Phycosaccharide Al, a Mixture of Alginate Polysaccharides, Increases Stem Cell Proliferation in Aged Keratinocytes

Alexandra Charruyer, Stephen Fong, Lili Yue, Sarah T. Arron, Ruby Ghadially

Correspondence:
Ruby Ghadially M.B.,Ch.B., FRCP(C)Derm
Professor, Dept. of Dermatology,
University of California, San Francisco
Co-Director Epithelial Section of the UCSF Eli and Edythe Broad Center of Regeneration Medicine and Stem Cell Research,

Email: ruby.ghadially@derm.ucsf.edu

Background
Keratinocyte stem cells (SCs) are responsible for tissue maintenance and regeneration. With age, SCs progressively lose proliferative capacity and ability to repair tissue injury (1,s1). Thus, increasing SC proliferation and self-renewal would be expected to benefit aged skin.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/exd.13051

This article is protected by copyright. All rights reserved.
SCs divide to produce either exact copies (undifferentiated SCs), or differentiated daughters (transit amplifying progenitors) that divide a few times before terminal differentiation. Only SCs self-renew, producing exact copies of themselves and thus maintaining an undifferentiated state. For most proliferation-inducing agents, whether hyperproliferation results from increased proliferation of SCs, or solely of transit amplifying progenitors is unknown.

Naturally occurring compounds are attractive new bioactive therapeutic agents. Plant oligosaccharides are potent signaling molecules in growth and development, acting via oligosaccharide-specific high-affinity receptors on cell membranes (2). Alginate oligosaccharides enhance growth of neonatal keratinocytes and alginate oligosaccharides are a possible cofactor for EGF-dependent stimulation of keratinocytes (3). Phycosaccharide Al (Barnett Products Corporation, Englewood Cliffs NJ) is a high molecular weight (3,500) alginate polysaccharide containing sodium mannuronate and sodium guluronate (5%, w/w in water) isolated from the brown algae *Laminaria digitata* (M/G ratio is >1, Fig. s1).

**Questions addressed**

Our purpose was to determine whether naturally occurring alginate polysaccharides affect proliferation of keratinocyte SCs from aged individuals (61-79 years).

**Experimental design**

SCs form large highly proliferative colonies *in vitro*(4,s2). We determined the effects of Phycosaccharide Al (a cosmetic ingredient; INCI name *Hydrolyzed align*, CODIF International, France), on the number of large highly proliferative colonies produced by aged human keratinocytes in vitro. The holoclone assay was used to study SC frequency/ self-renewal of Phycosaccharide-treated aged human keratinocytes (4,5,s3). Only SCs self-renew and this is accomplished predominantly by asymmetric SC divisions. Thus, finally, we treated aged keratinocytes with Phycosaccharide Al or vehicle and determined the number of SCs undergoing asymmetric self-renewal.
Results
To determine the number and size of colonies produced by keratinocytes from aged individuals, treated with Phycosaccharide AI, fresh keratinocytes were plated at clonal density with 0.1% Phycosaccharide AI or vehicle. After 3 weeks, the total number of colonies was not increased significantly (Fig. 1a). However, the number of colonies >2mm was increased significantly in Phycosaccharide AI-treated vs. vehicle-treated keratinocytes (6.9±1.3 vs. 1.9±2.4 colonies per 1000 cells, P = 0.01, n=4, Fig. 1b). No significant difference in the number of colonies <2mm was detected (Fig. 1c). While colonies arise from both SCs and from the more numerous transit amplifying progenitors, the increase in large highly proliferative colonies indicates an increase in highly proliferative, self-renewing SCs. In young keratinocytes, we found that, likewise, Phycosaccharide AI-treatment increased the number of large highly proliferative colonies (Fig. s2a-c). Interestingly, perhaps related to the relative deficit in large highly proliferative colonies in aging keratinocytes, the effect of Phycosaccharide AI on large highly proliferative colonies was more pronounced in aged than young (Fig. s2d-f).

For holoclone formation studies (Fig. 2a) single human keratinocytes are cultured individually. Individual colonies are then transferred to 100mm dishes. Assessment of secondary cultures allows identification of the primary clone as holoclone, meroclone or paraclone. Holoclone-forming cells (presumed SCs due to extensive proliferation/ self-renewal) produce large rapidly growing colonies in secondary culture (<5% terminal). Paraclone-forming cells (differentiated cells) produce small, terminally differentiating colonies. Meroclone-forming cells (intermediate growth potential) produce colonies intermediate between holoclones and paraclones(5). To determine whether Phycosaccharide AI increases the number of holoclones, single aged human keratinocytes, isolated from skin biopsies within hours of surgery, were individually plated in 96-well plates. After 3 weeks, colonies were transferred to secondary dishes for 4 weeks. Using the secondary dishes, primary clones were retrospectively classified as holoclones, meroclonos, or paraclones(5) (Fig. 2b,c and Tables s1, s2). Treatment with
Phycosaccharide AI vs. vehicle increased numbers of holoclones (32.8±10.1 vs. 2.4±2.4, \( P = 0.02 \), n=5, Fig. 2c). Notably, clones from freshly obtained aged keratinocytes did not achieve optimal size for assessment until 4 weeks, in contrast to previous studies that required only 12 days (4,5,9,s3). The varying size of secondary clones seen in our study and other studies potentially results from; aged vs. young keratinocytes, fresh vs. passaged keratinocytes, different media employed, and various sources of fibroblasts. Of additional interest, very few holoclones were formed from donors greater than 60 years (2.4% of total clones) in keeping with previous work [1.6%(5)].

