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Morphological changes in melanized and non-melanized *Cryptococcus neoformans* cells post exposure to sparsely and densely ionizing radiation demonstrate protective effect of melanin

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ABSTRACT

There is a need for novel and effective prophylactic treatments and radioprotective materials to protect civilians and military personnel from ionizing radiation in contaminated environments. Melanin, a naturally occurring, ubiquitous pigment, has been shown to confer radioresistance, acting as a potential radioprotective agent. We have demonstrated that melanized *Cryptococcus neoformans* (CN) cells had improved survival post ionizing irradiation than non-melanized ones. The goal of this study was to identify morphological changes in melanized and non-melanized CN cells following irradiation with densely-ionizing deuterons and alpha particles relative to sparsely-ionizing gamma radiation. We observed significant differences between the melanized and non-melanized CN cellular ultrastructure following irradiation. Melanized CN cells were relatively resistant to mid and max-dose levels of alpha particles and deuterons irradiation. Following irradiation the capsule was stripped, but the cell wall was intact and structural integrity was maintained. At the maximum dose, cytoplasmic vacuolization, and mitochondrial swelling started to occur. In contrast, the non-melanized CN strain was sensitive to the mid-dose radiation. Non-melanized cells presented two morphologies: small condensed, and swollen, lacking structural integrity. This morphological investigation provides the first direct evidence of the radioprotective properties of melanin in CN cells subjected to high RBE and high LET ionizing radiation.

Introduction

There is a growing need for novel and effective prophylactic treatments and radioprotective materials to protect civilians and military personnel from ionizing radiation in environments contaminated accidentally (e.g. nuclear power plant accidents) or maliciously (e.g. by terrorist attacks with radiological dispersion devices). In addition, there is a need to

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protect astronauts from harmful effects of cosmic radiation during the space travel. Particularly important is protection from/mitigation of delayed radiation effects such as carcinogenesis and damage to the central nervous system and cardiovascular system (Acharya et al. 2016; Parihar et al. 2016).

Melanins are pigments that are ubiquitous in nature (Meredith & Sarna 2006). These pigments confer a survival advantage to melanized fungi in extreme environments (Dadachova & Casadevall 2008). The resistance of melanized fungi exposed to cosmic radiation on the surface Mir Spacecraft and International Space Station and the presence of numerous melanized fungal species in the damaged nuclear reactor at Chernobyl suggest that melanins also play a pivotal role in protection from ionizing radiation (reviewed in Casadevall et al. 2017). Recently, several groups have demonstrated that internal administration of melanin protected mice against experimental sublethal and lethal doses of gamma radiation, possibly by protecting the gut and associated lymphatic tissues (Schweitzer et al. 2010; Kunwar et al. 2012; Revskaya et al. 2012; Rageh et al. 2014). These data suggest that radioprotective properties of fungal melanins can be exploited in mammalian systems.

Many melanized fungi are very radioresistant, requiring radiation doses exceeding 5 kGy to reduce cell survival to 10 % (Dadachova & Casadevall 2008). Such doses are roughly 1000-fold higher than the lethal dose for humans, showing that extreme radioresistance is not limited to prokaryotes such as *Deinococcus radiodurans*, and can be achieved by eukaryotic cells. One of our previous studies indicated that the radioprotective properties of fungal melanin result from a combination of physical shielding, chemical composition, spherical arrangement, and free radical quenching (Dadachova et al. 2008); another demonstrated that irradiation of melanin alters its oxidation-reduction potential (Turick et al. 2011). The transcriptomic investigation revealed that ionizing radiation stimulates protein biosynthesis with input from melanin (Robertson et al. 2012).

