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## **The wound healing effect of a glycoprotein isolated from aloe vera**

### **Keywords:**

- Ingredients in aloe vera
- Glycoprotein
- Wound healing effect

### **Summary:**

Aloe vera is well known for its wound healing effect. The objective of this study is to find out which of the ingredients in aloe vera is in fact responsible for this healing effect. The results show that it is a glycoprotein.

### **Source:**

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## The wound-healing effect of a glycoprotein fraction isolated from aloe vera

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### Summary

**Background** Aloe vera has been used as a family medicine for promoting wound healing, but it is not known which component of the plant is effective for this purpose.

**Objectives** To isolate and characterize the component effective in wound healing.

**Methods** Chromatography, electrophoresis and spectroscopic methods were used. The cell-proliferation activity of each component isolated was measured by a [<sup>3</sup>H]thymidine uptake assay. The cell-proliferation activity of the effective component was tested on a three-dimensional raft culture (cell culture technique by which artificial epidermis is made from keratinocytes). The effect of the active component on cell migration and wound healing was observed on a monolayer of human keratinocytes and in hairless mice.

**Results** A glycoprotein fraction was isolated and named G1G1M1D12. It showed a single band on sodium dodecyl sulphate–polyacrylamide gel electrophoresis, with an apparent molecular weight of about 5.5 kDa. It exhibited significant [<sup>3</sup>H]thymidine uptake in squamous cell carcinoma cells. The effect of G1G1M1D12 on cell migration was confirmed by accelerated wound healing on a monolayer of human keratinocytes. When this fraction was tested on a raft culture, it stimulated the formation of epidermal tissue. Furthermore, proliferation markers (epidermal growth factor receptor, fibronectin receptor, fibronectin, keratin 5/14 and keratin 1/10) were markedly expressed at the immunohistochemical level. The glycoprotein fraction enhanced wound healing in hairless mice by day 8 after injury, with significant cell proliferation.

**Conclusions** It is considered that this glycoprotein fraction is involved in the wound-healing effect of aloe vera via cell proliferation and migration.

**Key words:** *Aloe barbadensis* Miller, cell proliferation, epidermal growth factor receptor, fibronectin receptor, keratin, raft culture

Aloe vera is a perennial succulent belonging to the lily (Liliaceae) family. This plant has been known as the 'healing plant' or 'silent healer'. It has been claimed that aloe has several important therapeutic properties, including wound healing,<sup>1</sup> thermal injury healing,<sup>2</sup> anti-inflammation<sup>3</sup> and immunomodulation.<sup>4</sup> Using these effects, aloe is nowadays used in a variety of commercial products, including sun creams, cosmetics

and lotions. However, whole extracts of aloe are used, and the relationship between the various components and their effects has not been well elucidated. Therefore, in aloe research it is important to isolate single components with biological effects, to examine these effects, and to elucidate their functional mechanism.

There are several reports about the stimulatory effect on cell proliferation of whole extracts or components from aloe. Davis *et al.*<sup>3</sup> showed that aloe vera extract stimulated fibroblasts for growth and repair of the

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synovial model. Recently, Yagi *et al.*<sup>5</sup> found proliferation effects in a glycoprotein. Lee *et al.*<sup>6</sup> reported that aloesin stimulates proliferation of cultured human hepatoma SK-HEP-1 cells by upregulating cyclin E/cyclin-dependent kinase 2 activity. Davis *et al.*<sup>7</sup> reported that mannose-6-phosphate in aloe has wound-healing and anti-inflammatory effects in rats. Wolffe *et al.*<sup>8</sup> reported that aloe emodin resulted in a  $\approx 2.5$ -fold increase in DNA synthesis in primary rat hepatocytes. Moreover, Lee *et al.*<sup>9</sup> found an angiogenic activity of dichloromethane extracts of aloe vera gel. Despite these studies, the relationship between many aloe components and their effect has not yet been elucidated. We aimed to isolate components effective in cell proliferation and wound healing and to elucidate the effects using various tools.

For our research, we used a raft culture system, which is a three-dimensional culture designed to permit long-term culture at the air-liquid interface. Keratinocytes proliferate in a raft culture, and culture at the air-liquid interface enhances organization and differentiation of the air-exposed stratified epithelium.<sup>10</sup> Although several differences from its *in vivo* counterpart have been observed in this artificial skin, keratinocytes grown on a dermal equivalent at the air-liquid interface stratify and enter the terminal differentiation pathway, as (i) basal cells express bullous pemphigoid antigen and laminin, (ii) suprabasal cells synthesize involucrin and 67-kDa keratin, (iii) keratohyaline granules are present in a recognizable granular layer, and (iv) a horny layer is formed. Therefore, this organotypic skin construct is presently being used as a human *in vitro* skin model for dermatological and pharmacological research.<sup>11</sup>

