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Effects of Photodynamic Therapy Mediated by Liposomal Aluminumphthalocyanine Chloride on Chemically Induced Tongue Tumors

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Abstract

Human oral squamous cell carcinoma is one of the ten most frequently diagnosed cancer types and several researches are thus focused on improving therapeutic protocols against this disease. Particularly, photodynamic therapy has emerged as an effective treatment for oral carcinoma. It is mediated by a photosensitizer, a molecule that, after being light-activated, promotes the chemical reactions responsible for the production of cytotoxic species involved in photodynamic therapy. Despite of clinical approvals, several classical photosensitizers present important drawbacks. However, when associated to liposomes, these drugs have been shown to present improved characteristics, such as increased tumor accessibility and high photodynamic activity in physiological media. Therefore, this work aimed to investigate the effects of photodynamic therapy mediated by a liposomal formulation of aluminum-phthalocyanine chloride, a second generation photosensitizer, on tongue tumors induced in Swiss mice through topical application of the carcinogen 4-nitroquinoline-1-oxide. The treatment of these tumors consisted in injecting the liposomal phthalocyanine in the peritumoral area followed by irradiation of the tumor, three hours later, with laser (670 nm, 100 J/cm²). Single and double applications of this protocol were tested. The photodynamic therapy based on this liposomal aluminum-phthalocyanine, both in single and double applications, produced intense necrosis on tumor tissue accompanied by infiltration of polymorphonuclear cells and thrombi formation on tumor-associated blood vessels. These findings suggest that photodynamic therapy based on liposomal aluminum-phthalocyanine chloride is effective against chemically induced oral cancer.

Keywords: Oral squamous cell carcinoma; Cancer; Liposome; Necrosis; Aluminum-phthalocyanine chloride; Photodynamic therapy; 4-nitroquinoline-1-oxide; Tongue tumor

Abbreviations: 4-NQO: 4-Nitroquinoline-1-Oxide; PDT: Photodynamic Therapy; AlPcCl: Aluminum-Phthalocyanine Chloride; OSCC: Oral Squamous Cell Carcinoma; ROS: Reactive Oxygen Species; PS: Photosensitizer; PBS: Phosphate-Buffered Saline; HE: Hematoxilin and Eosin; ANOVA: One Way Analysis Of Variance; HpD: Hematoporphyrin Derivative

Introduction

Oral squamous cell carcinoma (OSCC) is one of the ten most frequently diagnosed cancers worldwide [1]. Despite the efforts in developing and improving anticancer therapies, the 5-year survival rate for oral cancer patients has not significantly increased over the past several decades [2], evidencing the need for more effective treatments for OSCC. In this context, photodynamic therapy (PDT) is a relatively safe and effective anticancer therapy, approved for cancer treatment in several countries and used as an alternative treatment for superficial malignant tumors, such as OSCC lesions [3,4].

PDT is based on light-induced generation of a series of potentially cytotoxic reactive oxygen species (ROS) through photochemical reactions involving molecular oxygen, light at specific wavelengths and a photosensitizer [5]. Despite good therapeutic performance and clinical approvals, some issues regarding the tolerability and safety of the photosensitizers remain to be solved. The majority of the clinical studies on PDT have been conducted with photosensitizers like Photofrin[®], hematoporphyrin derivative (HpD), aminolevulinic acid or Foscan[®] [2,6-8]. Skin phototoxicity, low accumulation in tumor tissue and lack of photodynamic activity in aqueous media are among the most important drawbacks presented by these conventional photosensitizers [9].

