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

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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-

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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.

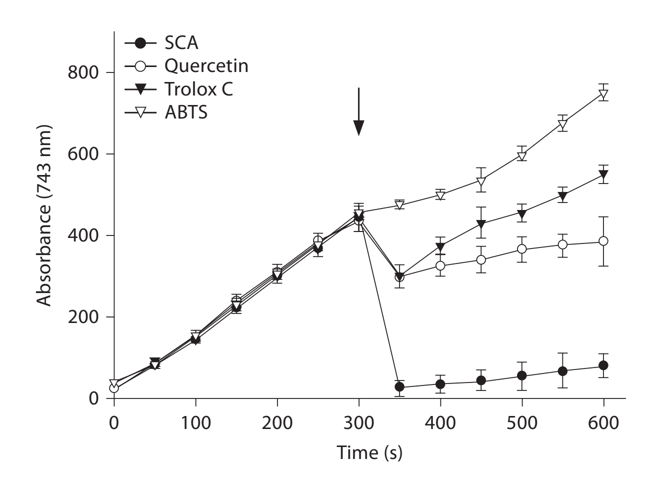

Fig. 1. Antioxidant properties of SCA. ABTS assay was carried out adding SCA (100 μg/ml), quercetin (2.5 μM), Trolox C (10 μM) or vehicle alone at 300 s (arrow); absorbance at 743 nm was measured at the time points indicated. Mean ± SEM of three independent experiments performed in triplicate are shown.

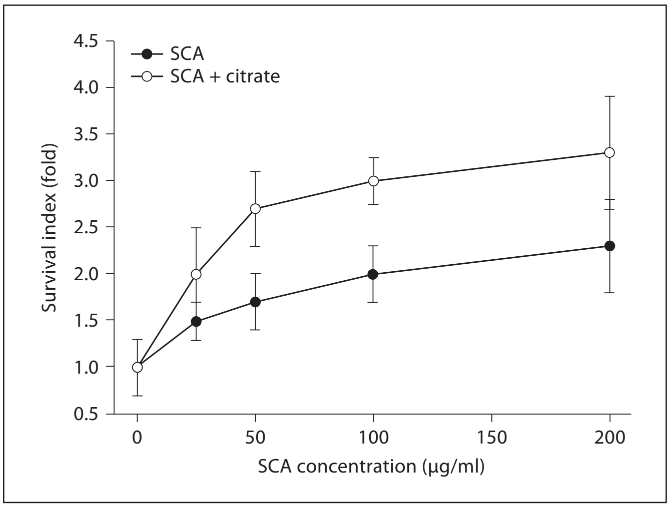

Fig. 2. SCA promotes fibroblast proliferation. Human dermal fibroblasts from healthy donors were incubated with the indicated doses of SCA in the presence or absence of 0.1 mM citrate pH 5.0. Cells were cultured in these conditions for a week and then cell numbers were determined spectrophotometrically 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

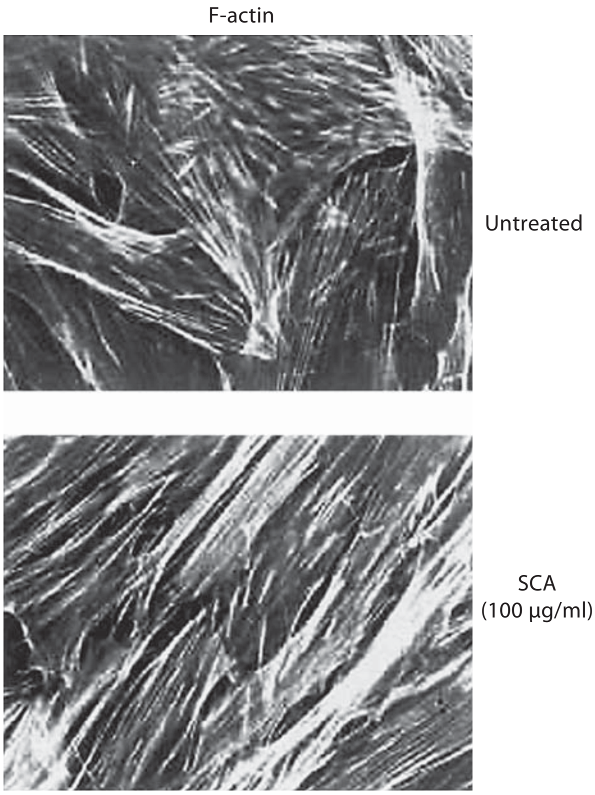

Fig. 3. SCA induces actin reorganization in human dermal fibroblasts. Human dermal fibroblasts were incubated for 24 h with 100 μg/ml SCA on glass coverslips, fixed and stained with Alexa488-phalloidin as indicated in Material and Methods. Representative images from three independent experiments are shown.

increased cell proliferation, which was further enhanced by addition of citrate, which has been shown to induce fibroblast proliferation [10] (fig. 2). This result was further reinforced by the fact that SCA increased cell survival upon irradiation with UVA light (data not shown), which suggests that the observed beneficial effect of SCA is due to combined antioxidant and proliferative activities. In addition, we found that SCA affected the morphology of human dermal fibroblasts, inducing actin reorganization, bundling and microfilament alignment, which resulted in cell elongation (fig. 3).

