings of low reserves of ascorbate in cancer patients and beneficial effects of ascorbate supplementation on their survival (Cameron et al., 1979). It has been known that carcinogenic transition metals, including nickel, can catalyze ascorbate destruction (Buettner and Jurkiewicz, 1996; Kaczmarek et al., 2007). Would this effect contribute to the mechanisms of nickel-induced carcinogenesis? In our previous publications (Salnikow and Kasprzak, 2005; Salnikow et al., 2004), we hypothesized that ascorbate depletion could, indeed, be a critical step in nickel carcinogenesis. The depletion is important because of the key role ascorbate plays in maintaining the enzymatic activity of specific non-heme dioxygenases that are responsible for DNA repair, gene expression regulation, collagen assembly, and the inhibition of hypoxic stress (a hallmark of cancer) (Arita and Costa, 2009; Salnikow and Kasprzak, 2005). Ascorbate depletion should also weaken the antioxidant cellular guard against metal-induced oxidative DNA, lipid and protein damage that is thought to play a mechanistic role in carcinogenesis (Bal et al., 2011; Kasprzak, 2011). The aim of the present study was to test the above hypothesis on muscle carcinogenesis induced by nickel subsulfide (Ni_3S_2).

Unlike humans the laboratory rats and mice, on which most of the nickel (and other metals) carcinogenicity has been studied thus far, synthesize ascorbate endogenously. It was thus impossible to design a study in which their tissue ascorbate level was changed following metal exposure. In this study, to achieve our research goal, we decided to use mice with inactivated *L-gulono-<gamma>-lactone oxidase gene* (Gulo−/− mice), which makes them unable to synthesize ascorbate.

Abbreviations: Gulo−/− mice, L-gulono-γ-lactone oxidase knock-out mice; WT, wild type; i.m., intramuscular injections.

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This allowed us to control dietary ascorbate uptake, as well as to evaluate changes in tissue ascorbate in nickel exposed animals. As a reference for comparison we used C57BL mice from which the Gulo−/− mice have been derived (Maeda et al., 2000).

Materials and methods

Animals. This study was conducted in compliance with the Intramural Animal Care and Use (ACU) program of the National Institutes of Health. The homozygous L-gulono-γ-lactone oxidase knock-out mice (Gulo−/− mice) and wild-type C57BL/6 background mice (WT mice) were derived from heterozygous BL6.129P2-*Gulo*$^{tm1/Unc}$/Ucd mice by in-house breeding at animal facilities of the Laboratory Animal Science Program, SAIC — Frederick (NCI at Frederick, Frederick, MD). The heterozygous breeders were purchased from the University of California Center for Comparative Medicine (MMRRC) at Davis, CA. All WT mice were kept on an ascorbic acid-free diet, PicoLab Mouse Diet 20/5058 (Quality Lab Products, Inc., Elkridge, MD) and HCl-acidified drinking water (pH 4). The Gulo−/− mice were offered 330 mg/L of ascorbic acid (Sigma-Aldrich, St. Louis, MO)-containing water starting at weaning at 3 weeks of age (full supplementation; Maeda et al., 2000; Telang et al., 2007). Some experimental groups were kept on reduced, 100 mg/L, ascorbic acid supplementation.

Genotyping was performed as described by Maeda et al. (2000), using three PCR primers: P2 (5′-CGCGCCTTAATTAAGGATCC-3′), P3 (5′-GTCGTGACAGAATGTCTTGC-3′), and P4 (5′-GCATCCCAGTGAC-TAAGGAT-3′).

The amplification conditions were the following: initial DNA denaturation 94 °C for 3 min; 35 cycles of 30 s denaturing at 94 °C, 30 s. annealing at 58 °C, 45 s extension at 72 °C and 8 min final extension at 72 °C. All experiments were performed on 6–10 weeks old male mice.

Treatments. For the carcinogenicity tests, the mice, divided randomly into groups of 15–22 animals received single intramuscular (i.m.) injections of 2.0 or 2.5 mg/site of Ni_3S_2 powder, 8.3 μm geometric mean particle size (volume-based; determined by Elzone analyzer), suspended in 50% aqueous glycerol, 0.05 mL/site, into the thigh musculature of both hind limbs, using gauge 25 needle. The control mice received 0.05 mL/site of the injection vehicle alone. The Ni_3S_2 powder was a generous gift of Dr. A. Oller (NiPERA, Durham, NC).

The mice were examined weekly by palpation for tumor development, starting at week 24 post-injection. They were sacrificed with CO_2 when a local tumor reached approximately 1 cm in diameter, or at termination of the bioassay at week 57.