Reports concerning the wound-healing effect of aloe in experimental animals or humans have been contradictory. Rodriguez-Bigas *et al.*<sup>2</sup> and Davis *et al.*<sup>1</sup> reported faster wound healing in experimental animals in wounds managed with aloe gel extract than in controls. However, Schmidt and Greenspoon<sup>12</sup> reported that the use of aloe vera gel on dermal wounds was associated with a significant delay in wound healing compared with a treatment based on an otherwise identical regimen that did not include aloe vera. There have been several reports recently on the wound-healing effects of aloe components. Acemannan isolated from aloe vera did not show a significant wound-healing effect,<sup>13</sup> while mannose-6-phosphate improved wound healing in mice.<sup>7</sup> Therefore, in the present study we isolated and studied

the effect of an active component from aloe vera upon cell proliferation and migration activity, and then examined its wound-healing effect in hairless mice.

## Materials and methods

### Materials

All reagents were of analytical grade purchased from Sigma-Aldrich (St Louis, MO, U.S.A.) unless otherwise mentioned. The freeze-dried aloe vera gel (designated as G1) was supplied by Aloecorp (Broomfield, CO, U.S.A.).

### Isolation of glycoprotein fraction G1G1M1D12

The aloe gel (25 g, G1) was suspended in 200 mL of distilled water and four volumes of 95% ethanol were added. The solution was allowed to stand for 12 h at 4 °C. The yellowish supernatant was decanted off from the white residue and the precipitate was centrifuged at 13,000 *g* for 30 min at 4 °C. The supernatants were combined, dialysed against phosphate-buffered saline (PBS, pH 7.2), evaporated and lyophilized (G1G1, 14.5 g). G1G1 (1 g) was applied to an Amberlite XAD-2 column (250 mL resin, 2.5 × 2.5 cm, Organo Co., Suwa, Nagano, Japan). The column was eluted successively with distilled water (500 mL), 50% aqueous methanol (200 mL) and methanol-acetone (1 : 1 by volume, 400 mL). The eluates with 50% aqueous methanol were dialysed against PBS pH 7.2, concentrated, and lyophilized to give a dark grey powder (G1G1M1, 53 mg). G1G1M1 (53 mg) was dissolved in PBS pH 7.2, then dialysed for 3 days (molecular weight cut-off 5000), and lyophilized (G1G1M1D, 23 mg). This fraction, G1G1M1D, was dissolved in 0.02 mol L<sup>-1</sup> NH<sub>4</sub>HCO<sub>3</sub> and applied to an ion-exchange column (2.5 × 20 cm) of DEAE-Toyopearl 650M (Tosoh Co., Tokyo, Japan). The column was equilibrated and eluted with 0.02 mol L<sup>-1</sup> NH<sub>4</sub>HCO<sub>3</sub> (600 mL), and then eluted with 0.3 mol L<sup>-1</sup> NaCl (600 mL). Each fraction of 15 mL was collected and detected at 210, 280 and 490 nm (phenol-H<sub>2</sub>SO<sub>4</sub> method to detect the carbohydrate moiety of glycoprotein)<sup>14</sup> by an ultraviolet-visible spectrophotometer (V-3210, Hitachi, Tokyo, Japan). Cell-proliferation activity was detected in the G1G1M1D12 fraction at 0.3 mol L<sup>-1</sup> NaCl elution.

*Sodium dodecyl sulphate–polyacrylamide gel electrophoresis, thin-layer chromatography and infrared spectroscopy/nuclear magnetic resonance analysis of sugar and amino acid components of G1G1MIDI2*

The purity and molecular weight of G1G1MIDI2 were determined by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) using a 20% separation gel. The carbohydrate moiety of the glycoprotein fraction G1G1MIDI2 was detected by periodic acid–Schiff's reagent.<sup>15</sup> After protein separation by PAGE, the SDS gel was incubated in 7.5% acetic acid for 1 h at 4 °C, then 0.2% periodic acid for 1 h, and finally stained by Schiff's reagent. Thin-layer chromatography (TLC) was performed on Kieselgel 60F<sub>254</sub> plates (Merck, Whitehouse Station, NJ, U.S.A.) and cellulose plates (Merck) using *n*-butanol–ethanol–water (5 : 5 : 7 by volume) as a developing agent. Detection was done by spraying 1% Ce(SO<sub>4</sub>)<sub>2</sub> in 10% aqueous H<sub>2</sub>SO<sub>4</sub> followed by heating at 110 °C (silica gel TLC plates) and by 5% aniline phthalate (cellulose TLC plates). Spectroscopic analysis was performed to detect glycoprotein-specific chemical properties of G1G1MIDI2: Fourier transform infrared (IR) spectroscopy (Bruker IFS48, Bruker, Germany), <sup>1</sup>H-nuclear magnetic resonance [NMR; Bruker AM-400; 400 MHz; solvent D<sub>2</sub>O; internal standard 3-(trimethylsilyl) propionic-2,2,3,3-d<sub>4</sub> acid, sodium salt], and <sup>13</sup>C-NMR (Bruker AM-400; 100 MHz; solvent D<sub>2</sub>O).