SCA Stimulates Fibronectin Secretion and Assembly

Since ECM assembly is a key event in tissue regeneration, we assayed the putative effect of SCA in this process. CHO-K1 cells were cultured on either untreated or 5 μg/ml human fibronectin in the presence of two different doses of SCA, and fibronectin assembly was monitored after 24 h by indirect immunofluorescence. We found that SCA induced fibronectin assembly compared to control cells (fig. 4). Interestingly, SCA seemed to increase fibronectin secretion since it increased fibronectin deposition by the cells in the absence of exogenous fibronectin (fig. 4). To demonstrate that the increase of fibronectin was not due to fibronectin present in the secretion, we analyzed SCA by ELISA and Western blot using a panel of antibodies anti-fibronectin; included that used for the experiments shown in figure 4. We found that SCA contained no fibronectin reactivity using any of the antibodies used neither by Western blot nor by ELISA (fig. 5, and data not shown). This demonstrates that the observed effect of SCA in fibronectin assembly and secretion is due to its effect on the cells, not on direct deposition of ECM included in the extract.

SCA Downregulates Matrix Metalloproteinase Expression in Dermal Fibroblasts

Increased metalloproteinase expression has been associated to impaired wound healing and regeneration. To investigate if SCA enhanced regeneration through the regulation of metalloproteinase expression, we assayed MMP expression by dermal fibroblasts in the presence of different concentrations of SCA. We found that SCA significantly inhibited MMP-1 and MMP-2 expression (fig. 6), suggesting that limitation of MMP expression contributes to its regenerative properties.

Discussion

SCA is a natural secretion from the mollusk *C. aspersa* that bears regenerative properties in experimental acute radiodermatitis produced in a rat model [7]. Furthermore, SCA application twice daily for 3 months also results in a significant improvement of clinical and histopathological photoaging signs [5]. However, the molecular foundations of the beneficial effect of SCA remained unknown. In this report, we have analyzed in vitro several possible contributions to the physiological effect of SCA. First, we found that SCA bear antioxidant activities as well as free radical scavenging capability. The role of oxidation in wound healing is controversial [11], but there is a clear correlation between enzymatic and non-enzymatic cutaneous antioxidants and its protection from ROS-mediated skin damage induced by UV radiation. Indeed, UV radiation can decrease endogenous antioxi-