As an additional measure of SC self-renewal, we determined the number of asymmetric SC self-renewal divisions after Phycosaccharide AI or vehicle treatment. Asymmetric SC divisions can be identified by segregation of cell fate determinants, including Numb, to the differentiated cell during cell division (Fig. 2d,e) (6-8). Phycosaccharide AI treatment resulted in increased numbers of asymmetric SC divisions (8.5±1.3% vs. 4.5±1.0% per 100,000 cells, \( P = 0.047 \), n=4, Fig. 2f).

These studies show that Phycosaccharide AI treatment of aged keratinocytes increases the numbers of keratinocytes producing large highly proliferative colonies and results in an increase in holoclone formation and asymmetric SC divisions, both indicators of increased SC self-renewal.

**Conclusions**

Previously we showed that the proliferative capacity of individual aged keratinocyte SCs was decreased compared with young adult keratinocyte SCs (1,s1,s4). The present study showed that aged keratinocytes almost completely lack holoclone formation, as previously observed(5). Phycosaccharide AI increased the number of aged keratinocytes with high proliferative potential and increased SC self-renewal (holoclone formation and asymmetric SC division), indicating
that alginate polysaccharides can improve some of the deleterious proliferative changes associated with aging and may be expected to improve epidermal homeostasis in aged skin.

While the hierarchical model of epidermal generation has been challenged in favor of a single type of interfollicular epidermal progenitor(s5), our current study, along with others, is consistent with the existence of stem and progenitor cells with different proliferative abilities(4,5,9,s6).

Most importantly, this work indicates that it is possible to manipulate keratinocyte SC divisions using naturally-occurring agents providing a novel approach to the treatment of aging, wounds, and other cutaneous disorders associated with hypo and hyperproliferation.

Figure Legends

Figure 1. Phycosaccharide Al treatment increases the number of cells producing large highly proliferative colonies in vitro. (a-c) Aged keratinocytes were treated with 0.1% Phycosaccharide Al or vehicle (n=4, quadruplicates. Each n corresponds to a single human donor). (a) The total number of colonies per 1000 cells plated, (b) the numbers of large highly proliferative colonies (> 2mm) per 1000 cells plated, and (c) the numbers of small colonies (<2mm) per 1000 cells plated, was determined. NS: not significant. **P ≤ 0.01. PAI: Phycosaccharide Al.

Figure 2: Phycosaccharide Al treatment increases SC self-renewal. (a-c) Phycosaccharide Al treatment increases holoclone formation. (a) Holoclone assay. Single human keratinocytes, isolated from aged individuals, were cultured with either 0.1% Phycosaccharide Al or vehicle. After 3 weeks, each primary clone was transferred to a secondary dish. After 4 weeks, secondary colonies were fixed and stained and the primary clones retrospectively classified as described in Methods and Results. As found by Barrandon and Green (5), three types of colonies were observed; rapidly growing, terminal, and intermediate wrinkled colonies. Primary clones were classified as holoclones if they produced <5% terminal colonies. (b) Secondary
colonies from individual Phycosaccharide AI and vehicle-treated aged keratinocytes.

Secondary cultures from holoclones show large colonies with smooth edges and central differentiation (Phycosaccharide AI-treated dishes on the right). (c) The number of holoclones produced by Phycosaccharide AI-treated vs. vehicle-treated keratinocytes was determined. (n=5. Each n corresponds to a single human donor). *P ≤ 0.05. (see also Tables s1 and s2.)

(d-f) Phycosaccharide AI treatment increases asymmetric stem cell self-renewal divisions. (d) Schematic of SC self-renewal divisions assessed by Numb staining. (e) Representative pictures showing immunostaining to assess asymmetric segregation of Numb in Phycosaccharide AI or vehicle-treated aged keratinocytes. BrdU incorporation was used to identify cell divisions. (f) The number of asymmetric self-renewal SC divisions per 100,000 total keratinocytes plated (n=4. Each n corresponds to a single human donor). *P ≤ 0.05. PAI: Phycosaccharide AI. ACD: asymmetric stem cell division. SCD: symmetric cell division.

Acknowledgements

We thank Prof. Bernice Krafchik, Dept. Dermatology, University of Toronto, for her critical reading of the manuscript.

Author contributions

Manuscript and data analysis: RG, AC. Experimental work: SF, LY, AC. Samples: SA.

Conflict of interest

The authors have declared no conflicting interests.

References


This article is protected by copyright. All rights reserved.