Original Paper

Skin Pharmacology and Physiology

Skin Pharmacol Physiol 2008;21:15–22
DOI: 10.1159/000109084

Received: March 9, 2007
Accepted after revision: June 30, 2007
Published online: October 2, 2007

Molecular Basis for the Regenerative Properties of a Secretion of the Mollusk *Cryptomphalus aspersa*

A. Brieva[a] N. Philips[c] R. Tejedor[b] A. Guerrero[a] J.P. Pivel[a]
J.L. Alonso-Lebrero[a] S. Gonzalez[d]

[a]R&D Department, IFC SA, and [b]Hospital Universitario de la Princesa, Universidad Autonoma, Madrid, Spain;
[c]Department of Biological Sciences, School of Natural Sciences, Fairleigh Dickinson University, Teaneck, N.J., and
[d]Dermatology Service, Memorial Sloan-Kettering Cancer Center, New York, N.Y., USA

Key Words

Cryptomphalus aspersa · *C. aspersa,* cellular and molecular effects · Extracellular matrix assembly · Mollusk · SCA, regenerative properties · Skin aging

Abstract

A screen for natural products bearing pharmacological properties has yielded a secretion of the mollusk *Cryptomphalus aspersa* (SCA), which possesses skin-regenerative properties. In this report, we outline some of the cellular and molecular effects underlying this observation. First, we found that SCA contained antioxidant SOD and GST activities. In addition, SCA stimulated fibroblast proliferation and rearrangement of the actin cytoskeleton. Additional mechanisms involved in the regenerative effect of SCA included the stimulation of extracellular matrix assembly and the regulation of metalloproteinase activities. Together, these effects provide an array of molecular mechanisms underlying SCA-induced cellular regeneration and postulate its use in regeneration of wounded tissue.

Introduction

Cutaneous aging is the result of a complex process where genetics as well as chronological and environmental factors (particularly UV radiation) are involved. Skin aging manifests as wrinkles, diminished structural integrity and impaired wound healing due to alterations in the remodeling process of the extracellular matrix (ECM). Collagen and elastin impart strength and their degeneration with the passing of time causes skin to become fragile, and aged in appearance [1–4].

Many factors can affect skin regeneration. The presence of pathogens in the lesion may impair regeneration, and other factors such as reactive oxygen species can also play a negative role in this process [5]. In addition, dermal fibroblasts must proliferate and migrate into the injured tissue, covering the lesion and manipulating the ECM ('matrix remodeling') to ensure scar formation and promote healing, a process compromised by skin aging [6].

The search of substances with regenerative properties has led many pharmaceutical companies to develop extensive search programs aimed to identify natural products that can induce skin regeneration or stimulate natural regeneration. In this regard, it has been noted that snails perceive radiation, retract their orientation organs, and se-

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

1660–5527/08/0211–0015$24.50/0

Accessible online at:
www.karger.com/spp

Salvador Gonzalez
Memorial Sloan-Kettering Cancer Center
160 East 53rd Street, New York, NY 10022 (USA)
Tel. +1 212 610 0185, Fax +1 212 308 0530
E-Mail gonzals6@mskcc.org

crete large amounts of mucous substances as a defensive response in order to protect themselves from harmful radiation. In addition, snails never suffer from skin infections, which directed our attention to the possibility of using this secretion as a possible treatment of skin-compromising diseases. In this regard, an early study showed that a secretion from the mollusk *Cryptomphalus aspersa* (SCA) induces skin regeneration after wound healing impairment from acute radiodermatitis [7]. However, the molecular basis underlying this effect is not known.

In this report, we have evaluated the regenerative properties of SCA using multiple in vitro approaches. We have found that SCA possesses antioxidant capabilities and induces fibroblast proliferation. A complementary mechanism is provided by the fact that SCA promotes ECM assembly, which is essential for wound healing and tissue plasticity. Finally, SCA inhibits MMP production, which limits the extent of the damage during wounding and scar formation. Together, these mechanisms contribute to the observed beneficial effects of SCA and postulate its employment in regenerative therapy.

Material and Methods

This study was performed in compliance with the guidelines of the Ethics Committee of the Hospital Universitario de la Princesa.

SCA Preparation

SCA was prepared according to US patent US 5538740. Briefly, the gastropod was physically stimulated by centrifugation to increase the secretions naturally produced by the mucinous, albuminous, and salivary glands. Then, the secreted fluids were separated and collected from the live gastropod, clarified by centrifugation and further clarified by filtration through 0.22-μm filters. Further dilutions were performed in aqueous solution (pH 7.4). SCA toxicity was assayed by trypan blue. The highest effect/toxicity ratio was achieved at 100 μg/ml SCA, thus we employed this concentration for most of the assays described.

