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Molecular effects of 1-naphthyl-methylcarbamate and solar radiation exposures on human melanocytes

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ABSTRACT

Carbaryl (1-naphthyl-methylcarbamate), a broad-spectrum insecticide, has recently been associated with the development of cutaneous melanoma in an epidemiological cohort study with U.S. farm workers also exposed to ultraviolet radiation, the main etiologic factor for skin carcinogenesis. We hypothesized that carbaryl exposure may increase deleterious effects of UV solar radiation on skin melanocytes. This study aimed to characterize human melanocytes after individual or combined exposure to carbaryl (100 μM) and solar radiation (375 mJ/cm^2). In a microarray analysis, carbaryl, but not solar radiation, induced an oxidative stress response, evidenced by the upregulation of antioxidant genes, such as *Hemeoxygenase-1 (HMOX1)*, and downregulation of Microphtalmia-associated Transcription Factor (MITF), the main regulator of melanocytic activity; results were confirmed by qRT-PCR. Carbaryl and solar radiation induced a gene response suggestive of DNA damage and cell cycle alteration. The expression of *CDKN1A*, *BRCA1/2* and *MDM2* genes was notably more intense in the combined treatment group, in a synergistic manner. Flow cytometry assays demonstrated S-phase cell cycle arrest, reduced apoptosis levels and faster induction of cyclobutane pyrimidine dimers (CPD) lesions in carbaryl treated groups. Our data suggests that carbaryl is genotoxic to human melanocytes, especially when associated with solar radiation.

1. Introduction

Carbaryl (1-naphthyl methylcarbamate, CAS 63–25-2), also known by the commercial name of Sevin®, is a broad-spectrum carbamate insecticide widely used in agriculture and in the domestic environment (CALEPA, 2014). Occupational exposure is the main concern regarding this pesticide. Recently, a cohort epidemiological study with a four year follow-up of agricultural workers demonstrated a significant association between the development of cutaneous melanoma and the use of carbaryl (≥56 exposure days; OR = 1.7; CI 95%, 1.1–2.5; trend $p = 0.013$) (Dennis et al., 2010). Additionally, a dose–response relationship was suggested between carbamate pesticides in general and prevalence of cutaneous melanoma. No linear relationship was found between sun exposure and melanoma development in that study cohort. Another publication from the same epidemiological study, with a two-year follow-up and fewer cases of cutaneous melanoma, also has indicated increased risk of melanoma associated to carbaryl exposure (Mahajan et al., 2007).

Carbaryl genotoxicity is reported as positive in several in vitro assays with different human cell lines (Bigot-Lasserre et al., 2003; Delescluse et al., 2001). Carbaryl 100 μM was reported to induce oxidative stress and high levels of DNA damage in human lymphoblastoid cells, and these results were confirmed in HepG2 cells; apparently, its genotoxic potential was indirect, exerted by reactive metabolites resulting from its biotransformation, as the genotoxic activity was demonstrated in CYP1A1-transfected cells, but not on the parental cell line (Delescluse et al., 2001). When exposed to carbaryl via the diet, in doses up to 4000 ppm for 180 days, heterozygous p53 knockout mice did not develop tumors and carbaryl was considered non-genotoxic (Bigot-Lasserre et al., 2003). Overall, the weight of evidence indicates that carbaryl is not an in vivo genotoxic agent (European Food Safety Authority, 2006).

In parallel, ultraviolet (UV) radiation is the main etiological factor to the development of skin tumors, squamous cell carcinoma, basal cell carcinoma and cutaneous melanoma, being classified as a human

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carcinogen group I by IARC (1992). Melanoma skin cancer is one of the fastest growing malignancies in incidence worldwide, with one of the worst prognoses (de Santis et al., 2014). It is well known that UVB radiation induces DNA damage directly in epidermal cells, leading to mutagenesis; UVA radiation induces indirect genotoxicity through reactive oxygen species (ROS) and oxidative stress, and eventually leads to mutations that can trigger the carcinogenic process (de Gruijl, 2000). Although less abundant, UVB radiation is considered to be more carcinogenic than UVA, inducing formation of cyclobutane pyrimidine dimers (CPDs) and 6,4-photoproducts (6-4PP) (Matsumura and Ananthaswamy, 2002). CPD lesions are the most difficult to be repaired and, among them, dimers formed between adjacent cytosines (C-C) or between thymine and cytosine (T-C) are considered the most mutagenic (Marrot and Meunier, 2008). UVA radiation is an important oxidative stress inducer in epidermal cells through single and/or double DNA strand breaks and formation of 8-oxo-7,8-dihydroguanine (8-oxo-dG), which is strongly associated with cutaneous carcinogenesis due to generation of high genomic instability (von Thaler et al., 2010; Ridley et al., 2009). It is noteworthy that melanocytes are slowly proliferating cells that persist in the epidermis for decades, and therefore are exposed to high cumulative levels of UV-induced ROS. In fact, the role exerted by ROS in melanomagenesis is well characterized in the literature (Wittgen and van Kempen, 2007).

Moreover, UVA radiation has been investigated with regard to its ability to intensify the suscetibility to carcinogenesis induced by UVB or by low doses of environmental chemicals, resulting in significant synergistic scenarios (Burke and Wei, 2009). These are called photosensitization reactions, where chemicals are capable of amplifying UV deleterious effects, leading to formation of DNA adducts and/or to induction of oxidative stress and damage (Marrot and Meunier, 2008).