G1G1MIDI2 (10 mg) was dissolved in 1 mol L<sup>-1</sup> H<sub>2</sub>SO<sub>4</sub> (10 mL) and hydrolysed at 100 °C for 6 h in a sealed tube. The hydrolysate was neutralized with BaCO<sub>3</sub>, and then filtered. The filtrate was passed through a column of Dowex 50WX8 resin (acidic form) with deionized water. Sugar components were analysed by high-performance liquid chromatography (HPLC; Spectra System P2000; Spectra Physics, Mountain View, CA, U.S.A.; detector UV 1000). The HPLC column Carbo Pak PA1 4 × 250 mm (Dionex, Sunnyvale, CA, U.S.A.) was eluted with water–0.2 mol L<sup>-1</sup> NaOH (92 : 8 by volume) at 1 mL min<sup>-1</sup> at room temperature.

G1G1MIDI2 (10 mg) was dissolved in distilled water (2 mL), then NaIO<sub>4</sub> (5 mg) was added. The reaction mixture was stirred at 20 °C for 20 h in the dark. The excess of NaIO<sub>4</sub> was decomposed by the addition of ethylene glycol (1 mL) with stirring for 1 h. The solution was dialysed overnight and lyophilized. The product was dissolved in distilled water (1 mL), and NaBH<sub>4</sub> (5 mg) was added slowly. The solution was stirred at room temperature for 7 h in the dark. The

excess of NaBH<sub>4</sub> was decomposed by the addition of 0.1 mol L<sup>-1</sup> acetic acid. After dialysis and lyophilization, a small amount of 0.05 mol L<sup>-1</sup> H<sub>2</sub>SO<sub>4</sub> was added and the mixture stored overnight at room temperature. The solution was then neutralized with 0.05 mol L<sup>-1</sup> NaOH. After dialysis and lyophilization, a carbohydrate-degraded product (3 mg) was obtained. G1G1MIDI2 was hydrolysed with 6 mol L<sup>-1</sup> HCl (10 mL) for 24 h at 110 °C in a sealed tube to analyse the composition of amino acids. After evaporation to dryness, the hydrolysate was dissolved in 1 mL of citrate buffer (pH 2.2) and then analysed by an amino acid analyser (L-8500A, Hitachi).

*Thymidine uptake assay*

Fractions isolated from aloe vera were evaluated for the effects on cell proliferation by measuring the thymidine uptake of human squamous cell carcinoma (SCC) 13 cells in the presence of each aloe fraction. SCC 13 cells were seeded on to a 96-well tissue culture plate at 5000 cells per well, and fed a mixture of Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Grand Island, NY, U.S.A.) and Ham's nutrient mixture F12 (Gibco-BRL) (3 : 1 by weight), supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, U.S.A.). When the cell population reached 80% of confluency, an aloe fraction was applied at 1 mg mL<sup>-1</sup> with serum-free medium and incubated for 24 h. Then [<sup>3</sup>H]thymidine (Amersham, Arlington Heights, IL, U.S.A.) was added at 5 µCi mL<sup>-1</sup> and incubated for 3 h. Unreacted thymidine was washed out by PBS. The nucleic acids were dried at room temperature for 15 min, and fixed in 10% trichloroacetic acid (TCA) at 4 °C for 1 h. TCA was removed by washing with PBS, then the nucleic acids were dried at room temperature for 15 min, solubilized in 0.5 mol L<sup>-1</sup> NaOH for 6 h, neutralized with 0.5 mol L<sup>-1</sup> HCl, collected, and mixed with scintillation cocktail (Packard, Downers Grove, IL, U.S.A.), and the amount of thymidine uptake was measured by a scintillation counter (Packard).

*Preparation of keratinocytes*

Foreskin keratinocyte cultures were prepared and maintained as described by Blanton *et al.*<sup>16</sup> Human foreskins were obtained from circumcised newborn babies. Foreskins were washed extensively with multiple changes of PBS, subcutaneous tissue was removed, and the remaining samples were enzymatically dissociated in multiple changes of 0.25% trypsin and



versene (50 : 50). Epidermal sheets were peeled from the dermis, minced and dispersed in trypsin solution by repeated pipetting. Cell suspensions were pelleted from the trypsin solution, sequentially resuspended, and washed with PBS by centrifugation at 1000 g for 5 min at 20 °C. These cells were raised in a tissue culture dish with J2 mouse fibroblasts as feeder cells (donated by Dr E. Fuchs, University of Chicago, IL, U.S.A.). Cultures were maintained in a growth medium consisting of DMEM and Ham's nutrient mixture F12 at a 3 : 1 ratio. The medium was supplemented with 10% fetal bovine serum,  $1 \times 10^{-10}$  mol L<sup>-1</sup> cholera toxin, 0.4 µg mL<sup>-1</sup> hydrocortisone, 5 µg mL<sup>-1</sup> insulin, 5 µg mL<sup>-1</sup> transferrin and  $2 \times 10^{-11}$  mol L<sup>-1</sup> triiodothyronine. Cultures were fed every 3 days and subcultured by dispersal in 0.025% trypsin in PBS and replated at a split ratio of 1 : 3. Cultures were used between passages 2 and 3.