Several researches are focused on the development of improved PS formulations in order to avoid these problems. Particularly, liposomal PS formulations have been widely investigated in the field of anticancer PDT and have shown to be effective against different cancer models [10]. Our group has previously demonstrated that a liposomal aluminum-phthalocyanine chloride (AlPcCl) formulation is effective in PDT against a model of mouse tongue tumor induced in Swiss mice by ectopic transplantation of Ehrlich cancer cells [11]. However, this liposomal formulation has not yet been applied in PDT against a model of chemically induced oral cancer. The result obtained with this kind of tumor can differ from that observed with transplantation models, as in chemical carcinogenesis some tumor characteristics may significantly vary for different individuals [12]. Thus, the present study aimed to investigate if liposomal AlPcCl-based PDT is effective against tongue tumors induced in Swiss mice by topic 4-nitroquinoline-1-oxide (4-NQO). This carcinogen is known to cause adduct formation and non-specific oxidative damage in the DNA, similarly to some tobaccoderived carcinogens, important inducers of OSCC development in humans [13]. Our results show that AlPcCl-based PDT induced tumor necrosis accompanied by formation of thrombi in tumor-associated

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blood vessels and infiltration of polymorphonuclear cells in tumor tissue.

Materials and Methods

Chemicals and materials

AlPcCl and 4-NQO were provided by Sigma-Aldrich Co. (Brazil). A diode laser (670 nm, BWF light source, Tech in) was used as the continuous light source.

Liposomal AlPcCl formulation

This formulation was prepared as described elsewhere [14].

Animals and tongue tumor induction

Female Swiss mice (6 weeks old, n=45), purchased from IQUEGO (Ribeirão Preto – SP, Brazil), were used for the studies. Mice were maintained under a 12-hour light/dark cycle and allowed food (standardized pellets) and filtered tap water *ad libitum* – with exception for the period of 2 hours after application of 4-NQO, when they had no access to water and food. All procedures involving animals were approved by the Animal Research Ethics Committee of University of Brasilia.

For tongue cancer induction, the carcinogen 4-NQO was applied as described elsewhere [15]. Briefly, for the tongue cancer-induced group (4-NQO group), a solution of 4-NQO (1 mg/ml in propylene glycol) was freshly prepared and applied with a microbrush three times a week for 16 weeks on the ventral surface of the tongue of 28 mice (Figure 1). The control group consisted of 3 mice receiving propylene glycol on the same area of the tongue and with the same timing as for the tongue cancer-induced group.

Photodynamic Therapy

Fifty-four weeks after the last application of the carcinogen 4-NQO, the animals presenting tongue tumors were randomly distributed in three experimental groups, accordingly to the treatment received: 1) treated with PDT on day 0 and sacrificed on day 3 (n=3); 2) treated with PDT on days 0 and 7, and sacrificed on day 14 (n=3); 3) no treatment, sacrificed on day 0 (n=2).

The PDT application protocol was based on the findings presented in our previous study [11]. Prior to PDT application animals were anesthetized with xylazine (5 mg/kg, ip) and ketamine (15 mg/kg, ip). Then, the tumor-bearing mice received an injection of 40 μ L liposomal AlPcCl suspension (5 μ M AlPcCl, in PBS) into the peritumoral area. Three hours later, the tumor was laser-irradiated (670 nm, 100 J/cm²).

Clinical evaluation

During experiments, animals were evaluated every three days for weight, tumor aspect/coloration and general clinical aspects.





Figure 2: Histopathological findings in 4-NQO induced tumors, characterized by (a) connective tissue infiltration (circle); (b) nuclear polymorphism (arrows); (c) keratinization of isolated cells (arrows); (d) reduction in epithelial intercellular adhesion, represented by an increase of interstitial space (arrows).

Histological analyses

Animal were sacrificed by cervical dislocation. Mice tongues were excised, fixed with 4% paraformaldehyde, dehydrated in ethanol, clarified in xylene, and embedded in paraffin wax. Histological sections of samples were then stained with hematoxilin and eosin (HE) for light microscopy analyses.

Statistical analyses

The weight of animals was expressed as mean \pm standard deviation. The data were compared using one way analysis of variance (ANOVA) and Scheffer's test ($\alpha = 0.05$).

