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Evaluation of Resveratrol and *N*-acetylcysteine for Cancer Chemoprevention in a Fanconi Anemia Murine Model

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Abstract

Fanconi anemia patients suffer from progressive bone marrow failure and often develop cancers. Previous studies showed that antioxidants tempol and resveratrol delayed tumor onset and reduce hematologic defects in Fanconi anemia murine models, respectively. Here we tested whether antioxidants *N*-acetylcysteine or resveratrol could delay cancer in tumor prone *Fancd2*^{-/-}/*Trp53*^{+/-} mice. Unlike tempol, neither compound had any significant chemopreventive effect in this model. We conclude that not all anti-oxidants are chemopreventive in Fanconi anemia. In addition, when given to *Fancd2*^{-/-} mice, *N*-acetylcysteine helped maintain *Fancd2*^{-/-} KSL cells in quiescence while tempol did not. The mechanisms behind the different actions of these antioxidants await further investigation.

Keywords

Chemoprevention; Fanconi Anemia; Antioxidants; Resveratrol; *N*-acetylcysteine

Introduction

Fanconi anemia (FA) is an autosomal recessive genetic disorder characterized by congenital malformations, progressive bone marrow failure, and cancer susceptibility. FA is caused by the disruption of FA-BRCA pathway and is genetically heterogeneous, with at least 16 complementation groups and genes identified so far [1]. Mortality from non-hematologic cancers is very high even in FA patients who have undergone successful bone marrow transplantation [2]. For this reason, the search for new small molecules that can delay or prevent cancer in FA is urgent.

There are multiple murine models of FA available [3]. Among them, *Fancd2*^{-/-} mice, *Fancp*^{-/-} mice, and *Fancc-Fancg* double knockout mice resemble most of the FA patient phenotypes. *Fancd2*^{-/-} mice under 129 background recapitulate the characteristic tumor susceptibility and hematopoietic defects of FA [4,5]. Prenatal lethality and premature death are notably milder in this strain, allowing the generation of large amounts of animals for drug screening. Heterozygosity for p53 accelerates the onset of epithelial tumors in

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Fancd2^{-/-} mice and makes *Fancd2*^{-/-}/*Trp53*^{+/-} mice an ideal model for testing chemoprevention regimes in FA [6].

FA is associated with elevated oxidative stress [7]. Previous animal tests using *Fancd2*^{-/-}/*Trp53*^{+/-} mice demonstrated that oral administration of the superoxide dismutase mimetic antioxidant tempol (4-hydroxy-2,2,6,6-tetramethyl piperidine-*N*-oxyl) reduced oxidative DNA damage and significantly delayed the onset of epithelial cancers [8]. We also found that antioxidant resveratrol (RV; 3,5, 4'-trihydroxyl-trans-stilbene) improved the hematopoiesis in FA mice [5]. Interestingly, it has been reported that resveratrol has cancer chemopreventive potential in some settings [9]. These observations suggest that RV might be beneficial in cancer chemoprevention as well as bone marrow function in FA. Another antioxidant *N*-acetylcysteine (NAC), which has long been hailed as a promising cancer chemopreventive agent [10], was recently reported to alleviate hematopoietic defects in some animal tests [11–13]. We therefore set to further explore the therapeutic potential of these clinically relevant antioxidants for tumor chemoprevention and hematopoiesis enhancement in Fanconi anemia and report the results here.

Methods

Mice

All *Fancd2* or *Trp53* mice were maintained on the 129S4 background. Drug treatment started from weaning (3–4 weeks) and lasted until the end of the study. *N*-acetylcysteine (Sigma) was administered through drinking water (1 mg/ml, protected from the light) and freshly made every week. Resveratrol (Orchid Chemicals & Pharmaceuticals Ltd, India) was added to common rodent chow at 2g/kg diet (Bio-Serv, New Jersey). Tempol (Sigma) was mixed with bacon-flavored mouse chow at the dose of 11g/kg diet (Bioserv), as described previously [8]. All animals were treated in accordance with the guidelines of the Institutional Animals Care and Use Committee at OHSU.

Histopathology

Tumor tissues along with liver, spleen, thymus, ovary and uterus samples were fixed in 10% phosphate-buffered formalin and stained with hematoxylin and eosin as reported previously [8].

Flow cytometry

The frequencies of cKit⁺Sca1⁺Lin⁻ cells and cell cycle profiles of these cells were measured by flow cytometric analysis on a LSR II flow cytometer (BD Biosciences) as previously described [5].

Statistical analysis

Kaplan-Meier survival curves were generated and statistically analyzed using Prism 4.0b software (GraphPad Software Inc.). For all statistical analyses, a *P* value less than 0.05 was considered significant.

Results

We reported previously that dietary RV administration in *Fancd2*^{-/-} mice partially corrected the abnormal cell cycle status of cKit⁺Sca1⁺Lin⁻ (KSL) hematopoietic stem and progenitor cells and helped maintain quiescence in this cell population. These effects on hematopoiesis results encouraged us to assess whether RV and another clinically applicable antioxidant NAC could prevent tumor formation in *Fancd2*^{-/-}/*Trp53*^{+/-} murine model. The NAC or RV diets were given long-term to *Fancd2*^{-/-}/*Trp53*^{+/-} mice and wildtype controls.