#### *Effect of G1G1MIDI2 on keratinocyte migration*

Normal human keratinocytes were cultured to confluence in a six-well culture plate, and the culture medium drained away. A wound (width 0.5 mm) was created on an area of cells by gentle scraping with a rubber stick and moving back and forth against the top of the culture.<sup>17</sup> The wells were washed four times with PBS to remove remaining cellular debris. Cultures were maintained with a medium supplemented with 5% fetal bovine serum. The aloe fraction G1G1MIDI2 was added to the culture at 100 µg mL<sup>-1</sup>, and the control culture received PBS. Wound restoration was photographed at 16, 25 and 40 h after injury.

#### *Effect of G1G1MIDI2 on epidermis formation from keratinocytes in raft culture*

Keratinocytes were raft cultured according to the method of Asselineau *et al.*<sup>10</sup> When the cells reached 90% of confluency, they were trypsinized on a collagen matrix for raft culture as follows. Mouse fibroblasts obtained from Dr E. Fuchs were mixed with type I collagen matrix (Cell Matrix, Nitta Gelatin, Tokyo, Japan) at a density of 17,000 cells per Millericell, and seeded in 12-mm Millericell (Falcon, Lincoln Park, NJ, U.S.A.). Keratinocytes were then seeded on the matrix at 17,000 cells per Millericell. Cells were cultured submerged in media for 7 days, transferred to the air-liquid interface and then raised for 21 days. The aloe fraction G1G1MIDI2 was applied to cells with serum-free media at 0, 0.05, 0.5 and 50 µg mL<sup>-1</sup> every 2

days. Part of the epidermal tissue formed by 3 weeks of culture was taken, fixed in Carnoy solution (ethanol-glacial acetic acid-chloroform 6 : 1 : 3 by volume) and washed with 60%, then 80% ethanol and put in paraffin blocks for morphological comparison.

#### *Immunohistochemical study*

Slices (5 µm) of paraffin blocks were deparaffinized and hydrated before immunohistochemical staining. Immunostaining was carried out as described by Choi and Fuchs.<sup>18</sup> Antisera and dilutions were as follows: mouse monoclonal antisera against human epidermal growth factor (EGF) receptor, 1 : 5 (Triton Diagnostics, Alameda, CA, U.S.A.); rabbit polyclonal antisera against human fibronectin receptor, 1 : 200 (Chemicon, Temecula, CA, U.S.A.); rabbit polyclonal antisera against human fibronectin, 1 : 100 (Biomedical Tech, Stoughton, MA, U.S.A.); rabbit polyclonal antisera against human keratin 5/14, 1 : 100 (Chemicon); mouse monoclonal antisera against keratin 1/10, 1 : 50 (Chemicon). After incubation with primary antisera, the sections were then subjected to immunogold enhancement (Amersham). The immunostaining intensity was estimated by an image analyser (BAS-2500, Fujifilm, Tokyo, Japan) with image analysis software (MCID ver. 3.0, Imaging Research Inc., Ontario, Canada) and expressed as a percentage of the control.

#### *Effect of G1G1MIDI2 on wound healing in hairless mice*

Ten male hairless mice (CrI:SKH1-hrBR, Charles River, Wilmington, MA, U.S.A.) were used for each control and experimental group in this study. Under pentobarbital sodium (1.92 mg kg<sup>-1</sup>) anaesthesia, epithelial wounds (approximately 154 mm<sup>2</sup>) down to subcutaneous fat were induced by using a biopsy punch on both sides of the gluteal region, approximately 1 cm distal from the vertebral column. Experimental animals were given G1G1MIDI2 (10 mg g<sup>-1</sup> ointment) with gentamicin 0.1% every day. Control animals received the vehicle only. The ointment was changed daily to permit evaluation of the size and appearance of each wound until the wound healed completely. The wound area was photographed, and the size of the wound was estimated using an image analyser (BAS-2500, Fujifilm) with image analysis software (MCID ver. 3.0, Imaging Research Inc.) and expressed as a percentage of wound size at day 0 after injury. On day 8, a 7-mm diameter punch biopsy of the skin was immersed in

