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Original article

Healing activity of proteolytic fraction (P1G10) from *Vasconcellea cundinamarcensis* in a cutaneous wound excision model.

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ABSTRACT

The proteolytic enzymes from *Vasconcellea cundinamarcensis* have demonstrated efficacy to accelerate healing of skin lesions. We report here the efficacy of the proteolytic fraction − P1G10 during repair of excisional wounds in rodent model and analyze possible mediators involved. Using 0.05% P1G10 we observed on day 3rd increased wound contraction accompanied by an increase in activated neutrophils and VEGF relative to the control. On day 7th neutrophils returned to normal levels, and at 0.01% P1G10, an increase in NAG activity used to monitor monocyte/macrophage, was observed. On the other hand, on day 7th, we observed a decrease in TGF-β at 0.05% P1G10, accompanied by an increased transformation of the latent TGF-β to its active form. Also, on day 7th a reduction in MMP-9 activity and the number of apoptotic cells was observed along with an increase in fibroblast levels. Morphometrically, it appears that treatment with P1G10 accelerates the decline of initial inflammatory phase and reduces some unwanted effects likely caused by remaining TGF-β or MMPs, thus enhancing the quality of scar. Overall, these data suggest that the active proteolytic fraction P1G10 enhances the efficacy of repair in excisional cutaneous wounds

1. Introduction

Tissue repair triggers a set of vascular, cellular and biochemical events to replace dead or defective cells with newly formed ones [1]. Initially, neutrophils migrate into the wounded area to neutralize invading organisms and secrete cytokines that recruit other inflammatory cells. The inflammatory phase is followed by proliferation and migration of keratinocytes and fibroblasts from the wound edges [2,3]. Fibroplasia begins with the formation of granulation tissue comprising macrophages, fibroblasts and new vessels which are supported by a porous matrix containing fibronectin, hyaluronic acid and collagen isoforms I and III [4]. Maturation and remodeling account for an increase in tissue tensile strength [5,6]. Tissue remodeling involves apoptosis, production of extracellular serine proteases, metalloproteases and collagenases released by cells of connective tissue [7].

To date, there are a number of procedures designed to improve the physiology of wound healing. The relevance of these procedures varies according to the type of wound, age, the condition of patient or impaired physiology of healing. Natural agents (secondary metabolites, essential oils and plant lattices) have been used from ancient times, and ethnopharmacological accounts support their use as healing products. Topical application of latex proteins from several sources prompt procoagulant and/or wound healing activity [8–10].

Our group has characterized biochemically and pharmacologically the cysteine proteinases in *Vasconcellea cundinamarcensis* (syn *Carica candamarcensis* L.) [11–15]. The species is a member of the Caricaceae family, common to many areas in South America and contains several isoforms of cysteine proteinases like in latex from *C. papaya*. It is being proposed that these enzymes play a role as protecting agents during insect predation and following physical aggression [16]. This plant is commercially grown in Peru and Chile, where the edible fruit is mostly consumed fresh or canned. *V. cundinamarcensis* has been ethnopharmacologically used to treat digestive disorders and to enhance wound healing [17]. Studies using a proteinase fraction − P1G10 obtained by gel filtration show that its topical application enhances skin healing, as demonstrated in dermabrasion [18] and burn [19] models. The proteolytic fraction also enhances protection and healing of induced gastric ulcers in animal model [14,15], and displays antitumor/antimetastatic effect *in vivo* [20,21]. P1G10 has low dermal retention and skin permeation [18], as well as low dermal and systemic toxicity,

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and lacks mutagenic and genotoxic activity [22].

We provide here evidence of the healing activity of *Vasconcellea cundinamarcensis* fraction P1G10 in an excisional wound model by evaluating biochemical and histological parameters relevant to healing progress.

2. Material and Methods

2.1. Animals

Male C57Bl/6J mice (8-10 weeks old) were obtained from Centro de Bioterismo, Instituto de Ciências Biológicas (CEBIO/ICB) at the Universidade Federal de Minas Gerais (UFMG). The experiments were carried out in accordance to internationally accepted guidelines on laboratory animal handling and were approved by the institutional Ethics Committee on Animal Experimentation, CETEA-Protocol 174/2010. The animals were housed in individual cages with controlled temperature, humidity and light–dark cycle, with unrestrained access to food and water.

2.2. Chemicals

EDTA, sodium acetate, sodium hydroxide, dimethylsulfoxide, absolute ethanol, potassium monophosphate and Triton X-100 were from Merck, Darmstadt, Germany. Milli-Q-water used in various assays was purified by Millipore®, System Bedford, MA, USA. Ethyl alcohol was from Synth (Sao Paulo, Brazil). All the raw materials used in formulations were from Galena (Campinas, SP, Brazil) or Clariant (Sao Paulo, SP, Brazil). Sephadex G-10 was from GE Healthcare, (formerly Amersham Biosciences); Coomassie G-250 was from Bio-Rad Laboratories, Hercules, California, USA; TdT-Fragel™ DNA-Fragmentation Detection Kits (CAT QIA33, Calbiochem®, San Diego, CA, USA).