Most research on radioresistance mechanisms and radioprotection involves the use of high energy photons (gamma- and X-rays). However, civilians and military personnel in contaminated areas will need to be protected not only from gamma-rays, but also from other, qualitatively different, types of ionizing radiation (Cassatt et al. 2008; Shin & Kim 2009; Maxwell et al. 2011). In particular, densely-ionizing radiations such as alpha particles, which are emitted by the decay of many radionuclides found in nuclear fuel and military-grade radioactive materials, can have much more severe biological effects per unit dose than sparsely-ionizing gamma rays (Hada & Georgakilas 2008; Stewart et al. 2011). Recently we investigated the effects of densely ionizing deuterons on two fungal species capable of melanization – fast growing *Cryptococcus neoformans* and slow growing *Cryomyces antarcticus* and found that melanin effectively protected both fungi from high energy deuterons (Pacelli et al. 2017). In this study our goal was to investigate any morphological changes to ultrastructure in melanized and non-melanized *Cryptococcus neoformans* cells irradiated with densely ionizing deuterons and alpha particles and to compare them to changes caused by sparsely ionizing gamma radiation.

Materials and methods

Microorganisms

Cryptococcus neoformans (CN) American Type Culture Collection (STCC, Rockville, MD) strain *CN* H99 and its non-melanized laccase mutant, *lac-* (kind gift from Dr. A Idnurm, Duke University, NC) were used in all experiments. *CN* was grown in Sabouraud dextrose broth (SAB: 20 g L^{-1} Dextrose, 10 g L^{-1} peptone; BD Company, Sparks, MD, USA) for 24 h at 30 °C with constant shaking at 150 rpm. *CN* SAB cultures were then used to inoculate 25 ml of minimal media (29.4 mM KH_2PO_4, 10 mM $MgSO_4$, 13 mM glycine, 15 mM dextrose, 3 μM thiamine) at a ratio of 1:1000 and grown for 5–10 d at 30 °C with constant shaking at 150 rpm in the dark. Melanization was induced by supplementing the minimal media with 1 mM 3,4-dihydroxyphenylalanine (L-DOPA) during the 5–10 d culture period. *CN* H99 grown in the absence of L-DOPA, and *CN lac*-grown in the presence of L-DOPA served as non-melanized controls. Fig 1 shows the cultures at the end of melanization period: a) absence of melanin in H99 culture grown without L-DOPA, b) high degree of melanization in H99 culture grown with L-DOPA, c) absence of melanin in the laccase deficient mutant *lac*-grown with no L-DOPA, and d) small degree of melanisation in the *lac*-mutant grown in presence of L-DOPA. Cultures depicted in Fig 1A, B and D were used in all irradiations. Prior to irradiation the cells were transferred to phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4).

Irradiation conditions

Gamma irradiations were performed at the University of Saskatchewan, Saskatoon, Canada. Alpha particle and deuteron irradiations were performed at the Radiological Research Accelerator Facility, Columbia University, New York, USA. Fungal cell concentration was adjusted to 10^8 ml^{-1} in PBS prior to irradiation. The deuteron irradiations were performed as previously described (Pacelli et al. 2017) at a dose rate of 120 Gy min^{-1}, 2.2 MeV energy, and a linear energy transfer (LET) of 38 keV μm^{-1}. Alpha particle irradiation was performed using the same method as deuterons, at a dose rate of 72 Gy min^{-1}, 8.5 MeV energy, and a LET of 83 keV μm^{-1}. The gamma irradiations were performed in a 1 ml volume in a 5 ml Eppendorf tube, in a Cobalt 60 source Gammacell 220 Irradiator (AECL Industrial Irradiators) at the University of Saskatchewan, Saskatoon, Canada, at a dose rate of 2.1 Gy min^{-1}.

Transmission electron microscopy (TEM)

Following irradiation, *CN* cells were fixed in 2 % glutaraldehyde/0.1 M sodium cacodylate buffer, pH 7.2, at room temperature for 1h, and then transferred to 0.1 M sodium cacodylate buffer and stored at 4 °C. Fixed cells were pelleted and extra buffer was removed and replaced with warm 1 % w/v agarose and allowed to set. Excess agarose was removed from settled cells and the remaining pellet was washed with 0.1 M sodium cacodylate buffer and stored overnight at 4 °C. Samples were post-fixed in fresh 1 % Osmium tetroxide/0.1 M sodium cacodylate buffer

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