The doses of the compounds were chosen based on previously published results [5,13]. Both drugs were well tolerated in this long-term study and did not change the experimental mice's weights. In either of the drug tests, the tumor spectrum of placebo-treated mice was identical to that of test drug-treated mice and was the same as what we previously reported [8]. NAC-treated animals displayed an average tumor-free survival time that was exactly the same as their placebo-treated genotype-matched counterparts (Figure 1A), indicating no significant chemoprevention from NAC. It is important to note that the dose of NAC used here is the same as in other studies that have shown tumor preventive benefit [13]. RV-treated *Fancd2*^{-/-}/*Trp53*^{+/-} and *Fancd2*^{+/-}/*Trp53*^{+/-} mice showed an average tumor-free survival time of 411 days and 641 days, respectively, as compared with an average tumor-free survival time of 383 days and 622 days for placebo-treated *Fancd2*^{-/-}/*Trp53*^{+/-} and *Fancd2*^{+/-}/*Trp53*^{+/-} mice respectively (Figure 1B). Statistical analysis showed no significant changes between placebo and RV treatments for either *Fancd2*^{-/-}/*Trp53*^{+/-} or *Fancd2*^{+/-}/*Trp53*^{+/-} mice. Our previous study has demonstrated that RV treatment at the same dose partially corrects hematopoietic defects in *Fancd2*^{-/-} mice, arguing against the possibility that the chosen RV dose in the current study is too low to be effective [5]. Together, these results indicate that neither NAC nor RV delays tumor formation in this FA murine model.

We also tested whether tempol and NAC could enhance hematopoiesis in *Fancd2*^{-/-} mice. Both of the compounds were given to *Fancd2*^{-/-} mice via oral diet for 3 months. Flow cytometric analysis revealed no significant changes in the size and quiescent status of HSPC pool after tempol treatment (Figure 2A). The similar test with NAC oral administration showed no significant difference between the size of HSPC pool of NAC-treated mice and that of placebo-treated mice, either. However, a significant higher proportion of KSL cells (36.7±4.1%) in NAC-treated *Fancd2*^{-/-} mice were in G0 phase of the cell cycle, as compared to 24.8±1.4% for placebo-treated *Fancd2*^{-/-} KSL cells (Figure 2B, *P* < 0.05). This observation indicates that NAC administration helps maintain *Fancd2*^{-/-} KSL cells in a quiescent state, similar to the effects of RSV on cell cycle status in this cell population.

Discussion

Fanconi anemia is associated with elevated oxidative stress. In this study, we tested a few antioxidants NAC, RV, and tempol for chemoprevention or hematopoiesis enhancement. Neither NAC nor RV had any significant chemopreventive effect in tumor prone *Fancd2*^{-/-}/*Trp53*^{+/-} mice. Furthermore, NAC helped maintain *Fancd2*^{-/-} KSL cells in quiescence while tempol did not.

Combining these findings with our previous observation on tempol and RV in the same mouse model [5,8], we now have an overall picture of how these three antioxidants perform in FA mice. Both NAC and RV show no effects on tumor formation but help maintain quiescence in *Fancd2*^{-/-} KSL cells. In contrast, tempol substantially delays tumor onset in *Fancd2*^{-/-}/*Trp53*^{+/-} mice but has no beneficial effects on hematopoiesis in various tests ([8] and Figure 2A). Tempol, NAC and RV are chemically distinct anti-oxidants: tempol is a stable free radical nitroxide, NAC is a thiol precursor of intracellular cysteine and glutathione, and RV is a polyphenol. They could target different aspects of oxidative stress. Our data indicate that different kinds of oxidative damage play a role in the pathophysiology of bone marrow failure and tumor evolution in FA. Alternatively, the mechanisms of action of the drugs may be occurring via other cellular processes unrelated to oxidative stress response. Some of these antioxidants are known to elicit distinct cellular signaling events. Tempol activates Raf-1 protein kinase by tyrosine phosphorylation, whereas RV inhibits cAMP-degrading phosphodiesterases and regulates metabolism [14–16]. The precise

molecular mechanisms behind the different actions of these compounds in FA await further investigation.

Acknowledgments

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Figure 1. Neither NAC nor RV delayed tumor formation in $Fancd2^{-/-}/Trp53^{+/-}$ mice

Statistical analyses were performed with Prism 4.0 software using log rank test. ns denotes not significant ($P > 0.05$), and * denotes $P < 0.001$. (A) Tumor-free survival curves for NAC treatment. In the $Fancd2^{+/+}/Trp53^{+/-}$ group, $n=14$ for placebo treatment and $n=19$ for NAC treatment; in the $Fancd2^{-/-}/Trp53^{+/-}$ group, $n=10$ for placebo treatment and $n=19$ for NAC treatment. (B) Tumor-free survival curves for RV treatment. In the $Fancd2^{+/+}/Trp53^{+/-}$ group, $n=19$ for placebo treatment and $n=22$ for RV treatment; in the $Fancd2^{-/-}/Trp53^{+/-}$ group, $n=21$ for either placebo treatment or RV treatment.

A

B

Figure 2. NAC helped *Fancd2*^{-/-} KSL cells maintain quiescence while tempol did not
 (A) Frequencies and cell cycle profiles of bone marrow KSL cells before and after tempol treatment. ns denotes not significant ($P > 0.05$). $n=5$ for either group in all the assays. (B) Frequencies and cell cycle profiles of bone marrow KSL cells before and after NAC treatment. $n=7$ for either group in all the assays.