Cells

After informed consent was given, normal skin tissue was obtained from surgical specimens of the forearm. Dermis from each subject was cut into small pieces, and the adherent outgrowth cells from the minced skin were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) containing 10% fetal calf serum (FCS) (Invitrogen). Each sample was incubated at 37°C in a 5% CO_2 humidified incubator and the culture medium was changed every 3 days. The cells were passaged with use of trypsin, maintained in a subconfluent state, and expanded for three passages before the experiments. Six different normal skin surgical specimens were used in this study. CHO-K1 cells were obtained from ATCC (Manassas, Va., USA) and maintained in DMEM supplemented with 10% FCS.

Reagents and Assay Kits

Human fibronectin and the GST kit were from Sigma Chemical Co. (St. Louis, Mo., USA). The ABTS antioxidant kit was from Vector Labs (Burlingame, Calif., USA). The SOD, MMP-1, MMP-2 kits were from Calbiochem Co. (San Diego, Calif., USA). The elastase ELISA kit was from Alpco Diagnostics (Salem, N.H., USA). All other reagents were from Sigma unless otherwise indicated.

Antioxidant Assays

GST and SOD activities were determined according to the kits manufacturers' protocols in 1 mg/ml SCA suspensions. Positive and negative controls were provided in the kits and employed as indicated. The ABTS assay was performed adding SCA (100 μg/ml), quercetin (2.5 μM) and Trolox C (10 μM) or vehicle alone at 300 s. Absorbance at 743 nm was measured at different time points before and after addition of the reagent.

Fibroblast Proliferation Assay

Twenty-five thousand human dermal fibroblasts per well were plated into 12-well plates and treated for a week with the indicated doses of SCA together with 0.1 mM citrate pH 5.0, 100 U/ml LMW heparin or 1 μM inositol hexasulfate. Monolayers were washed with PBS, fixed in 10% formalin, and rinsed with distilled water. Cells were then stained with 0.1% crystal violet (Sigma) for 30 min, rinsed extensively, and dried. Cell-associated dye was extracted with 2.0 ml 10% acetic acid. Aliquots were diluted 1:4 with H_2O, transferred to 96-well microtiter plates, and the optical density at 590 nm was determined. Values were normalized to the optical density at day 0 for untreated cells. Within an experiment, each point was determined in triplicate; each growth curve was performed at least twice.

Fibronectin Assembly Assay

CHO-K1 cells were cultured on glass coverslips using Ham's F-12 (Gibco-BRL, Life Technologies Ltd, Paisley, UK) medium supplemented with penicillin/streptomycin and 10% fetal bovine serum. Cells were cultured for 24 h and then incubated for another 24 h in the presence of fibronectin (5 μg/ml) and/or SCA (50 or 100 μg/ml). Cells were then fixed in chilled methanol for 10 min. Fibronectin was stained with the anti-80 kDa pAb followed by incubation with Alexa488-conjugated anti-rabbit antibody (Invitrogen). Samples were mounted in Mowiol (Calbiochem, La Jolla, Calif., USA), and were examined in a Leica DMR (Leica, Mannheim, Germany) photomicroscope with 63× and 100× immersion objectives. Images were processed in a Leica Q550CW Workstation (Leica Imaging Systems, Ltd, Cambridge, UK), using Leica QFISH software V1.01. At least 50 fields from three independent experiments were examined.

Cell Morphology Assay

For assessment of fibroblast morphology, human dermal fibroblasts from healthy volunteer donors were cultured for 24 h in the presence or absence of 100 μg/ml of SCA, fixed with 4% formaldehyde in PBS and permeabilized using 0.1% Triton X-100. For F-actin staining, coverslips were incubated with 1 μg/ml Alexa488-conjugated phalloidin (Invitrogen) in PBS for 1 h at room temperature. Samples were mounted and examined as described previously. At least 200 cells/condition from three independent experiments were examined.

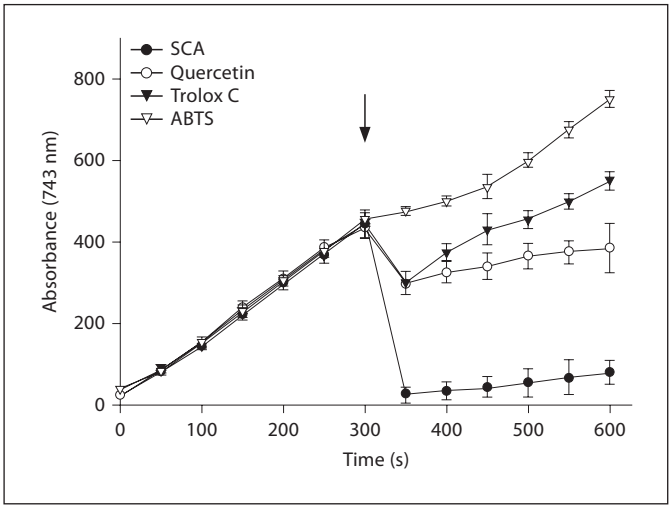

Fig. 1. Antioxidant properties of SCA. ABTS assay was carried out adding SCA (100 μg/ml), quercetin (2.5 μM), Trolox C (10 μM) or vehicle alone at 300 s (arrow); absorbance at 743 nm was measured at the time points indicated. Mean ± SEM of three independent experiments performed in triplicate are shown.