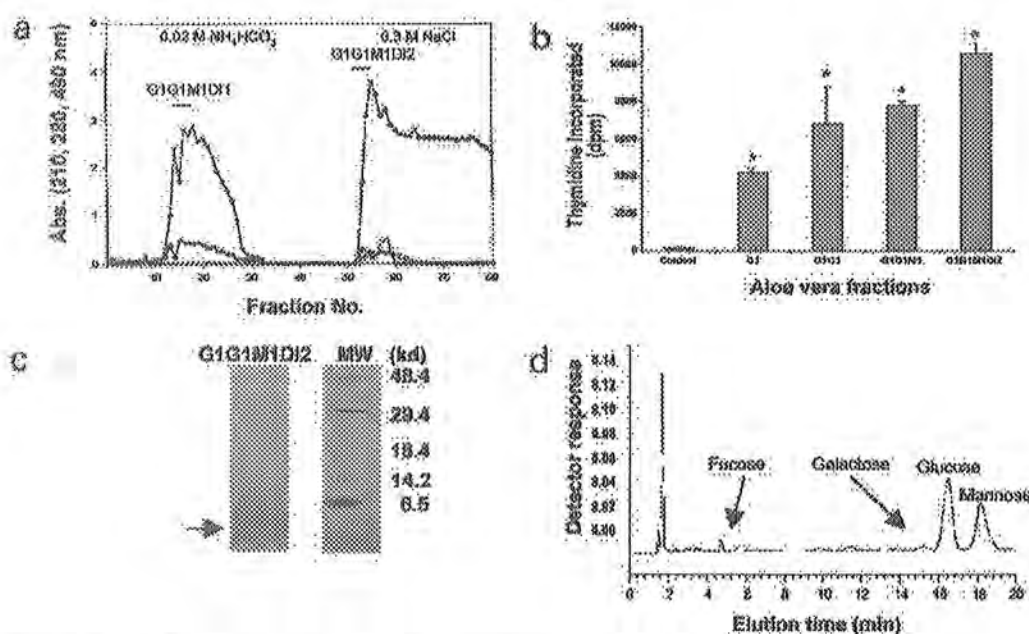


Figure 1. Characterization of glycoprotein fraction G1G1M1D12. (a) Elution profile of G1G1M1D12 by DEAE-Toyopearl 650M column chromatography. The sample was dissolved in  $0.02 \text{ mol L}^{-1} \text{ NH}_4\text{HCO}_3$ , and the column was eluted with  $0.02 \text{ mol L}^{-1} \text{ NH}_4\text{HCO}_3$  and then  $0.3 \text{ mol L}^{-1} \text{ NaCl}$  at  $4^\circ\text{C}$ . Filled circles, absorbance at  $210 \text{ nm}$ ; open circles, absorbance at  $280 \text{ nm}$ ; filled triangles, absorbance at  $490 \text{ nm}$  (phenol- $\text{H}_2\text{SO}_4$  method). Abs., absorbance. (b) Effect of various fractions of aloe vera on [ $^3\text{H}$ ] thymidine uptake by squamous cell carcinoma 13 cells. Data represent mean  $\pm$  SD of three experiments. \* $P < 0.05$  compared with control, in which an aloe fraction was not included in the thymidine uptake assay (Student's *t*-test). G1, G1G1, G1G1M1 and G1G1M1D12 described in Materials and methods. dpm, disintegrations per min. (c) Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of G1G1M1D12. Protein was detected by Coomassie brilliant blue staining. (d) High-performance liquid chromatogram of acid hydrolysate obtained from carbohydrate moiety of G1G1M1D12. The column (Carbo Pak PA1  $4 \times 250 \text{ mm}$ ) was eluted with water- $0.2 \text{ mol L}^{-1} \text{ NaOH}$  (92 : 8) at  $1 \text{ mL min}^{-1}$  at room temperature.

cold neutral-buffered formalin for 24 h, embedded in paraffin, sectioned into  $5\text{-}\mu\text{m}$  slices, and stained using haematoxylin and eosin.

## Results

### Isolation and characterization of proliferation-stimulating glycoprotein fraction G1G1M1D12

The G1G1 fraction obtained from the gel (G1) of aloe vera was applied to an Amberlite XAD-2 column. The eluate (G1G1M1) with 50% aqueous methanol showed activity in the thymidine uptake assay. It was subjected to ion-exchange column separation and gave a non-binding fraction (G1G1M1D11) and a binding fraction (G1G1M1D12) (Fig. 1a). G1G1M1D11 did not show any activity in the thymidine uptake assay, but the main activity was found in the binding fraction G1G1M1D12 (Fig. 1b). The amount of [ $^3\text{H}$ ]thymidine taken up by SCC 13 cells was measured to screen and pick out the most effective fraction. Increased activity was observed from highly purified aloe fractions: aloe components

prepared from fraction G1 showed a steady increase in thymidine uptake when further purified, and G1G1M1D12 showed about a 2.5-fold increase in thymidine uptake compared with the G1 fraction.

