

Aloe-emodin inhibits proliferation of adult human keratinocytes *in vitro*

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Synopsis

Aloe-emodin (AE) is a plant-derived hydroxyanthraquinone with several biological activities. It is present in a variety of skin-conditioning agents containing aloe extracts, but its influence on keratinocyte growth was not examined so far. We investigated the influence of AE on human keratinocyte proliferation and apoptosis *in vitro*. AE significantly inhibited proliferation of cultivated human keratinocytes at 5 μM concentration, as revealed by incorporation of radioactive thymidine. The antiproliferative effect of AE was accompanied with induction of apoptosis, but not necrosis, as demonstrated by flow cytometric analysis and lactate dehydrogenase release assay. Based on the half maximal inhibitory concentration values, we demonstrated that AE may impair proliferation of keratinocytes at concentrations far below the industry standards for commercial products containing aloe extracts. Therefore, further research of AE effects on the human skin and proper labeling of products are necessary for maximizing benefits from aloe extracts and to avoid undesired responses.

INTRODUCTION

Since ancient times, *Aloe vera* has been used to help wound and burn healing. Several reports emphasize the stimulatory effect of whole extracts or components from aloe on skin cell proliferation and collagen synthesis (1). By the virtue of these effects, aloe is currently used in a variety of commercial products, including sunscreens, cosmetics, and lotions. Aloe crude extracts typically present in topical formulations contain a variety of active substances differing considerably between manufacturers and from batch to batch (2). Moreover, the functional relationship between the various compounds and their net effects on human cells has not been completely established.

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Aloe-emodin (AE), a hydroxyanthraquinone from *Aloe vera* leaves has been recognized for a long time as a potent laxative, while in the last decade, several other biological activities are reported including its antiproliferative effect (3). On the contrary, there are data indicating that AE stimulates growth and increases DNA synthesis in some primary cell cultures (4). Although it is present in topical formulations that are applied to the skin, the influence of AE on normal keratinocytes has not been examined so far. In this study, we investigated the effect of AE on primary adult keratinocyte monolayer cultures. Our data indicates that AE significantly reduces proliferation capacity of the keratinocytes cultivated *in vitro* at doses as low as 5 μM , and this effect can be attributed to the induction of apoptosis.

MATERIAL AND METHODS

CELL CULTURE AND REAGENTS

Skin samples were obtained from 10 subjects undergoing cosmetic surgery, who all gave the informed consent, and processed as described previously (5). Cells were used for experiments after the third or fourth passage. AE was dissolved in dimethylsulfoxide (DMSO) (both from Sigma, St. Louis, MO). Control cell cultures contained same amount of DMSO as cultures with the 5 μM concentration of AE used in the particular experiment.

CELL PROLIFERATION AND LACTATE DEHYDROGENASE (LDH) RELEASE ASSAY

Keratinocytes were cultivated in 96-well plates (3×10^3 cells/well) for 48 h, then washed and cultivated for an additional 72 h in fresh medium containing 1.25, 2.5, or 5 μM AE or DMSO as a control. During the last 24 h of incubation, keratinocytes were pulsed with 1 μCi of [^3H]thymidine per well and proliferation was determined as described previously (5). The half maximal inhibitory concentration (IC_{50}) values for the inhibition of proliferation were calculated using Calcsyn software (Biosoft, Cambridge, UK) (5). LDH release assay was employed to assess cell necrosis. Keratinocytes were cultivated under the same conditions as for the proliferation assay and LDH assay was performed as previously described (6).

DETERMINATION OF APOPTOSIS

For the assessment of apoptosis, keratinocytes were cultivated in 24-well plates (3×10^4 /well) for 48 h, washed and incubated for an additional 6 h in fresh medium containing 5 μM of AE or DMSO as a control. Following trypsinization, the cells were stained with fluorescein diacetate (FDA) and trypan blue (TB), and analyzed as described previously (5).

STATISTICAL ANALYSIS

Statistical significance of the differences in keratinocyte proliferation in the group of 10 donors was determined by the analysis of variance (ANOVA) for repeated measures.