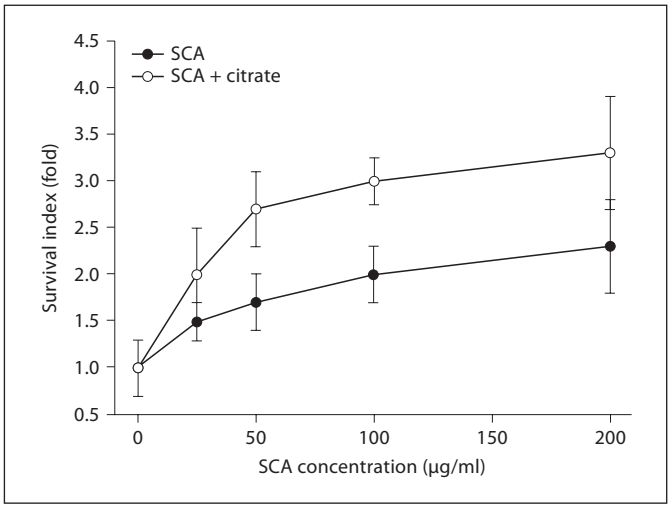

Fig. 2. SCA promotes fibroblast proliferation. Human dermal fibroblasts from healthy donors were incubated with the indicated doses of SCA in the presence or absence of 0.1 mM citrate pH 5.0. Cells were cultured in these conditions for a week and then cell numbers were determined spectrophotometrically by crystal violet staining. Data are referred to cells cultured in medium in the absence of SCA or other additives. Mean ± SEM of three independent experiments performed in triplicate are shown.

Western Blot

Fibronectin (0.5 and 1 μg/lane) and SCA samples (5 and 10 μg/lane) were mixed with 3× Laemlii buffer, boiled for 5 min at 100°C, and resolved by 7.5% PAGE/SDS under reducing conditions. Resolved proteins were transferred to nitrocellulose membranes, which were blocked using 5% non-fat milk for 1 h at room temperature. Membranes were incubated for 90 min with the corresponding antibodies (0.5 μg/ml mAb anti-FN80 and 1933, 1:2,500 dilution of the Telios and anti-FN80 pAb), washed three times with TBS-0.2% Tween 20 and incubated with HRP-conjugated anti-mouse (mAb anti-FN80 and 1933) or anti-rabbit (Telios and anti-FN80 pAb) antibodies for 60 min. After three additional washes, the membranes were incubated with ECL reagent (Amersham) and revealed using Kodak XB-1 film (Rochester, N.Y., USA).

MMPs ELISA Determination

Human dermal fibroblasts from healthy volunteer donors were incubated in the presence of increasing doses of SCA, and cell culture supernatants were collected after 24 h. MMP-1 and MMP-2 expression was assessed by specific ELISA according to the manufacturer's protocol.

Results

Antioxidant Properties of SCA

To determine if SCA possessed antioxidant properties, we assayed SCA for different antioxidant activities. This assay revealed that SCA cannot antagonize cytochrome C oxidation (data not shown), but possesses superoxide dismutase (SOD) as well as GST activities (4.1 ± 2.1 and 3.4 ± 1.5 U/ml, respectively). SOD is a key enzyme during inactivation of the superoxide anion (O_2^-) radical and hydrogen peroxide (H_2O_2) [8], whereas GST is a typical phase 2 enzyme responsible for detoxification of both ROS and electrophilic xenobiotics [9].

In addition, the capability of SCA to scavenge free radicals was assayed by the ABTS method (fig. 1). We found that SCA sequestered $ABTS^+$, the free radical generated in the assay more potently than 10 μM Trolox C, used as a positive antioxidant control. In addition, SCA also inhibits the production of $ABTS^+$, as determined by the slope of the absorbance curve (fig. 1). The extent of the inhibition was more extensive to that exerted by 2.5 μM quercetin. Together, these data demonstrate that SCA possesses multiple modes of antioxidant action, acting at the level of free radical production and also sequestering free radicals.

SCA Induces Fibroblast Proliferation in vitro and Regulates Fibroblast Cytoskeleton Reorganization

To investigate if the regenerative properties of SCA are related to enhanced cell proliferation, we assayed its effect on fibroblast proliferation in vitro. Interestingly, SCA