Results and Discussion

The protocol of tumor induction through topical 4-NQO application was adapted from previous work [13,16] to Swiss mice, giving an efficiency of tumor induction of 28% at 54 weeks after the last application of the carcinogen. This relatively low incidence of tumors is expected in this model [12,13]. The induced tumors were highly polymorphic in macroscopic aspect, varying in size and anatomical position in the tongue. Histopathological findings showed squamous cell carcinoma characterized by connective tissue infiltration, nuclear polymorphism, keratinization of isolated cells, and reduction in epithelial intercellular adhesion, intense mitotic activity and increase in nuclear/cytoplasmic ratio (Figure 2). These tumors were diagnosed as invasive squamous cell carcinoma and their morphological pattern was similar to that observed in naturally developed human squamous tongue carcinoma [17]. These similarities may be related to the fact that tobacco carcinogens share their mechanism of action with 4-NQO [13]. Tobacco is known as the major risk factor for oral cancer development and contains more than 60 different carcinogens [18]. Therefore, the mechanistic similarity between 4-NQO and tobacco-derived carcinogens, along with the morphological aspect of 4-NQO-induced tongue tumors in Swiss mice, makes this model useful for evaluating new anticancer therapies designed for OSCC.

Some clinical characteristics of human oral cancer were also observed in 4-NQO-induced tumor-bearing Swiss mice. Weight loss (p < 0.05, Figure 3) and alopecia (Figure 4) were the most important clinical findings. Weight loss is commonly observed in patients with oral cancer and can be due to the clinical status of dysphagia, meaning difficulty in swallowing, which is associated with impairment in masticatory ability observed in oral cancer patients [5]. Particularly, dysphagia may be even more intense when tumors are located in the tongue because of the central role of this organ in the swallowing



Figure 3: Mice weight at the 70th week of the experimental protocol (mean \pm standard deviation). Control = mice that received only propylene glycol. 4-NQO = mice that received 4-NQO and developed tongue tumors. p<0.05 for Control vs 4-NQO groups.



4-NQO (b) groups. 4-NQO-treated mice presented visible hair loss (arrow) after the tumor induction period.

process. On the other hand, the observed alopecia (Figure 4) can be a consequence of low protein intake [19] observed in dysphagic individuals.

Once the tumor model was successfully adapted to Swiss mice, the next step in this work was to investigate the effects of liposomal AlPcClbased PDT on chemically induced tongue tumors. PDT has proven to be an effective approach against several kinds of cancer [6,8]. However, the effectiveness of this liposomal AlPcCl formulation has not yet been tested in chemically induced tongue tumors, a more realistic model for OSCC [12,18]. For the PDT treatment, liposomal AlPcCl was injected in the peritumoral tissue and three hours later the tumors were irradiated. The histological analysis showed major structural changes in PDT-treated tumors.

All the tongue tumors treated with liposomal AlPcCl-based PDT presented significant macroscopic and microscopic structural alterations indicating extensive cancer cell death, both after single and double PDT treatments. Ulcers and exposure of submucosal tissue were clinically observed after both single and double PDT application. These clinical findings were corroborated by microscopic analysis, which indicated superficial necrosis in treated tumors accompanied by exposure of connective tissue (Figure 5). After the double PDT treatment, a discrete macroscopic reepithelization of superficial ulcers was also observed; microscopically, epithelial cells were found recovering the exposed connective tissue. A few viable tumor cells were found in the deeper layers of connective tissue (Figure 5).