2.3. Production of P1G10 and P1G10-IAA

Unripe fruits of females *V. cundinamarcensis* grown commercially were the source of latex used in this study. A voucher specimen of the plant was deposited at the herbarium of the Universidad de La Serena, Chile, with #15063. Latex was collected by making incisions onto the surface of unripe fruits with a steel blade. Following collection, latex was stored in the dark, at −20 °C until lyophilized. The isolation of P1G10 was described previously [14]. Briefly, freeze-dried latex was dissolved in buffer containing 25 mM L-cysteine, 5 mM DTT and 10 mM EDTA pH 5.0 in 1 M sodium acetate solution. After low speed centrifugation and filtration of the supernatant (Whatman #1, Wilmington, MA, USA), the clear solution was chromatographed through Sephadex G-10 previously equilibrated with 1 M sodium acetate pH 5.0 at room temperature. The first protein fraction (P1G10) containing the bulk of proteolytic activity was pooled and concentrated by ultrafiltration (10,000 Da pore size) and stored at −20 °C until use. An aliquot of the concentrated fraction was analyzed by SDS-PAGE, reverse phase HPLC, its proteolytic activity and microbial counts routinely assessed. Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-MS) was used to characterize the molecular mass of proteolytic components in P1G10. Samples (25 µg) were mixed with α-cyano-4-hydroxy-cinnamic acid (matrix) dissolved in 0.1 g/100 mL trifluoracetic acid and processed in a UltrafleXtrem MALDI-TOF mass spectrometer (Bruker Daltonics, Leipzig, Germany), using Protein Calib Standart 2 (Bruker Daltonics) as standard. To gain insight into the relevance of the proteolytic activity on the healing effect, we used a P1G10 fraction previously inhibited with iodoacetamide (IAA) as described previously [23].

2.4. P1G10 formulation

The vehicle used was an hydrosoluble base containing 8% Polawax®, 6% Vaseline, 0.05% butyl-hydroxytoluene, 0.1% Propylaparaben, 5% Propilenglycol, 0.15% Methylparaben, 0.1% EDTA, 0.03% Aminomethyl propanol, 0.3% Imidazolidinyl-urea, 2% Cyclomethicone, prepared at the Faculty of Pharmacy-UFMG, Belo Horizonte, Brazil. The P1G10 protein sample was fully suspended in sterile water 10% w/v and dispersed into the hydrosoluble vehicle to a final concentration of 0.01 − 0.05% w/v. The control sample contained a similar volume of saline dispersed directly in the vehicle. The formulations were stored at 4 °C.

2.5. Wound Healing Assay

C57Bl/6J mice were anesthetized with 80 mg/kg of ketamine and 10 mg/kg of xylazine. After shaving the dorsal area and cleaning the exposed skin, four full-thickness (including the *Panniculus carnosus*) excisional wounds were punched in the middle of the dorsum using sterile 5 mm Ø biopsy needles [24]. Each lesion was covered with a thin layer of each formulation; 0.01% P1G10, 0.05% P1G10, 0.05% P1G10-IAA (proteolytically inhibited) or the vehicle alone (control-saline) dispersed in Polawax™ for 3, 7 and 14 days (n = 7 each treatment). A basal group not receiving any treatment or manipulation was included. Wounds were photographed at different intervals and their larger (R) and smaller (r) contours measured with a digital caliper. The area (A) was then calculated by Eq. (1)

$$A = \pi \times Rr \quad (1)$$

The healing rate was monitored by comparing the initial area (A_0) with the area after 3, 7 and 14 days (A_1) using Eq. (2)

$$M \pm SD = (A_0 - A_1)/A_0 \quad (2)$$

Following euthanasia with an overdose of anesthetics, wounds and surrounding skin were removed on different intervals (8 mm biopsy needles) and perpendicularly divided into two halves. One half was immediately frozen for biochemical analysis while the other half was fixed in 4% buffered formalin solution.

2.6. Measurement of VEGF and TGF-β

Wounded tissue was removed on days 3rd, 7th and 14th, homogenized (Tekmar TR-10, Ohio, USA) in PBS pH 7.4 containing 0.05% Tween-20 and centrifuged at 10,000 x g for 30 min [25]. The supernatant was collected to determine endogenous levels of VEGF and TGF-β1 (total and active) by ELISA. The assays were performed using kits from (DuoSet®, R & D Immunoassay kit, Minneapolis, MN, USA) according to the manufacturer's instructions. For dosage of active TGF-β1, samples were first acid-treated as described by Khan et al. [26].

2.7. Determination of myeloperoxidase (MPO) and N-acetylglucosaminidase (NAG) activities

The sediments recovered after centrifugation of tissue homogenates (see measurement of VEGF and TGF-β1 levels method) were divided into two portions and suspended with buffers specific for assays of myeloperoxidase (MPO) or N-acetylglucosaminidase (NAG) activities, used as markers of neutrophil and macrophage indexes, respectively, as described previously [25].