The differences in apoptosis of control versus treated keratinocytes were analyzed by paired *t*-test. The value of *p* < 0.05 was considered significant.

RESULTS

AE DECREASES PROLIFERATION OF THE KERATINOCYTES CULTIVATED *IN VITRO*

We first assessed the influence of AE on keratinocyte proliferation (Figure 1A). The proliferation of control cultures containing only DMSO was 9194 ± 3101 counts per

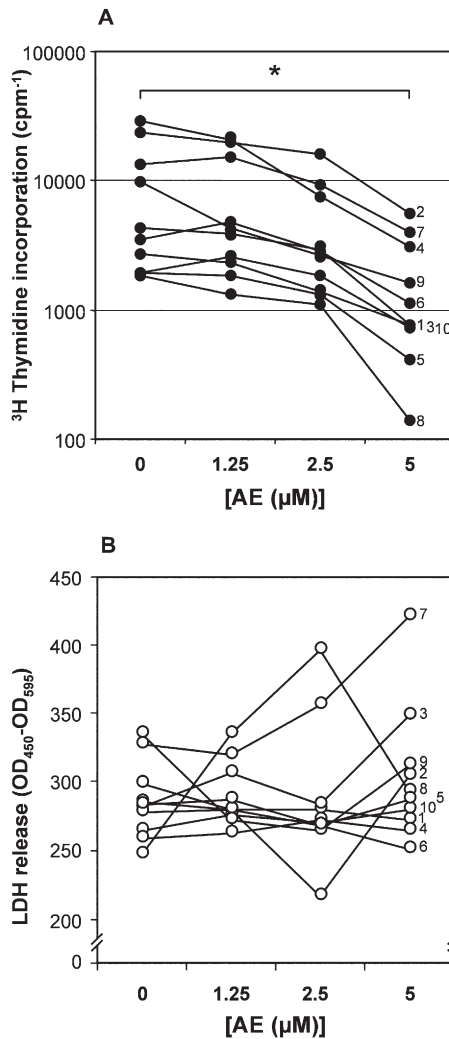


Figure 1. Aloe-emodin (AE) downregulates keratinocyte proliferation. Keratinocytes were incubated without (0) or with AE at indicated concentrations for 72 h. The incorporation of [³H]thymidine was determined and results are presented as counts per minute (cpm). (A) Each value is a mean of triplicate cultures of one sample. (B) The observed antiproliferative effect of AE was not followed by an increase in lactate dehydrogenase release. Keratinocytes from 10 different donors are labeled 1–10 (Figures 1A and 1B). **p* < 0.05.

minute (cpm), in cultures treated with 1.25 μM AE $7647 \pm 2\,423$ cpm, in cultures treated with 2.5 μM AE 4680 ± 1535 cpm, and in those treated with 5 μM AE 1815 ± 566 cpm (mean \pm SEM). The results of ANOVA showed that AE significantly inhibited keratinocyte proliferation (Figure 1A) in a dose dependent manner ($p = 0.019$), with the maximal inhibitory effect achieved at 5 μM AE ($p = 0.021$ compared to control). Although AE was able to significantly inhibit keratinocyte proliferation at 5 μM concentration, considerable differences in keratinocyte response were observed among the cultures obtained from different individuals (Figure 1A). The observed individual differences were confirmed by comparing the IC_{50} values between different donors (Table I), which revealed that keratinocytes from some donors display markedly different sensitivity to the antiproliferative action of AE (e.g., compare donors No. 8 and 10). The observed antiproliferative effect of AE was not accompanied by a significant increase in LDH release during a 72 h incubation period (Figure 1B) (ANOVA $p = 0.66$), as well as at earlier time points (6, 24, and 48 h; data not shown), indicating that the inhibitory effect of the drug on keratinocyte proliferation was probably not due to necrotic cell death.

THE INFLUENCE OF AE ON KERATINOCYTE APOPTOSIS

In order to explore the mechanisms underlying the observed inhibition of keratinocyte proliferation, we assessed whether AE induces apoptotic cell death (Figure 2). A significant increase in the percentage of apoptotic (TB/FDA⁺) keratinocytes could be observed 6 h upon addition of 5 μM AE (16.7 ± 1.3 , mean \pm SEM) compared to the untreated cultures (10.4 ± 0.1 , mean \pm SEM, $p = 0.001$) (Figure 2). The number of necrotic cells in keratinocyte cultures did not significantly change upon treatment with AE (Figure 2), thus further supporting results of the LDH release assay (data not shown).

