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Targeting melanocyte and melanoma stem cells by 8-hydroxy-2-dipropylaminotetralin

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ABSTRACT

Monobenzyl ether of hydroquinone (MBEH) is cytotoxic towards melanocytes. Its treatment efficacy is limited by an inability to eradicate stem cells. By contrast, 8-hydroxy-N,N-dipropyl-2-aminotetralin (8-DPAT) affects melanocyte stem cell survival. MBEH and 8-DPAT were added to melanocytes and melanoma cells to compare cytotoxicity. Stem cell content among viable cells was determined by fluorocytometry using markers CD34, Pax3, and CD271. Immunostaining was used to identify stem cells in skin explants treated with MBEH or 8-DPAT *ex vivo*. Mice were exposed to MBEH or 8-DPAT and scanned for depigmentation before harvesting skin. MBEH exposure prompted a relative increase in stem cells among cultured melanocytes and melanoma cells, as treatment preferentially eliminated differentiated cells and spared the stem cells. Viability of this remaining, enriched stem cell population was however rapidly reduced by exposure to 8-DPAT within melanocyte and melanoma cell cultures. In human skin explants, the abundance of melanocyte stem cells was also visibly reduced after 8-DPAT treatment, in contrast to tissue exposed to MBEH. Meanwhile, significant depigmentation of the mouse pelage and loss of differentiated melanocytes was observed *in vivo* in response to topical application of MBEH, but not 8-DPAT. Prolonged application of the latter agent instead appeared to effectively reduce the abundance of melanocyte stem cells in the dermis. This furthers the idea that MBEH and 8-DPAT target complementary cell populations. Results indicate that combination treatment may demonstrate superior therapeutic activity by eliminating both differentiated and tumor initiating populations.

Introduction

The incidence of melanoma has gradually increased since the 1980s at a rate of approximately 2.8% annually [1]. There were more than 75,000 new cases in the United States in 2013, accounting for almost 5% of all new cancers. It is estimated that more than 920,000 people are living with melanoma in the U.S, where the incidence is about 21 per 100,000 per year [2]. The incidence of melanoma in the United Kingdom and Australia is 12.6 and as high as 46.7 per 100,000, respectively [1]. Approximately 20% of those diagnosed with cutaneous malignant melanoma will die secondary to metastatic disease [3].

Conventional chemotherapy is largely ineffective for the treatment of metastatic melanoma. There has been increased focus on the use of immunotherapy agents which demonstrate much greater success in disease regression. High-dose Interleukin-2 therapy was approved by the FDA in 1998 for metastatic disease, but response rates were less than 20% [4]. However, approximately 8% of treated patients do achieve complete disease remission. Cytotoxic T-lymphocyte antigen 4 (CTLA-4) has been shown to suppress T-cell activation. Antibodies to CTLA-4 potentiate the immune response against tumor cells. A monoclonal antibody against CTLA-4 has demonstrated improved survival in patients with stage 3 and 4 melanoma [5], and in 2011 became the first new FDA-approved treatment for advanced melanoma in more than 10 years. The approval of BRAF inhibitors for melanoma

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treatment is revealing some spectacular remissions in late stage melanoma patients, however, these tumors do recur [6]. Clearly, it is important to understand the process of drug resistance that evolves within tumors under these circumstances.

It was previously believed that melanoma arose from mature melanocytes, but growing evidence suggests that a subpopulation of melanoma stem cells is mostly responsible for disease initiation and proliferation [7]. Melanoma stem cells demonstrate self-renewal ability and give rise to the full heterogeneous composition of the tumor. Only a unique subset of tumor cells possesses these qualities, though the proportion of stem cells reportedly found within individual tumors is inconsistent, ranging from 0.1% to 53% in several studies [8,9]. Nevertheless, there is much interest in identifying markers of melanoma stem cells, as the ability to specifically target these cells may lead to development of new therapies. CD271 represents the low-affinity receptor for neural growth factor and is a neural crest stem cell marker found on human multipotent melanoma cells. These stem cells have been shown to establish the full heterogeneity of parental melanoma when xenotransplanted to nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice [9]. CD34 has likewise been identified as a marker of melanoma progenitor cells in mouse models [10]. Melanocyte stem cells provide pigmented cells during tissue homeostasis. Melanoma is thought to arise from transformed mature melanocytes, melanoma-specific stem cells, or both [7]. Melanocyte stem cells are found in hair follicles primarily. Similar to melanoma stem cells, they are influenced by other cell types and are affected by various factors that promote or restrict multipotency and self-renewal [11]. Melanocyte stem cells can be found in hair follicles, but also derive from neural crest cells in the dermis [12].