The effect of ascorbate on acute toxicity of Ni_3S_2-treated mice was assessed based on one-week mortality data from the two carcinogenicity experiments and an additional short-term test in which five groups of 18 mice were injected with 2.5 mg Ni_3S_2/site as in the carcinogenicity experiments and observed for one week. Kidneys of mice dying after nickel exposure were fixed in 10% formalin for microscopic examination.

To check the effect of ascorbic acid on transplanted tumor growth, two groups of six Gulo−/− plus two groups of five WT mice each, supplemented with different levels of ascorbic acid (as in the carcinogenesis experiment) were grafted subcutaneously on both flanks with 2–3 mm chunks of a muscle tumor induced with Ni_3S_2 in a Gulo−/− mouse kept on 330 mg/L of ascorbate in drinking water. The tumor to be transplanted, approximately 1 cm in size, free of necrosis, was collected at sacrifice and minced with scissors in normal saline. The transplants were inserted with fine forceps, randomly, under ether anesthesia, through 5 mm incisions, which were closed with metal clips (removed after 3 days). The growing tumors were measured in weekly intervals for up to 3 month post-transplantation.

To determine tissue levels of ascorbic acid in blood, kidneys, lungs, and muscles, groups of 12–14 Gulo−/− and WT mice were injected i.m. with 2.0 mg doses of Ni_3S_2 in the same way as described for the carcinogenicity bioassays. For analysis, surviving mice were sacrificed with CO_2 and autopsied 1 or 7 days after the injection.

Ascorbic acid analysis. Tissue ascorbate levels were measured using Lykkesfeldt's procedure (Lykkesfeldt, 2000) with HPLC separation and electrochemical detection as described elsewhere (Kaczmarek et al., 2007).

Statistical evaluation. The results of the carcinogenicity bioassay were assessed by testing the end point results (final survival populations) with Fisher's exact test (Enderlein, 1987). The results of the Ni_3S_2 acute toxicity testing were evaluated statistically using a number of non-parametric analyses. The possibility of pooling the results of analogous groups from different experiments was examined using an ANOVA table with a Kruskal–Wallis non-parametric test (Hollander and Wolfe, 1999).

The transplanted tumors growth was evaluated considering four possible growth profiles. In profile 1, the tumors were considered to begin growing immediately after grafting in a linear progression. In profile 2, the tumors began to grow immediately in an exponential progression. In the third and forth profiles, an initial delay in the tumor growth (incubation period) followed by either a linear or exponential growth progression were considered. Different detailed descriptions of the transplanted tumor growth were unified by noting that there was a delay followed by a growth rate.

The results of the growth delay and growth rate parameters from non-linear regression analysis of each tumor were used to search for a linear relationship between the tumor growth and mouse flank. A correlation analysis showed the extent of the tumor growth dependence on individual mice.

An ANOVA analysis of the non-linear regression analysis results was used to search for significant differences in the rate and delay of tumor growth for the four mouse groups (Hogg and Ledolter, 1987).

A global nonlinear regression was used to look for differences in tumor growth delay among the four mouse groups (Seber and Wild, 1989). Significance of differences in tissue ascorbate levels was established using Student's *t*-test.

Results

Carcinogenesis

The survival data and incidence of tumors in the carcinogenesis bioassays are presented in Tables 1 and 2. All the injection site tumors developed only in one leg. They readily invaded the adjacent muscles and bone marrow. The tumors grew at uneven rates, reaching the terminal size of 1 cm in 4 to 8 weeks from the time of detection by palpation. The time of appearance of the first tumor (latent period) in each group varied from 30 to 40 weeks after injections, and the final tumor incidence range varied from 11–67% of mice that survived until the occurrence of the first tumor in this study (30 weeks). Control mice developed no tumors. Histologically, all the injection site tumors were fibrosarcomas. No other tumors, either primary or metastatic, were found.

The observed differences in the development of the injection site tumors appeared to depend on the treatments and/or mouse strain, but due to the high initial mortality among Ni_3S_2-treated mice (see below) the number of induced tumors was small and the real importance of these differences had to be confirmed by statistical means. The end point statistical analysis, using Fisher's exact test, considered mice that survived past the acute stage of nickel toxicity, i.e., long enough to develop tumors. This analysis disclosed that the differences in tumor development between Gulo−/−, 100 mice and Gulo−/−, 330 mice and between WT and WT 330 mice in each experiment taken separately were not significant. Likewise non-