Photodiagnosis and Photodynamic Therapy 23 (2018) 154–164

Contents lists available at ScienceDirect

Photodiagnosis and Photodynamic Therapy

journal homepage: www.elsevier.com/locate/pdpdt

Effects of methylene blue-mediated photodynamic therapy on a mouse model of squamous cell carcinoma and normal skin

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ARTICLE INFO

Keywords:
Photodynamic therapy
Methylene blue
Squamous cell carcinoma
Normal skin
Mice

ABSTRACT

Background: Photodynamic therapy is used to treat a variety of cancers and skin diseases by inducing apoptosis, necrosis, immune system activation, and/or vascular damage. Here, we describe the effects of a single photodynamic therapy session using methylene blue on a mouse model of squamous cell carcinoma and normal skin.
Methods: The photodynamic therapy protocol comprised application of a 1% methylene blue solution, followed by irradiation with a diode laser for 15 min at 74 mW/cm^2, for a total dose of 100 J/cm^2. Morphological changes, cell proliferation, apoptosis, collagen quantity, immune system activity, and blood vessel number were analyzed 24 h and 15 days after photodynamic therapy.
Results: In the squamous cell carcinoma group, photodynamic therapy reduced tumor size and cell proliferation and raised cytokine levels. In normal skin, it decreased cell proliferation and collagen quantity and increased apoptosis and blood vessel numbers.
Conclusions: The effects of photodynamic therapy were greater on normal skin than squamous cell carcinoma tissues. The reduced epithelial thickness and keratinization of the former are factors that contribute to the efficacy of this treatment. Adjustments to the treatment protocol are necessary to potentiate the effects for squamous cell carcinoma therapy.

1. Introduction

Photodynamic therapy (PDT) is used for the treatment of various types of cancers, precancerous lesions, and dermatological disorders. PDT uses dyes or pigments, referred to as photosensitizers, that absorb visible light and induce or participate in photochemical reactions. The photosensitizer is injected into the target tissue under visible light irradiation of an appropriate wavelength, which results in cell damage. Photochemical reactions of types I and II may occur separately or simultaneously to generate cytotoxic products, such as singlet oxygen ($^1O_2$), a highly reactive molecule [1,2].

The photosensitizer methylene blue (MB) can trigger the production of high levels of $^1O_2$ or reactive oxygen species. Skin photosensitivity is a common problem after PDT; however, use of MB reduces the risk of developing this condition because it is hydrophilic, rapidly absorbed, and quickly removed from target tissue. MB also absorbs photons at wavelengths within the therapeutic window (600–800 nm), at which light penetration of the target tissue is greatest [3]. The efficacy of MB-mediated PDT (MB-PDT) in inactivating microorganisms has been demonstrated previously [4,5]. Moreover, in vitro studies have verified the effectiveness of MB as a photosensitizer for the induction of tumor cell death [6,7].

Squamous cell carcinoma (SCC) is a type of non-melanoma skin cancer (NMSC). Its incidence, which is increased by exposure to

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https://doi.org/10.1016/j.pdpdt.2018.06.012
Received 24 March 2018; Received in revised form 8 May 2018; Accepted 13 June 2018

Table 1
Description of the experimental design and each treatment group.

SCC (DMBA + TPA)		Procedure	Normal skin (acetone)	Procedure
Untreated (controls)	**SCC + 24 h (n = 10)**	Animals euthanized together with the treated groups 24 h after PDT	**N (n = 5)**	Animals euthanized together with the treated groups 15 days after PDT
	SCC + 15 days (n = 6)	Animals euthanized together with the treated groups 15 days after PDT		
Treated	**SCC + PDT + 24 h (n = 15)**	Animals euthanized 24 h after PDT	**N + PDT + 24 h (n = 7)**	Animals euthanized 24 h after PDT
	SCC + PDT + 15 days (n = 15)	Animals euthanized 15 days after PDT	**N + PDT + 15 days (n = 8)**	Animals euthanized 15 days after PDT

SCC: squamous cell carcinoma; DMBA: 7,12-dimethylbenz[*a*]anthracene; TPA: 12-*O*-tetradecanoylphorbol-13-acetate; PDT: photodynamic therapy; N: normal skin; n: number of animals.

ultraviolet radiation, is higher than that of melanoma among Caucasians [8]. SCC is associated with a high mortality rate because it is a risk factor for the development of other cancers. It is also associated with various other pathological conditions, such as chronic obstructive pulmonary disease, cardiovascular diseases, acute infections, pneumonia, and immune system dysregulation [9]. PDT has been used for the treatment of NMSC, as well as that of acne, actinic keratosis, and viral warts, and for photorejuvenation, in which its promotion of tissue remodeling yields cosmetic benefits [10].