Neural crest cells, after migrating from the neural plate, can differentiate into a wide array of cell types, including bone, cartilage, and melanocytes. Melanoblasts derive from glial-melanoblast progenitors, whose fate is in part determined by the Wnt signaling pathway [13]. Many important genes have been identified as crucial in the development of melanocytes. Pax3, a gene in the paired box family of transcription factors, also plays a role in melanoblast development, possibly by balancing differentiation to melanocytes, versus maintaining an undifferentiated state [13]. Mutations in Pax3 are responsible for the Waardenburg syndrome phenotype, a rare auditory-pigmentary syndrome. c-Kit is a tyrosine kinase receptor which, when ligated, activates signaling implicated in melanocyte differentiation, migration, and differentiation. Multiple important signaling pathways work downstream from the Kit receptor, including PI3K and RAF/MEK/ERK pathways [14]. Though no single marker is currently universally accepted as an identifier of all melanocyte or melanoma stem cells, their typical location and a combination of CD271, Pax3 and CD34 expression may be considered suitable to identify these populations under different conditions.

Mutations in the oncogene BRAF, which is dependent on RAF/MEK/ERK, are common in melanoma, and drugs targeting this pathway have recently been FDA approved for metastatic disease [6]. Studies of the zebrafish model have led to a deeper understanding of melanocyte biology and the complexity of these pathways. Melanocytes are visible both in the embryo and in the mature zebrafish. This transparent quality, in addition to their small size and ability to produce numerous embryos, makes zebrafish an ideal model for large-scale screening studies of small molecules. In this regard, 8-hydroxy-2-dipropylaminotetralin (8-DPAT) has been shown to modify kit-dependent melanocyte migration and survival in drug-screening studies on zebrafish, resulting in a markedly reduced number of migrated melanocytes when compared to the untreated zebrafish population [14]. Interestingly, in response to 8-DPAT the migrated melanocytes are smaller and appear to fragment similar to that seen in zebrafish treated with (2-morpholinobutyl)-4-thiophenol (MoTP), a melanocytotoxic drug dependent on tyrosinase activity [14,15].

Radiolabeled 8-DPAT is commonly used to delineate the expression and distribution of 5-hydroxytryptophan (serotonin) 1A receptors in human brain. The drug is also used as a 5HT1a receptor agonist to study effects on serotonin reuptake inhibition and prevention of depression [16,17]. Taken together, the role of 8-DPAT is best established in relation to neuronal function. These studies suggest that responses to 8-DPAT observed in zebrafish may well translate to mammals including mice and men.

Targeting of adult melanocytes is readily accomplished by monobenzyl ether of hydroquinone (MBEH),[2] an FDA-approved melanocytotoxic drug for depigmentation therapy in vitiligo. Our previous works have explored how MBEH mediates depigmentation, demonstrating that this compounds induces melanocyte necrosis while another depigmenting compound, 4-TBP (4-tertiary butyl phenol), induces cell death by apoptosis [15]. We have subsequently shown that MBEH treatment leads to a CD8 T-cell response which targets sites distant from the site of topical application, making it a potentially useful agent for melanoma prophylaxis [18]. As MBEH-induced depigmentation likely requires tyrosinase which is not expressed by undifferentiated stem cells, we hypothesized that treatment will leave a population of stem cells that is resistant to treatment, and agents targeting the stem cell population are thus predicted to enhance the efficacy of MBEH treatment in either disease.

Throughout our studies, we have engaged antibodies to CD34, CD271 and Pax3 to identify the stem cell population. We studied the potential of 5HT-1A agonist 8-DPAT to affect melanocyte and melanoma stem cell viability *in vitro* by MTT assays using a range of 8-DPAT concentrations, evaluated morphologic changes induced by 8-DPAT exposure, assessed topical application of 8-DPAT to mouse and human skin *in vivo* and in organotypic explant cultures, respectively, and evaluated its effects on the abundance of melanocyte and melanoma by scanning and immunohistochemistry. Taken together, these experiments outline the opportunities for stem cell targeting presented by 8-DPAT exposure and its potential synergism with MBEH for the treatment of pigmentation disorders.

Materials and methods

Mouse model

C57BL/6 mice from The Jackson Labs (Bar Harbor, Maine, USA) were used for all of the depigmentation and organotypic culture experiments. In the topical treatment study 5 mice per group were used. All experiments were approved by Loyola University Medical Center's Institutional Animal Care and Use Committee.

Preparation of bleaching agents and treatment

Both 8-DPAT (R-(+)-8-hydroxy-DPAT) (Sigma–Aldrich, St. Louis, MO, USA) and MBEH (Sigma) were prepared as stock solutions of 250 mM in 1:5 mixture of DMSO and 70% EtOH respectively. This 1:5 DMSO–EtOH mixture was also used as the vehicle control. These stocks were diluted to a final concentration of 50–250 mM to treat organotypic cultures and further diluted to 50–500 μM to treat cell cultures. Additional stocks of 900 mM 8-DPAT (8-hydroxy-DPAT hydrobromide) (Tocris, Bristol, United Kingdom) and 450 mM MBEH (Sigma) were prepared for topical treatment of mice. After these stocks were diluted 1:1 with Eucerin lotion (Beiersdorf, Wilton, CT, USA), resulting in 450 mM and 225 mM concentrations, they were topically applied in 100 μl volumes to

[2] *Abbreviations used:* MBEH, monobenzyl ether of hydroquinone; 8-DPAT, 8-hydroxy-N,N-dipropyl-2-aminotetralin.