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“ROS-generating mitochondrial DNA mutations can regulate tumor cell metastasis”—a critical commentary

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Abstract

In a recent publication (K. Ishikawa et al., 2008, *Science* **320**, 661–664), the authors described how replacing the endogenous mitochondrial DNA (mtDNA) in a weakly metastatic mouse tumor cell line with mtDNA from a highly metastatic cell line enhanced tumor progression through enhanced production of reactive oxygen species (ROS). The authors attributed the transformation from a low-metastatic cell line to a high-metastatic phenotype to overproduction of ROS (hydrogen peroxide and superoxide) caused by a dysfunction in mitochondrial complex I protein encoded by mtDNA transferred from the highly metastatic tumor cell line. In this critical evaluation, using the paper by Ishikawa et al. as an example, we bring to the attention of researchers in the free radical field how the failure to appreciate the complexities of dye chemistry could potentially lead to pitfalls, misinterpretations, and erroneous conclusions concerning ROS involvement. Herein we make a case that the authors have failed to show evidence for formation of superoxide and hydrogen peroxide, presumed to be generated from complex I deficiency associated with mtDNA mutations in metastatic cells.

Keywords

Reactive oxygen species; Fluorescent probes; Metastasis; Mitochondria; Dichlorodihydrofluorescein; Hydroethidine; Hydrogen peroxide; Superoxide; Free radicals

Recently, Ishikawa et al. [1] reported that the replacement of endogenous mitochondrial DNA (mtDNA) in a weakly metastatic mouse tumor cell line with the mtDNA derived from a highly metastatic cell line enhanced the progression of tumor mediated by overproduction of reactive oxygen species (ROS), namely superoxide radical anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2). This transformation from a weakly metastatic tumor cell line to a highly metastatic phenotype was attributed to a dysfunctional mitochondrial complex I encoded by mtDNA transferred from the highly metastatic cells. The overall conclusion was that an overproduction of ROS generated from mitochondrial complex I was responsible for increased tumor cell metastasis. The authors employed the flow cytometry technique using two fluorogenic probes, 2',7'-dichlorodihydrofluorescein diacetate (DCFH₂-DA) and mito-hydroethidine (Mito-HE or MitoSOX red), to quantify the levels of H_2O_2 and $O_2^{\bullet-}$. In this critical commentary, we point out that the authors [1] have failed to show evidence for the involvement of ROS in the tumor cell transformation owing to a lack of appreciation of the complex radical chemistry of the fluorescent dyes. Our conclusions are supported by the following findings.

Intracellular DCF fluorescence arising from DCFH₂-DA cannot be used to quantify intracellular hydrogen peroxide

DCFH₂-DA is cell-permeative and is taken up into several cell types. DCFH₂-DA ester is hydrolyzed inside the cells by esterases, forming DCFH₂ (2',7'-dichlorodihydrofluorescein, 2',7'-dichlorofluorescein). DCFH₂ is not strongly fluorescent; however, DCFH₂ is oxidized to a green-fluorescent molecule, 2',7'-dichlorofluorescein (DCF), a two-electron oxidation product of DCFH₂. Contrary to common belief, DCFH₂ does not readily react with H₂O₂ to form DCF [2,3]. Trace levels of redox-active metal ions (e.g., ferrous iron or ferric ion and reductants) or heme proteins (cytochrome *c* or peroxidase) are required to catalyze the conversion of DCFH₂ to DCF [2–8]. This is mediated by hydroxyl radicals or iron intermediates of higher oxidation states. In this reaction, DCFH₂ is initially oxidized to a radical (DCFH[•]/DCF^{•-}) that reacts with molecular oxygen to form superoxide that dismutates to form additional hydrogen peroxide (Fig. 1) [3,9–11]. Moreover, the fluorescent product, DCF, can undergo further oxidation to a phenoxy-type radical as well as acting as a photosensitizer for oxidation of intracellular reductants, such as GSH or NADH, yielding additional superoxide and hydrogen peroxide (Fig. 1) [12,13]. Clearly, DCFH₂ itself can induce intracellular H₂O₂ formation. Thus, the green fluorescence observed by fluorescence microscopy or flow cytometry under conditions of intracellular oxidative stress is not a measure of intracellular H₂O₂ or any other form of intracellular oxidant, but it is the result of combination of several factors including, but not limited to, reduced GSH level, altered iron uptake, increased peroxidase activity, and cytochrome *c* release from mitochondria. Consequently, the statement “H₂O₂ formation generated by mtDNA mutations was quantitatively measured by DCFH dye” [1] is incorrect.