Although comprehensive and well designed, the epidemiological study published by Dennis et al. (2010) has certain limitations. For example, it could not accurately estimate the UV dose and the accumulated carbaryl dose to which workers were exposed. Consequently, it is insufficient to determine each factor's direct contribution in the development of melanoma tumors found in that study. Therefore, we aimed to characterize human melanocytes after individual or combined exposure to carbaryl (100 μM) and solar radiation (375 mJ/cm^2). The following parameters were analyzed: global gene expression (microarray), cell growth curve (Trypan Blue cell viability assay), genotoxicity (CPDs and 8-oxo-7,8-dihidroguanine quantification) and cell cycle evaluation. Our hypothesis is that carbaryl is associated with initiation of melanocyte transformation and that this event occurs synergistically with solar radiation.

2. Material and methods

2.1. Cell culture

This study was approved by the Ethics Committee of the School of Pharmaceutical Sciences – University of Sao Paulo (Process n. 943/09).

Experiments were performed with human primary skin melanocytes, extracted from the foreskin of two unrelated Caucasian donors (4- and 6-years of age) as described by Pennacchi et al. (2015). Melanocytes were cultured with 254CF medium supplemented with calcium chloride 0.2 M and Human Melanocyte Growth Supplement (HMGS, Gibco, Invitrogen Cell Culture, USA), with the addition of Ampicillin and Streptomycin 100 mg/L. After plating, cells were maintained in a humidified incubator at 5% CO_2 and 37 °C. Cells were cultured separately and pooled with equal proportions of both donors at the time of assay plating, in an effort to increase genetic diversity.

2.2. Treatment and irradiation

Twenty-four hours after plating, cells were at 80% confluency and were subjected to the following experimental treatment groups: Group 1: No treatment; Group 2: Irradiation and no treatment; Group 3: Carbaryl treatment; Group 4: Irradiation and carbaryl treatment; Group 5: Vehicle treatment; Group 6: Irradiation and vehicle treatment.

Treatment regimen consisted of melanocyte incubation with carbaryl 100 μM (CAS No. 63-25-2; Sigma-Aldrich, St Louis, USA) for 6, 24, 48 or 72 h (depending on the analysis performed) after single dose exposure to 375 mJ/cm^2 of solar radiation using a solar simulator (SS2.5 kW, Sciencetech Inc., Ontario, Canada) with a global air mass filter (A.M 1.5G, Sciencetech Inc., Ontario, Canada); duration of radiation exposure was 16 s, with a potency of 0.9 kW. For the irradiation assays, culture medium was replaced by PBS buffer without Ca^{2+} or Mg^{2+} (PBS-A). All experiments were performed in triplicates.

The solar simulator lamp profile using the AM 1.5G filter, which lets through UVB, (280–320 nm), UVA (320–400 nm), visible light (400–700 nm) and infrared (700–1000 nm) irradiations, is similar to the mean global solar radiation that reaches the surface of the earth in the USA region.

2.3. Trypan blue viability and cell growth assays

Cells were cultured in 24-well plates, 3×10^4 cells/well, and after 24, 48 and 72 h of treatment, cells were trypsinized, resuspended in PBS-A with 3% fetal bovine serum and incubated with Trypan Blue 0.4% (Sigma-Aldrich, St. Louis, MO, USA) for 3 min. Cell counting was performed in a Neubauer chamber using a light microscope after 24, 48 and 72 h of treatment. Cell viability was assessed by cell counting excluding blue stained cells. Cell growth was assessed by summing numbers of viable and non-viable cells.

2.4. Mechanisms of cell death characterization by flow cytometry

Cells were cultured in 6-well plates, 20×10^4 cells/well and, after 24 h of treatment, cells were trypsinized, resuspended in binding buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 1.8 mM $CaCl_2$) with 3% Annexin-APC (BD Biosciences, San Jose, CA, USA) and incubated for 20 min at room temperature, in the dark, for labelling of apoptotic cells. Subsequently, a Propidium Iodide (PI, Life technologies, USA) solution (8 μg/mL) was added to the cell suspension for labelling of exposed DNA (necrotic cells). Cells were further analyzed by flow cytometry (FACSCanto, BD Biosciences, San Jose, CA, USA) using FlowJo software (Tree Star Inc., Ashland, OR, USA). Cisplatin (90 μM) was used as positive control for apoptosis induction (Liu et al., 2006) and DMSO (10%) was used as positive control for necrosis induction.

2.5. Cell cycle evaluation by flow cytometry

Cells were cultured in 6-well plates, 20×10^4 cells/well, and after 48 h of treatment, cells were trypsinized, resuspended in lysis buffer (Triton X-100 0.1%, Trisodium Citrate 0.1%) with Ribonuclease-A 10 mg/mL, and incubated for 30 min at 37 °C. Subsequently, a PI solution (10 μg/mL) was added to the cell suspension, which was further analyzed by flow cytometry (FACSCanto, BD Biosciences, San Jose, CA, USA) using FlowJo software (Tree Star Inc., Ashland, OR, USA). Cell cycle evaluation was performed by quantification of DNA content measured as the intensity of PI fluorescence.

2.6. 8-Oxo-7,8-dihydroguanine quantification

Cells were cultured in 100 mm plates, 10^6 cells/plate. Labelling with primary antibody, anti-8-Hydroxiguanine (8-oxo-dG) MAb (2E2 Clone - RandD Systems Inc., Minneapolis, MN, USA), was performed according to the manufacturer's protocol with some adaptations. Briefly, after 1 and 24 h of treatment, cells were trypsinized, fixed in ethanol 70% and stored at −20 °C. Cells were rehydrated with PBS-A followed by incubation with Ribonuclease-A 10 mg/mL for 1 h and incubation with HCl