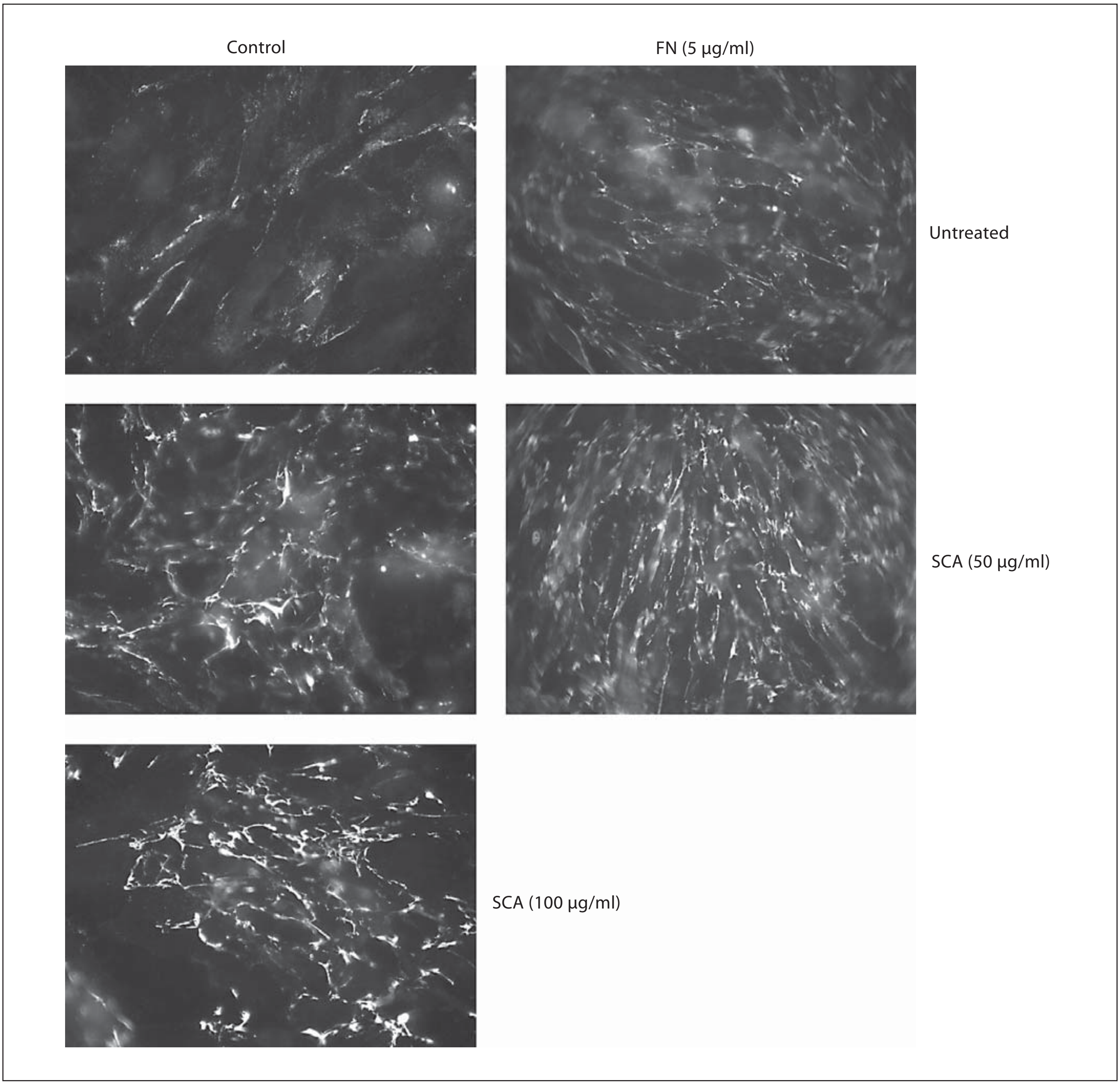

Fig. 4. SCA induces fibronectin assembly in CHO-K1 cells. CHO-K1 cells were cultured for 24 h on glass coverslips, and then SCA at the indicated concentrations and/or human fibronectin (5 µg/ml) were added for another 24 h. Fixed coverslips were stained for fibronectin. Representative images from four independent experiments are shown for each condition.

dant levels and lead to increased damage [12]. In these cases, antioxidant compounds can modulate abnormal remodeling after trauma or prevent or reverse clinical signs associated with photoaging secondary to ROS.

In addition, we found that SCA promoted fibroblast survival and proliferation. Proliferation is essential for wound healing, since the formation of the scar relies not only on cell migration (i.e. invasion of the wound by

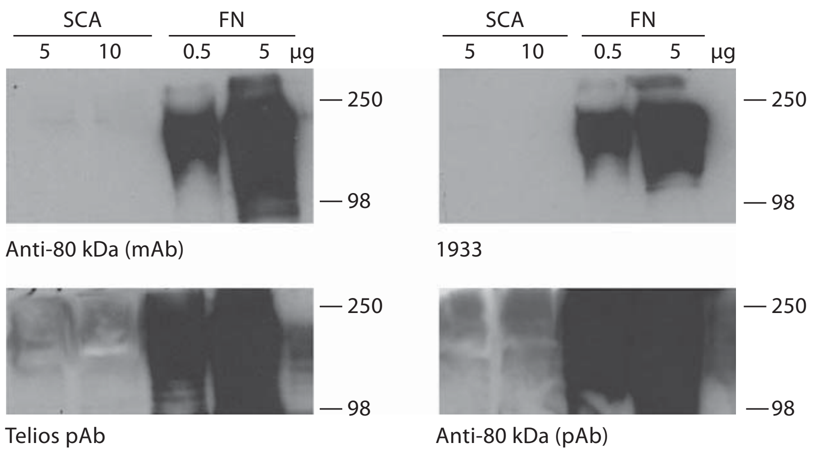

Fig. 5. SCA does not contain fibronectin reactivity recognized by a panel of anti-fibronectin antibodies. Samples containing the indicated amounts of SCA or human fibronectin were separated by PAGE/SDS, transferred to nitrocellulose membranes and blotted with the indicated anti-fibronectin antibodies as stated under Material and Methods. Representative blots from three independent experiments are shown.

border cells), but also on cell proliferation to replenish the tissue zones that provide the migrating cells invading the wound. The effect of SCA was similar to that observed in dermal fibroblasts treated with bFGF [13]. Thus, we hypothesized that SCA might contain an FGF-like activity responsible for fibroblast proliferation. However, the fact that the secretion belongs to a mollusk impaired the analysis of bFGF, since the only kits for mammalian FGFs are currently available, and these did not recognize any FGF-like activity in SCA (data not shown). Thus, we devised an indirect strategy, adding well-characterized stabilizers and enhancers of bFGF to SCA to modulate its effect on proliferation. These included citrate [14–16], which had no effect when added to the cultures alone (data not shown). However, in conjunction with SCA, it boosted its pro-proliferative effect, suggesting that citrate enhanced the putative FGF-like activity contained in SCA.