G1G1M1D12 was seen as a single band with an apparent molecular weight of about  $5.5 \text{ kDa}$  estimated by SDS-PAGE (Fig. 1c). It was stained by glycoprotein-specific periodic acid-Schiff's reagent and showed a reddish-pink colour at about  $5.5 \text{ kDa}$  (data not shown). G1G1M1D12 showed a positive reaction with 1%  $\text{Ce}(\text{SO}_4)_2$  in 10% aqueous  $\text{H}_2\text{SO}_4$  on a silica gel TLC plate ( $R_f = 0.67$ ) and with aniline phthalate on a cellulose TLC plate ( $R_f = 0.35$ ).

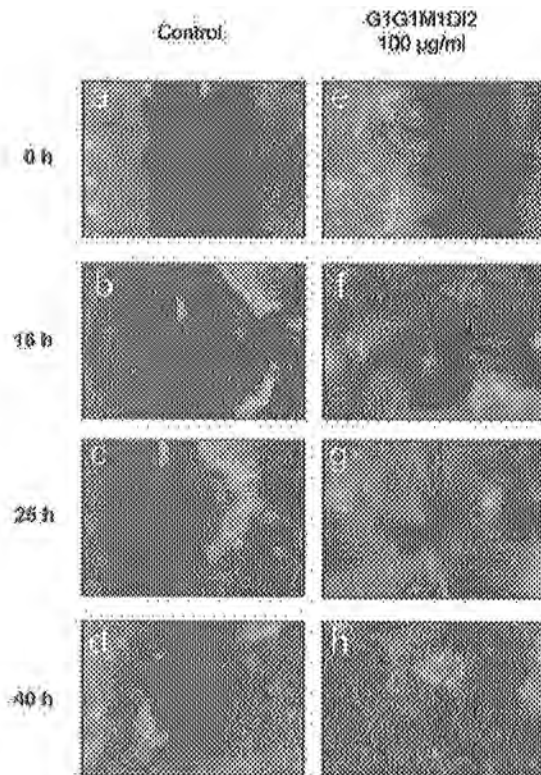
Table 1. Monosaccharide composition of glycoprotein fraction G1G1M1D12, based on high-performance liquid chromatography detector response ratio

Monosaccharide	Composition (%)
Fucose	0.5
Galactose	2.4
Glucose	48.8
Mannose	48.3

**Table 2.** Amino acid composition of glycoprotein fraction G1G1M1D12, estimated using an amino acid analyser

Amino acid	Composition (%)
Asp	12.68
Thr	0.72
Ser	0.94
Glu	22.70
Gly	18.47
Ala	10.96
Val	8.75
Met	0.62
Ile	2.98
Leu	8.50
Tyr	1.70
Phe	2.47
Lys	5.24
Arg	2.16
His	1.18

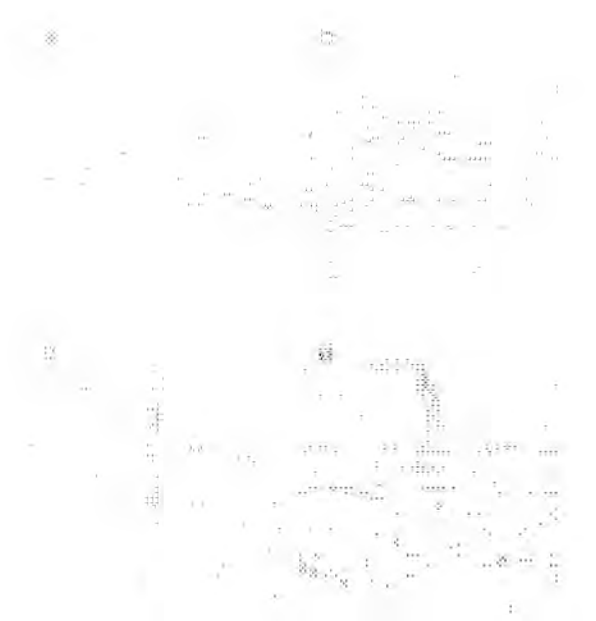
The IR spectrum of G1G1M1D12 showed absorption bands at 3359, 1601, 1382 and 1077  $\text{cm}^{-1}$ , indicating the presence of carbohydrate.  $^{13}\text{C}$ -NMR spectrum (100 MHz,  $\text{D}_2\text{O}$ ) showed signals ascribable to aliphatic carbons ( $\delta_{\text{c}}$  12.7, 12.9, 16.2, 21.4, 24.0, 32.8), carbonyl carbons ( $\delta_{\text{c}}$  160.5, 171.8, 186.5), germinal carbons of oxygen function ( $\delta_{\text{c}}$  61.2, 61.6, 62.0, 69.6, 70.6, 72.1, 75.7, 76.5) and anomeric carbons ( $\delta_{\text{c}}$  100.9, 101.1).  $^1\text{H}$ -NMR spectrum (400 MHz,  $\text{D}_2\text{O}$ ) also showed signals assignable to a carbohydrate moiety ( $\delta_{\text{c}}$  3.4–4.5). The carbohydrate moiety contained fucose, galactose, glucose and mannose at a ratio (%) of 0.5, 2.4, 48.8 and 48.3, respectively (Fig. 1d, Table 1). To determine the amino acid composition and content, the HCl hydrolysate of the peptide moiety of G1G1M1D12 was analysed by an amino acid analyser. Fifteen kinds of amino acids were detected, of which glutamic acid and glycine comprised 41.2 molar percentage of the total detected amino acids (Table 2). These results indicate that G1G1M1D12 is a glycoprotein.



