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Original article

Evaluation of the effects of thalidomide-loaded biodegradable devices in solid Ehrlich tumor

Bruno Gonçalves Pereira [a,b], Silvia Ligorio Fialho [a], Cristina Maria de Souza [c], Geovanni Dantas Cassali [c], Armando Silva-Cunha [b,*]

[a] Pharmaceutical and Biotechnological Development, Fundação Ezequiel Dias, Belo Horizonte, Brazil
[b] Faculty of Pharmacy, Federal University of Minas Gerais, Belo Horizonte, Brazil
[c] Department of General Pathology, Federal University of Minas Gerais, Belo Horizonte, Brazil

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ABSTRACT

Regarding thalidomide's effects in cancer and the problems related to its physicochemical characteristics and toxic effects, we proposed a new biodegradable polymeric implant to this drug. In this paper, we evaluate the antiangiogenic activity and antitumor effect of thalidomide when incorporated in poly-lactide-co-glycolide (PLGA) implants in an animal model for Ehrlich tumor. This dosage form permits the prolonged drug release. The biodegradable implants could reduce the blood vessel in a chorioallantoic membrane (CAM) model. When applied to the Ehrlich tumor model, implant also showed to reduce the number of vessels. It was also observed to reduce areas of inflammation and increases the area of necrosis in the group of thalidomide implant. A 47% reduction in tumor volume was observed in the thalidomide implant group, which is discussed in relation to literature reported results of thalidomide conventional administration ways.

1. Introduction

Thalidomide was introduced in 1957 as a sedative drug with low toxicity and relative freedom from undesirable effects such as hangover; but, probably as a result of its antiangiogenic effect, its usage was limited [1]. Recently, there has been a great interest in its reintroduction for the use as antitumor agent [2] and it has been considered a potent inhibitor of angiogenesis. In previous studies, the inhibition of angiogenesis was also demonstrated in murine cancer models, like for Ehrlich tumor [3]. In other studies, the drug reduces expression of ICAM-1, which was thought to be involved in monocyte adherence to epithelial cells and cancer cell invasion [4], and it has important anti-inflammatory properties as inhibiting TNF-α expression [5]. In murine breast cancer models, some successes in reducing tumor volume have been described [4,6,7]. For example, Zahran et al. [7] observed tumor necrosis, increase in apoptosis and decrease in mitosis in tumor. These authors also reported Fas-L increased and reduction of VEGF and Ki67.

Giving their promising biological activities, unfortunately thalidomide has unfavorable physicochemical characteristics: practically insoluble in water and presents a rapid and spontaneous hydrolysis [8]. As a result, it could exhibit a problematic pharmacokinetic, with erratic and incomplete absorption after oral administration [9]. Moreover, it has severe adverse effects that limit its use, resulting sometimes in treatment withdrawal [10]. Besides, the diverse activities of the parent drug and their products (hydrolysis or hepatic metabolism) need further explanation.

Biodegradable implants may represent an innovative and effective dosage form for the delivery of thalidomide in cancer. The active substance is incorporated into a biocompatible carrier and by its application, the drug can be release in a prolonged rate at therapeutic levels directly to the targeted site, avoiding undesirably targeting to healthy tissues and harmful side effects [11,12]. Because these systems are biodegradable, they do not need to be removed by surgical process after complete drug release, contributing to a great adherence of the patient to the treatment [13].

In this study, we aimed to develop a thalidomide loaded poly-lactide-co-glycolide (PLGA) implant and to evaluate its therapeutic potential as local controlled release in Ehrlich tumor model in mice.

2. Materials and methods

Thalidomide (THD) was purchased from Microbiológica Química e Farmacêutica LTDA (Rio de Janeiro, Brazil). PLGA (PLGA 50/50, PURASORB® PDLG 5004, inherent viscosity midpoint of 0.4 dl/g) was

* Corresponding author. Faculty of Pharmacy, Federal University of Minas Gerais, Avenida Antônio Carlos, 6627, Pampulha, CEP 31270-901, Belo Horizonte, MG, Brazil. Tel.: +55 31 3409 6949; fax: +55 31 3409 6753.
E-mail address: armando@ufmg.br (A. Silva-Cunha).

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a gift from Purac Biomaterials (São Paulo, Brazil). Acetonitrile HPLC grade was from Merck Brasil (São Paulo, Brazil). Ultrapure water was obtained from Milli-Q, Millipore (Massachusets, USA). All other chemicals were of analytical grade.

2.1. Preparation of the devices containing thalidomide

The implants were developed according to the technique previously described by Fialho and Silva-Cunha [14]. Briefly, drug and polymer at a ratio of 1:4 were dissolved in a mixture of ultrapure water and acetonitrile and lyophilized. Implants with about 5 mg were obtained by hot molding (Fig. 1).

