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Original Contribution

Maintenance of immune hyporesponsiveness to melanosomal proteins by DHICA-mediated antioxidation: Possible implications for autoimmune vitiligo

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ABSTRACT

Melanocyte destruction in the skin of vitiligo patients has been considered to be a consequence of an autoimmune response against melanosomal proteins. However, little is known about the molecular mechanisms by which the immune system recognizes these sequestered intracellular self-proteins, which are confined in specialized organelles termed melanosomes, and is provoked into an autoimmune response to melanocytes. Here, we utilize a sucrose density-gradient ultracentrifugation protocol to enrich melanosomal components from dopachrome tautomerase (Dct)-mutant or wild-type melanocytes exposed to a pulse of hydrogen peroxide at a noncytotoxic concentration to evaluate their immunogenicity in mice challenged with the corresponding melanosomal proteins. The results demonstrate that enhanced humoral and cellular immune responses to a challenge with late-stage melanosomal proteins, especially with those derived from Dct-mutant melanocytes, are found in the immunized mice. To elucidate whether a reduced 5,6-dihydroxyindole-2-carboxylic acid (DHICA) content in melanin might cause a loss in antioxidative protection to the proteins, we incubated these melanosomal proteins in vitro with synthetic 5,6-dihydroindole (DHI)–melanin or DHI/DHICA (1:1)–melanin and then used them to immunize mice. T cell proliferation and IgG antibody responsiveness to the challenges were significantly induced by melanosomal proteins treated with DHI–melanin, but not by those treated with DHI/DHICA (1:1)–melanin. Moreover, we observed that melanosomal proteins derived from Dct-mutant melanocytes are subject to oxidative modifications that alter their antigenic configurations to attain an enhanced immunogenicity compared with those derived from wild-type melanocytes. From these results, we conclude that DHICA-mediated antioxidation plays a critical role in the maintenance of immune hyporesponsiveness to melanosomal proteins.

Functional melanocytes disappear from the skin of vitiligo patients by a mechanism that is not fully understood [1–4]. It is generally accepted that the loss of histochemically recognizable melanocytes in the involved skin is the result of their destruction, caused at least in part by altered humoral and/or cellular immune reactions against the melanocytes per se [2]. Skin-homing autoreactive T cells [3] and autoantibodies [4] to melanocytes have been detected in the lesions and/or the sera of vitiligo patients, directed against various melanocyte antigens such as tyrosinase, tyrosinase-related protein 1 (Tyrp1), and dopachrome tautomerase (Dct/Tyrp2). However, it deserves further scrutiny to characterize how immunoglobulins recognize intracellular self-proteins to mediate cellular destruction and how these sequestered organelle antigens, which are confined in specialized compartments termed melanosomes, are exposed to the immune system at the onset of the disease [5]. The emerging view is that vitiligo melanocytes may have an intrinsic defect that makes them more susceptible to reactive oxygen species (ROS) and to overproduction of hydrogen peroxide (H_2O_2) found in the vitiligo epidermal milieu, which possibly induces the intracellular melanosomal antigens to be released subsequent to oxidative damage of melanocytes [6–8]. Despite the fact that exposure of melanosomal proteins to antigen-presenting cells might occur, an immune response to these normal self-antigens would presumably be difficult to provoke as a consequence of immune tolerance. It is possible that the occurrence of protein oxidative modification in vitiligo melanocytes is indispensable for the efficient induction of those immune responses [9].

A recent study demonstrated that inactivation of Dct lessens the radical-scavenging potential in Dct-deficient melanocytes and increases their vulnerability to oxidative damage [10]. Dct is a critical enzyme in the melanogenesis pathway that isomerizes the intermediate dopachrome to 5,6-dihydroxyindole-2-carboxylic acid (DHICA) and influences the proportion of the DHICA monomer incorporated

Abbreviations: Dct, dopachrome tautomerase; Tyr, tyrosinase; Tyrp1, tyrosinase-related protein 1; DHICA, 5,6-dihydroxyindole-2-carboxylic acid; DHI, 5,6-dihydroindole; ROS, reactive oxygen species; H_2DCFDA, 2′,7′-dichlorofluorescin diacetate.