**Figure 2.** Effect of glycoprotein fraction G1G1M1D12 ( $100 \mu\text{g mL}^{-1}$ ) on the migration of human keratinocytes on a monolayer. (a–d) Control; (e–h) treated with G1G1M1D12. This experiment was repeated in duplicate in two independent experiments. Photographs were taken at 0, 16, 25 and 40 h after injury (original magnification  $\times 100$ ).

#### *Effect of G1G1M1D12 on migration of keratinocytes*

Because cell migration is involved during wound healing, we evaluated cell migration in the presence of an aloe fraction to investigate whether G1G1M1D12 enhances cell locomotion (Fig. 2). Prior to wounding, cells appeared generally flat, polygonal in shape and relatively regular in size. Immediately after wounding, there were no cells within the defective area. G1G1M1D12 was applied at  $100 \mu\text{g mL}^{-1}$  and then cleavage recovery was observed. At 16, 25 and 40 h after incision, cells in the G1G1M1D12-treated group multiplied and became more compact. Membranes of adjacent cells became tightly juxtaposed, indicating close cell-to-cell contact. Under this condition, the leading edge of the closing wound was preceded by a number of migrating elongated cells. Cells more distal to the leading edge appeared confluent and generally polygonal in shape. There appeared to be a larger population of migrating cells throughout the original wound area than in the controls. After 40 h the cell migration finally filled the cleaved area (Fig. 2h). However, in the control group, cells were quite slow to rearrange and form permanent cell–cell contacts and did not restore the cleaved area. This result shows that G1G1M1D12 exerted its effect in cleavage recovery by attracting cells to the cleaved site as well as stimulating the division of the cells. The migration assay was repeated in duplicate, in two

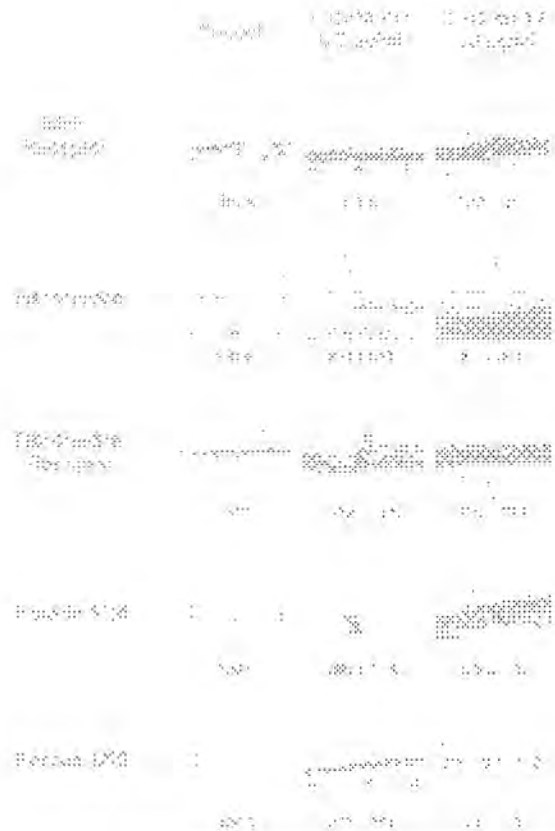


**Figure 3.** Effect of glycoprotein fraction G1G1M1D12 on the formation of epidermis from keratinocytes in raft culture. Cultures were treated with G1G1M1D12 for 21 days immediately after exposure to air. Cultures were grown (a) in the absence of G1G1M1D12, or in the presence of (b) 0.05, (c) 0.5 or (d) 50  $\mu\text{g mL}^{-1}$  G1G1M1D12 (haematoxylin and eosin, original magnification  $\times 200$ ).

independent experiments. In both cases, G1G1M1D12 treatment gave accelerated migration of keratinocytes. Next, G1G1M1D12 was evaluated for its capability to augment epidermal tissue formation from keratinocytes and to increase the expression of cell proliferation-related factors.