Ma et al. [20] found similar results. The authors reported that ALAbased PDT produced superficial necrosis on 4-NQO-induced tongue tumors followed by tissue regeneration 10 days after the treatment. Moreover, the same authors also observed remaining viable tumor cells in the deeper layers of connective tissue [20]. The persistence of viable cancer cells probably lies on the fact that PDT acts more strongly on the superficial cells of the tumor, while having limited action on malignant cells located at deeper layers of the tissue. Indeed, as observed in our experimental model, deeply located cancer cells are often observed in highly aggressive squamous oral cell carcinoma in humans [17]. The limited penetration of light in biological tissue is the main factor reducing the efficacy of PDT in the deeper structures of a tissue [9,21]. Light at wavelengths between 650 and 800 nm can potentially increase the efficacy of PDT against cells found deeply into a tissue, as this kind of light is less absorbed by biological molecules and thus penetrates deeper in biological tissues [21].

Besides tumor necrosis, this work evidenced that liposomal AlPcClbased PDT is effective in inducing other important events responsible for the anticancer activity of PDT. After both single and double treatments, a microvasculature collapse and an intense concentration of polymorphonuclear cells was observed in treated tumors. Histological observations showed that the treated tumor presented vascular shutdown as a result of thrombus formation and an increased number of innate immune cells, markedly neutrophils (Figure 6). These results are aligned with the literature. It is well established that the efficacy of anticancer PDT lies on three different and interrelated mechanisms of action [22,23]: 1) direct damage in cancer cells 2) induction of tumor ischemia through microvasculature collapse 3) immune system activation and/or boosting against tumor cells.

The formation of thrombi was observed in all the PDT-treated tumors (Figure 6). After the application of PDT, formation of thrombi generally occurs due to oxidative damage in endothelial cells and platelets, leading to a collapse of the microvasculature [9,24,25]. The efficiency in inducing tumor microvasculature collapse through



Figure 5: Clinical and histopathological aspects of control tongue tumors (a and d), tongue tumors treated with a single application of PDT (b and e), presenting superficial necrotic tissue; and tongue tumors treated with two applications of PDT (c and f), showing discrete superficial tissue reepithelization and viable cancer cells in inner tissues.



Figure 6: Vascular damage and infiltrated polymorphonuclear cells (circle) in PDT-treated tumors (a). In (b) is shown a thrombus in a tumor-associated blood vessel (arrow).

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PDT can be enhanced by PS delivery systems [26]. It is known that, in comparison with normal tissues, malignant tumors have more permeable blood vessels, allowing for the accumulation of PS-loaded liposomes in the tumor tissue [27,28]. The presence of thrombi in tumor-associated blood vessels, observed in this work, suggests that liposomal AlPcCl-based PDT may induce tumor ischemia, contributing to the efficacy of this anticancer therapy.

On the other hand, the presence of polymorphonuclear cells in PDT-treated tumors suggests that liposomal AlPcCl-based PDT elicits an immune response in tumor tissue, which can be directed against tumor cells. One of the major advantages of anticancer PDT is its ability in activating the immune system, in contrast to the strong suppression promoted by conventional antitumor therapies, such as chemo- and radiotherapy. The marked presence of innate immune cells observed in PDT treated tumors (Figure 6) can be due to the release of immunomodulatory molecules by stressed cancer cells or to the presence of cellular debris in necrotic tumor tissue [10,29]. It is known that damaged/stressed cells, particularly those undergoing oxidative stress, release the so-called danger signals. The danger signals are strong immune adjuvants and may elicit or boost adaptive immune responses against tumor antigens [29]. Adaptive immune responses might be important for the elimination of remaining viable malignant cells at the site of PDT application, as well as of distant metastatic focus [30,31].

In conclusion, the experimental model of 4-NQO-induced tongue tumors in Swiss mice shows histological characteristics close to those found in human OSCC, thus being a good model for testing anti-OSCC treatments. Liposomal AlPcCl-based PDT induced key features of an effective anticancer PDT, namely the necrosis of tumor tissue, the formation of thrombi in tumor-associated blood vessels and the activation of an innate immune response in the treated area. Therefore, the observed effects of liposomal AlPcCl-based PDT on chemically induced tongue tumors suggest that this therapy is effective against oral cancer.

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