DISCUSSION

In this study, we demonstrated antiproliferative effect of AE on the primary human keratinocytes from 10 different donors cultivated *in vitro* (Figure 1A). The antiproliferative effect of AE was previously reported in various human cell cultures at 20–80 μM concentration (6). It was a consequence of induction of p21, p53 and subsequent G₁ cell cycle arrest (7), or increased number of cells within S phase (8). In some experiments, increased proportion of cells cycling at a higher ploidy level (>G₂/M) was observed (8). The latest may explain increased DNA synthesis in the rat hepatocytes reported by Wolfe *et al.* in this particular experimental setup (4). In line with previously presented data (6), our

Table I
The Half Maximal Inhibitory Concentrations of Aloe-emodin for Proliferation of Keratinocyte Cultures from 10 Different Donors

Sample	1	2	3	4	5	6	7	8	9	10	Mean	SEM
IC_{50} (μM)	2.96	3.91	3.02	3.46	2.86	2.14	1.79	0.96	3.22	5.40	2.97	0.38
IC_{50} (ppm)	1.10	1.45	1.12	1.28	1.06	0.79	0.66	0.36	1.19	2.00	1.10	0.14

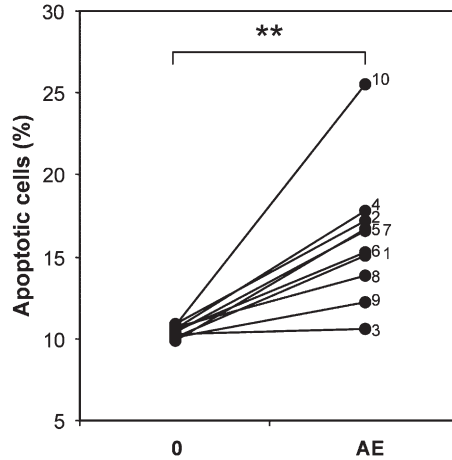


Figure 2. Aloe-emodin (AE) induces keratinocyte apoptosis. Keratinocytes from 10 different donors (labeled 1–10) were incubated for 6 h in the absence (0) or presence of $5 \mu\text{M}$ AE and the number of apoptotic cells was determined by flow cytometry. $**p < 0.01$.

results demonstrate that growth inhibition in keratinocyte cultures was not a consequence of cell necrosis, as demonstrated by LDH release assay (Figure 1B). Clearly, the mechanisms of AE antiproliferative effect are complex and cell-specific.

It seems that keratinocytes are more sensitive to antiproliferative effect of AE than commonly used tumor cell lines. We find this information particularly relevant, having in mind the fact that aloe extracts containing anthraquinones including AE are frequently applied to the skin. We demonstrated antiproliferative effect of AE at the $5 \mu\text{M}$ concentration, which corresponds to 1.35 ppm w/w in the cultivation medium (Figure 1A). In fact, the average AE IC_{50} value for the tested keratinocyte cultures was calculated to be 1.1 ppm (Table I). The industry-established standard for the anthraquinone contents in the products for the nonmedicinal use, frequently marketed as wound or burn healing promoting products, is 50 ppm (3) exceeding by far demonstrated IC_{50} . Therefore, AE present in such preparations may be detrimental in all conditions requiring epithelization including burns, wounds, and ulcers. On the other hand, one can keep in mind that observed antiproliferative effect of the AE can in fact be beneficial in hyperproliferative skin diseases such as psoriasis.

To determine beneficial and adverse effects of AE present in the aloe extracts, further experiments should be conducted, preferentially on the skin explants. Another set of experiments with head-to-head comparison of aloe extracts preparations with and without AE, possibly in clinical setting, could be warranted to delineate the net effect of AE on human skin. Also, the manufacturers should carefully screen raw materials and final products for the AE contents and label them properly.

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