2.2. Evaluation of vascular effects of angiogenesis from thalidomide in a chorioallantoic membrane model

For this study, the method previously described by Nowak-Sliwinska et al. [15] with some modifications was used. The fertilized chicken eggs were placed into a hatching incubator (air humidity of 60%, temperature of 37 °C) and 3 days after fertilization, a hole of approximately 2 cm in diameter was opened in the eggshell in order to provide access to the chorioallantoic membrane (CAM). Two days after, biodegradable implants containing or not thalidomide were applied over the CAM surface in a well-defined part. After 48 hrs the CAMs were removed from the eggs and analyzed with an optical microscope (Leica, model DM4000B, Germany) coupled to a Leica digital CCD camera model DFC 280 (Software Leica Application Suite V 3.3.0, Germany). The obtained images were used for the quantification of the capillary features (size, and quantity) using the image processing program ImageJ (version 1.44p; National Institutes of Health; USA). As a negative control (NC), the same procedure was realized and phosphate-buffered-saline (PBS, pH 7.4) was applied over the CAM surface; as positive control (PC) Avastin® (Bevacizumabe 100 mg/4 ml, Produtos Roche Químicos e Farmacêuticos S.A., Brazil) was applied over the CAM surface.

2.3. In vivo study in solid Ehrlich tumor

2.3.1. Animals

Ten female Swiss mice weighing approximately 30 g, 6 to 7 weeks of age, were used in this study. They were kept in a quiet and climatically controlled environment with free access to standard mice chow and water. The study was approved by the Ethics Committee in Animal Experimentation of the Ezequiel Dias Foundation (Protocol 027/2011, Belo Horizonte, Brazil).

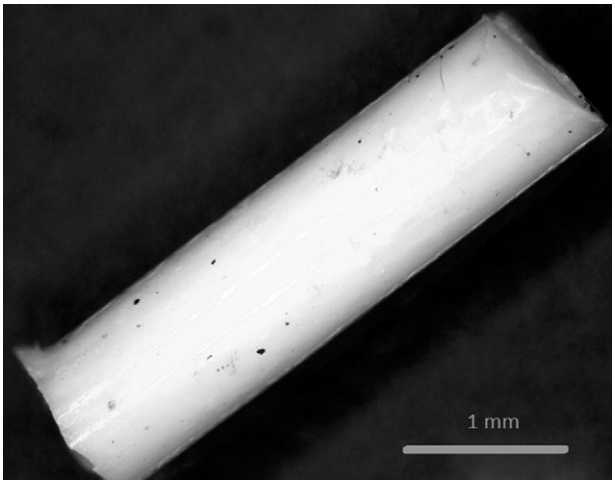

Fig. 1. Photography of the poly-lactide-co-glycolide (PLGA) implant containing thalidomide.

2.3.2. Experimental tumor model and treatment

The animals were inoculated in dorse with an injection (50 μL) containing 2.5×10^6 of Ehrlich tumor cells taken from ascites from Swiss mice. Five days after inoculation animals were divided into two groups of five animals each: Group 1 (control) implant without drug and group 2 (treated) implant containing thalidomide. Mice were anesthetized by intraperitoneal injection of 0.05 mL xylazine 2% and 0.05 mL of ketamine 10%, their dorsal hair was shaved, and their skin was wiped with 70% ethanol in preparation for implantation. The implants were placed into a subcutaneous pouch (through 1 cm long dorsal incision).

The protocols were designed to determine the effect of the thalidomide implant on tumor growing. The volume of the tumor was calculated using the following equation: tumor volume (mm^3) = (length × width^2)/2, where the length and width are in mm [16]. Body weights of animals were determined periodically and tumor size was measured at the end of the experiment. After 60-days postimplant administration, necropsy was performed with tumor removal of each animal for histopathological analysis.

2.3.3. Histopathological and immunohistochemical studies

Tissues were fixed in formalin (10% w/v in phosphate-buffered saline, pH 7.4) and sections (4 μm) were stained with hematoxylin and eosin (HE) and processed for light-microscopic studies. All staining were performed in paraffin-embedded sections mounted in glass slides. For the vessels number HE stainings were used to count microvascular density (MVD) in hot spot areas [17]. Microscopic images of cross-sections were obtained with a planapochromatic objective 40 in light microscopy. To perform morphometric analysis, images of cross-sections obtained from 15 fields per slide (8533 μm^2/field) were visualized with a planapochromatic objective 40× in light microscopy (final magnification = 1000×). The images were digitalized through a JVC TK-1270/JGB microcamera and transferred to an analyzer (Kontron Eletronics, Carl Zeiss–KS300 version 2).

In histological sections of tumor stained with HE the percentage of necrotic area, viable neoplastic tissue and inflammation were determined using a graticule of 25 dots. The images were captured with a microcamera Spot Insigh Color adapted to an Olympus Microscope (BX-40). Image analysis was performed using SPOT® software version 3.4.5 and Corel DRAW® version 7.468.

2.3.4. Statistical analysis

Results are presented as mean ± SEM. Comparisons between groups were carried out using the unpaired *t*-test and Kruskal–Wallis test. For all tests, the level of significance was set at $\alpha = 0.05$.

3. Results

3.1. Evaluation of vascular effects of angiogenesis from thalidomide in a chorioallantoic membrane model

The mean percentage of blood vessels remaining in the CAM after application of thalidomide-loaded PLGA implants (75.20 ± 5.04) were significantly lower ($P < 0.05$) than the negative control (set as 100%) but significantly higher than that of the positive control (70.07 ± 5.41) for $P < 0.05$. There was no significantly difference between PLGA implant without drug (98.95 ± 4.98) and the negative control ($P < 0.05$). It was observed that the procedure for implants fabrication did not alter the characteristics of the drug and that thalidomide present in the biodegradable system was able to reduce angiogenesis. PLGA implants without drug did not reduce the