PDT selectively affects tumor cells without injuring neighboring normal tissues [11], directly and/or indirectly causing cell death by activating an immune response or damaging tumor vasculature [2,12–15]. The tumor microenvironment is critical to the success of PDT, and tumors with a high collagen content respond better to treatment, owing to the vascular damage inflicted by this therapy [16].

In the present work, we evaluated the effects of MB-PDT on normal mouse skin and an experimentally induced mouse model of SCC at two arbitrary time points after treatment (24 h and 15 days). SCC was induced using the chemical carcinogens 7,12-dimethylbenz[*a*]anthracene (DMBA) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA). This particular model was chosen due to its similarities to SCC development in humans, with initiation, promotion, and progression stages. Our aim was to investigate the mechanisms involved in tissue damage during MB-PDT. We showed that this treatment was able to reduce tumor size, but did not result in complete remission. PDT also decreased cell proliferation and induced cytokine activation in SCC tissues. Its effects on normal skin were more pronounced, with cell death and tissue damage being increased. Thus, given that the same MB-PDT protocol was employed for the treatment of normal skin and SCC, the tissue microenvironment appears to interfere with treatment responses.

2. Material and methods

2.1. Animals

Sixty-six female albino mice (Swiss Webster, Unib:SW) were obtained from the breeding colonies of the Multidisciplinary Centre for Biological Research (CEMIB, UNICAMP, Brazil). The experiments were conducted in the Department of Pathology of the College of Veterinary Medicine and Zootechny at the University of São Paulo. The animals, which were 6–7 weeks old and weighed an average of 27 ± 2 g, were divided into separate cages (5 animals/cage) according to their weight. They were kept in polycarbonate cages at a controlled temperature (20 ± 4 °C) and relative humidity (45–75%) under a 12-h light/dark cycle, and had ad libitum access to food and filtered water (pH 7.0). The study design and all experimental procedures were approved by the Bioethics Committee for Animal Research of the Institute of Biomedical Sciences of the University of São Paulo (number 101), and were carried out in accordance with Brazilian legal guidelines concerning animal experiments (number 11.794/2008) and Decree 6.899/2009.

2.2. Experimental design

The hair on the dorsal region of each animal was removed 1 day before topical application of DMBA and TPA, both of which were obtained from Sigma-Aldrich (St. Louis, MO, USA). DMBA (200 nmol diluted in 200 µL acetone) was applied to the skin only once. In contrast, 1 week after the initial application, TPA (8 nmol diluted in 200 µL acetone) was topically administered twice per week for 28 weeks. Animals in the normal skin group received only topical acetone (200 µL), applied to the same region and at the same time intervals as those used in the induction of SCC.

PDT was administered 24 h after the final dose of TPA. The animals were anesthetized with an intraperitoneal injection of ketamine (150 mg/kg) and xylazine (20 mg/kg). Subsequently, 1% MB (Labsynth Products, São Paulo, Brazil) in saline solution was injected into normal and tumor tissues, with the latter receiving intratumoral and peritumoral injections. After 5 min to allow for dye absorption, the tissues were irradiated for 15 min with a gallium-aluminum-arsenide diode laser (Inova, Laserline, São Paulo, Brazil) at 74 mW/cm^2 with a spot area of 0.66 cm^2 and a wavelength of 650 nm, for a fluence of 100 J/cm^2. All PDT sessions were performed by the same operator.

Mice in which SSC had been induced were divided into 4 groups (2 untreated and 2 treated), and those with normal skin were divided into 3 groups (1 untreated and 2 treated). Twenty-four hours and 15 days after treatment, animals were euthanized with a lethal dose of anesthetic, namely, twice the dose used in a previous study [17]. The experimental design is summarized in Table 1.

2.3. Tissue processing

For histopathological evaluations and immunohistochemical tests, tissue samples were fixed in methacarn solution (60% methanol, 30% chloroform, and 10% acetic acid) for 12 h. For collagen content analyses, the specimens were fixed in Bouin fixative (50 mL formalin, 20 mL acetic acid, and 430 mL saturated picric acid solution) for 6 h. Following fixation, the samples were processed using routine histological techniques and embedded in Paraplast (Sigma-Aldrich). Normal and tumor tissues were cut into 5-µm sections and stained with hematoxylin and eosin (HE) for histopathological evaluation and Picrosirius red for collagen quantification. Fibrotic liver tissue was used as the positive control for collagen staining.

2.4. Tumor size measurement, grading, and histopathological evaluation

Tumors were photographed before and after PDT, and the diameter of each was measured using a caliper. SCC tissue slides were evaluated by two independent pathologists in a blinded manner, and the samples were classified according to their histological grade [18,19]. For standardization, only well-differentiated SCC samples were used in further tests. Owing to variations in size and morphology among the tumors,