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into melanin with the 5,6-dihydroxyindole (DHI) polymer, thereby generating a DHICA-rich eumelanin that confers a potent hydroxyl radical scavenging activity [10]. The spontaneous *slaty* mutation in the mouse Dct gene dramatically decreases the catalytic activity of the mutant enzyme compared with wild-type Dct [11,12]. In this study, we attempted to define the sensitivity of Dct-mutant melanocytes to oxidative stress, the immunogenicity of melanosomal proteins derived from Dct-mutant melanocytes after treatment with H_2O_2, as well as the protection from oxidative insults by synthetic DHICA-rich melanin. Our results show that oxidative stress significantly enhances the immunogenicity of melanosomal proteins derived from Dct-mutant melanocytes and that DHICA-mediated antioxidation plays a role in the maintenance of immune hyporesponsiveness to melanosomal proteins.

Materials and methods

Cell lines and cell culture

Murine melan-a melanocytes were a kind gift from Professor Dorothy C. Bennett (St. George's Hospital, London, UK) [13]. They were originally derived from C57BL/6J (black, a/a) mice and are routinely passaged in complete RPMI 1640 medium with 5% heat-inactivated fetal calf serum, 50 U/ml penicillin, 50 μg/ml streptomycin, 100 μM mercaptoethanol, 2 mM L-glutamine, and 200 nM phorbol myristate acetate (PMA). Murine Dct-mutant (Dct^{slt}/Dct^{slt}) melanocytes were generously provided by Dr. Vincent J. Hearing, (Laboratory of Cell Biology, National Cancer Institute, National Institutes of Health (NIH), Bethesda, MD, USA) [11]. They were originally derived from dorsal skins of 1-day-old mice carrying the *slaty* mutation in the Dct gene. Dct-mutant melanocytes are cultured in the same medium used for melan-a melanocytes, as previously described [14].

H_2O_2-pulse treatment and intercellular ROS assay

Both types of melanocytes (2×10^5) were cultured in six-well plates in complete RPMI 1640 medium. For the H_2O_2-pulse treatment [15], the medium was changed to D-PBS with or without 100 μM H_2O_2 (Sigma–Aldrich, St. Louis, MO, USA) for 1 h. Intercellular ROS levels were measured using the oxidation-sensitive fluorescent dye 2′,7′-dichlorofluorescin diacetate (H_2DCFDA) as described previously [10]. Briefly, equal numbers of cells were loaded with 10 μM H_2DCFDA (Sigma) for 20 min at 37 °C after exogenous H_2O_2-pulse treatment and then were washed with D-PBS twice. It should be noted that the nonfluorescent ester H_2DCFDA penetrates into cells and undergoes deacetylation to DCFH by cellular esterases. The DCFH probe is rapidly oxidized to the highly fluorescent compound 2′,7′-dichlorofluorescin (DCF) by ROS. ROS levels are expressed as the fluorescence intensity of DCF measured at an excitation of 495 nm and an emission of 527 nm using a Hitachi F4500 spectrofluorimeter (Hitachi Ltd., Tokyo, Japan).

Fontana–Masson staining

Fontana–Masson staining was performed to assess the amount of melanin in Dct-mutant *slaty* melanocytes and in melan-a melanocytes, as described previously [10,16]. Monolayer melanocytes grown on coverslips were fixed with cold acetone for 10 min and then were stained with a 10% silver nitrate solution for 2 h in a dark chamber. After being rinsed in water, the coverslips were placed in 0.1% gold chloride solution for 5 min, rinsed in water, and then placed in 5% sodium thiosulfate for an additional 5 min. After being rinsed with water, the coverslips were counterstained with eosin red.