We also found that SCA increased ECM assembly when added alone or in conjunction with exogenously added fibronectin. This is a very important phenomenon during wound healing since matrix remodeling is a key step in fibroblast motility for wound healing and scar formation. In fact, matrix secretion and rearrangement is absolutely required during in vitro wound healing assays, since scratching the surfaces also depletes ECM from the wound, which is required for lamellipodial extension and net cell migration into the wound. The first obvious conjecture was that SCA contained fibronectin. However, none of the antibodies assayed against fibronectin recognized any component of SCA. However, this provided an

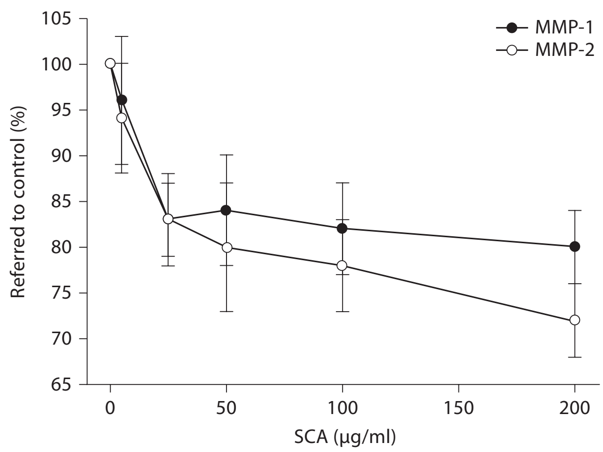

Fig. 6. Regulation of matrix metalloproteinases by SCA. Human dermal fibroblasts were incubated for 24 h with the indicated doses of SCA and MMP-1 and MMP-2 expression was assessed by specific ELISA. Results shown are the mean ± SEM of three independent experiments performed in triplicate.

excellent internal control since these antibodies could not detect SCA added to the cultures, and increases observed are likely to be due to increased fibronectin expression and/or secretion by the cultures. Indeed, bFGF has been shown to increase fibronectin fibrillogenesis [17]. The effect of SCA on fibronectin assembly seems more dramatic as shown by enhanced bundling of fibronectin fibers, but its effect on secretion cannot be ruled out without more experimentation, which is underway in our laboratory.

Another possible mechanism for fibronectin remodeling was an increase in integrin activation induced by SCA. Indeed, integrin activation has been linked to matrix remodeling [18]. However, we found that SCA did not activate β_1 integrins by itself nor did it affect Mn^{2+}-induced conformational changes, as determined by the use of HUTS-21, a conformation-dependent antibody (data not shown). This result suggests that the effect of SCA on ECM assembly relies on mechanisms other that integrin-fibronectin binding.

Finally, SCA has been shown to slightly inhibit the expression of MMP-1 and MMP-2. ECM proteinases are upregulated during wound healing and scar formation, and they are believed to be essential for wound closure and scar formation [19, 20]. This effect of SCA is apparently at odds with the fact that bFGF enhances MMP-1 and MMP-2 expression [21, 22]. We speculate that this apparent contradiction can be explained by SCA containing at least two active principles, an FGF-like activity and an inhibitor of MMP expression. Alternatively, SCA could enhance the expression of TIMPs (tissue inhibitor of metalloproteinases). This can also help explaining this dual effect of SCA, since bFGF has been shown to inhibit or increase TIMP expression depending on the cell model [23–25]. This is a very interesting topic, and the presence of TIMP-like molecules; stimulation of TIMP expression or the presence of other non-related MMP inhibitors in SCA is being currently studied in the laboratory.

Together, our data provide evidence for the molecular mechanism underlying the observed regenerative effect of SCA and open an interesting avenue of research aiming to clarify these effects and isolate the active principles responsible of the effects herein described.

Conflict of Interests

The authors declare that the main subject of this research (SCA) is subject to US patent US 5538740. Except commercial exploitation of the aforementioned patent by IFC, which provides funding for the authors as indicated, the authors declare that they have no other competing financial interests. S.G. is a consultant for IFC.

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