Reaction between superoxide and hydroethidine (HE) or Mito-HE (MitoSOX red) does not result in the formation of ethidium or mito-ethidium

Existing literature data unambiguously show that superoxide/ hydroethidine and superoxide/ mito-hydroethidine reactions do not result in the formation of ethidium (E⁺) or mito-ethidium (Mito-E⁺) [14–21]. Mito-HE, a triphenylphosphonium-conjugated mitochondria-targeted analog of HE, essentially reacts with superoxide in the same manner as does HE [17,21]. Both HE and Mito-HE react with superoxide at similar rates ($k = 2 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ [17,18]) to form the corresponding hydroxylated products, 2-hydroxyethidium (2-OH-E⁺) and 2-hydroxy-mito-ethidium (2-OH-Mito-E⁺) (Fig. 2) [14,16,20,21]. The fluorescence parameters of these hydroxylated products are different from those of ethidium and mito-ethidium [14,16,17,20,21]. In their paper, Ishikawa et al. report that the fluorescence spectrum was attributed to ethidium formed from the reaction between MitoSOX (Mito-HE) and superoxide [1]. The notion that superoxide directly oxidizes HE or Mito-HE to the corresponding ethidium or mito-ethidium is erroneous.

Intracellular red fluorescence derived from HE cannot be used to quantitate superoxide formation

Ishikawa et al. state in their *Science* paper that the red fluorescence derived from Mito-HE is a quantitative measure of intracellular superoxide formation [1]. Both HE and Mito-HE are also oxidatively converted to their ethidium analogs (exhibiting red fluorescence) [15,16,20,21]. The fluorescence spectra of E⁺ and 2-OH-E⁺ (2-OH-Mito-E⁺ and Mito-E⁺) have a significant overlap and it is nearly impossible to deduce the individual contributions of the superoxide/Mito-HE reaction product (2-OH-Mito-E⁺) and superoxide-independent product, Mito-E⁺. Thus, the fluorescence microscopy results obtained from Mito-HE do not give quantitative information on intracellular superoxide levels. In addition, both HE and

Mito-HE undergo one-electron (e.g., peroxidatic) oxidation in cells, forming several dimeric products that are not fluorescent (Fig. 2) [20–23]. Even for qualitative estimation of intracellular superoxide, one has to perform an HPLC analysis of cell extracts for the isolation and quantification of the superoxide-specific oxidation product of HE or Mito-HE [15,16,20,21].

Conclusion

In their *Science* paper, Ishikawa et al. did not appreciate the various methodological pitfalls for detecting ROS in cells using the fluorescent dyes DCFH₂-DA and Mito-HE (MitoSOX red). We submit that Ishikawa et al. failed to provide evidence in support of enhanced ROS formation in cells overexpressing mitochondrial DNA mutations derived from metastatic cells and that their paper is one of many papers wherein the fluorescent probes have been used indiscriminately for ROS detection.

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Fig. 1. Intracellular redox chemistry of 2',7'-dichlorodihydrofluorescein (DCFH₂) [2-13,24,25].

Fig. 2. Redox chemistry of hydroethidine (HE) and its mitochondria-targeted analog (Mito-HE) [14,16–21,26].