Purification of melanosomes by sucrose density-gradient ultracentrifugation

Preparation of cellular homogenates and purification of melanosomes were performed according to the method of Kushimoto et al. [17]. Briefly, confluent monolayers of Dct-mutant melanocytes or melan-a melanocytes were treated with D-PBS containing 100 μM H_2O_2 for 1 h. After the pulse treatment with H_2O_2, the cells were harvested with 0.25% trypsin/0.02% EDTA and washed once in 0.25 M sucrose by centrifugation at 1000*g* for 5 min at 4 °C. They were then homogenized on ice using 20 strokes of a Dounce glass–glass homogenizer and were centrifuged at 1000*g* for 10 min at 4 °C. The cellular homogenate was then layered on a discontinuous gradient of 1.0, 1.2, 1.4, 1.5, 1.6, 1.8, and 2.0 M sucrose (in 10 mM Hepes, pH 7.4) and centrifuged at 100,000*g* in a Beckman SW28 swinging-bucket rotor for 1 h at 4 °C. Early and late melanosomes were recovered from the 1.2–1.4 M and the 1.6–1.8 M sucrose interfaces, respectively. Fractions were collected and analyzed for enzyme activity, ultrastructure, melanogenic protein expression, and immunogenicity in vivo, as detailed below.

Transmission electron microscopy

Dct-mutant *slaty* melanocytes and melan-a melanocytes in the exponential growth phase were harvested and fixed with 2.5% (v/v) glutaraldehyde for 24 h at 4 °C, collected by centrifugation, and then washed twice with cold PBS. All samples were postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.2) for 1 h, dehydrated through a graded ethanol series, and embedded in Epon at 60 °C for 48 h. Ultrathin sections were cut and stained with uranyl acetate and lead citrate and then examined in a transmission electron microscope (Tecnai G2, FEI Co., Eindhoven, The Netherlands) [18].

Western blot analysis

For Western blotting, the cells were washed in PBS and lysed in extraction buffer containing 1% Nonidet P-40, 0.01% SDS, and a protease inhibitor cocktail (Roche, Indianapolis, IN, USA). Protein contents were determined with a BCA assay kit (Pierce, Rockford, IL, USA). Equal amounts of each protein extract (10 μg per lane) were resolved using 10% SDS–polyacrylamide gel electrophoresis (SDS–PAGE). After transblotting onto Immobilon-P membranes (Millipore, Bedford, MA, USA) and blocking in 5% nonfat milk in saline buffer, the membranes were incubated with αPEP7, αPEP1, or αPEP8 (gifts from Dr. Vincent J. Hearing at the NIH), each at a 1:2000 dilution except for αPEP7 (1:1000), or with an anti-β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-VADC1/porin antibody (Abcam, Cambridge, MA, USA) (used as loading controls for whole-cell lysates or subcellular mitochondrial components) for 1 h at room temperature. The membranes were then washed and incubated with horseradish-peroxidase-conjugated anti-rabbit IgG (Pierce) at a dilution of 1:2000 for 1 h at room temperature. The membranes were then washed and specific bands were visualized by a chemiluminescent reaction (ECL; Amersham, Piscataway, NJ, USA) [19].

Enzyme activity analyses of tyrosinase, Dct, and catalase

Tyrosinase activity was assayed as dopa oxidase activity using a modification of the method described by Ando et al. [20]. Approximately 10^7 cells were pelleted and lysed with extraction buffer, and 100 μl of each cell lysate was pipetted into a 96-well plate on ice in triplicate. One hundred microliters of 0.1 M phosphate buffer (pH 6.8) containing 0.1% 3,4-dihydroxy-L-phenylalanine (L-dopa; Sigma) was then added into each well and mixed with the lysates at 37 °C in the dark for 5 min. The plates were read at 475 nm using a microplate reader (Wallac1420; PerkinElmer, Waltham, MA, USA) to estimate the amount of dopachrome generated from dopa. Corrections for the