#### *Effect of G1G1M1D12 on the architecture of epidermis in raft culture*

The stained paraffin sections of the artificial epidermis raised in raft culture were compared by staining with haematoxylin and eosin (Fig. 3). In the control, most basal cells died, with a few cells surviving. A very thin spinous layer and a granular layer were observed, with a stratum corneum. As the G1G1M1D12 concentration increased from 0.05 to 50  $\mu\text{g mL}^{-1}$ , basal cells multiplied and formed thick epithelial coverings. At 0.05  $\mu\text{g mL}^{-1}$ , three to four spinous and granular layers were observed. The number of cells containing a nucleus increased. From 0.5 to 50  $\mu\text{g mL}^{-1}$ , the number of cells with a nucleus further increased, and a thicker stratum corneum was formed.

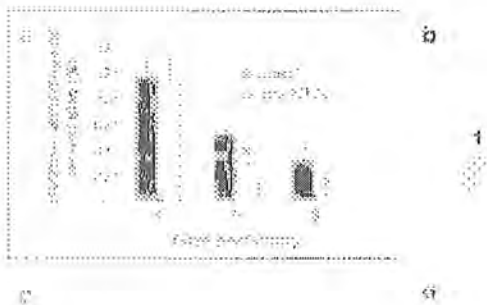


**Figure 4.** Immunohistochemistry of the artificial epidermis raised in the raft culture. Immunohistochemical staining was used to detect epidermal growth factor (EGF) receptor, fibronectin, fibronectin receptor, keratin 5/14 and keratin 1/10 in the artificial epidermis raised in the absence or presence of 0.5 or 50  $\mu\text{g mL}^{-1}$  G1G1M1D12. Immunostaining is indicated by arrowheads. Data below each picture represent mean  $\pm$  SD of relative percentage of optical density of three experiments. The immunostaining intensity was estimated by an image analyser with image analysis software (immunogold staining, original magnification  $\times 200$ ).

#### *Immunohistochemical comparison*

The effect of G1G1M1D12 on the expression of protein markers related to cell proliferation was studied using immunohistochemical methods (Fig. 4). Immunostaining against an EGF receptor showed a very low level of EGF receptor expression in the basal layer of the control. As the G1G1M1D12 concentration increased, EGF receptor expression increased on the cell membrane of the basal layer and even in the innermost spinous layer cells. Consistent with the increase in basal cells shown by haematoxylin and eosin staining from 0.5 to 50  $\mu\text{g mL}^{-1}$  of G1G1M1D12, EGF receptor expression increased in a dose-dependent manner: its





**Figure 5.** Effect of glycoprotein fraction G1G1M1D12 on the wound healing of hairless mice. (a) Data represent relative percentage of wound size (mean  $\pm$  SD;  $n = 10$ ). The wound area was photographed and the size of the wound was estimated using a BAS-2500 Image analyser with image analysis software MCID ver. 3.0 and expressed as a percentage of wound size at day 0 after injury. \* $P < 0.05$  compared with control (Student's *t*-test). (b) Wound area on day 10 after treatment: 1, control; 2, treated with G1G1M1D12 (1.0 mg g<sup>-1</sup> ointment). (c,d) Morphology of the wound area at day 10 after treatment: (c) control (d) treated with G1G1M1D12 (haematoxylin and eosin, original magnification  $\times 200$ ).

mean  $\pm$  SD expression increased to 113% and to 220  $\pm$  8% at 0.5 and 50  $\mu\text{g mL}^{-1}$ , respectively, of G1G1M1D12 compared with the controls. In normal skin, fibronectin is essentially located at the level of the papillary dermis. In our study, mouse fibroblasts embedded in the collagen matrix produced fibronectin, which made it possible for cells to locomote through the interaction with fibronectin receptors. Fibronectin was expressed in a dose-dependent manner: its expression increased to 294  $\pm$  34% and to 408  $\pm$  80% at 0.5 and 50  $\mu\text{g mL}^{-1}$ , respectively, of G1G1M1D12 compared with the controls. Fibronectin receptor was also expressed in a dose-dependent fashion: an increase of immunostaining to 159  $\pm$  11% and 220  $\pm$  19% at 0.5 and 50  $\mu\text{g mL}^{-1}$ , respectively, of G1G1M1D12, was observed. Cells expressing the fibronectin receptor were located at the basal and suprabasal layer of the artificial tissue. Fibronectin receptor was also found between the collagen matrix and the basal layer. Expression of keratins was examined in the artificial tissue. Keratin 5/14 was expressed in basal and spinous layers in a dose-dependent manner: an increase to 260  $\pm$  11% and 308  $\pm$  17% at 0.5 and 50  $\mu\text{g mL}^{-1}$  of G1G1M1D12, respectively. Keratin 1/10 was expressed in suprabasal and granular cells, and its expression increased to 228  $\pm$  24% and 174  $\pm$  19% at 0.5 and 50  $\mu\text{g mL}^{-1}$  of G1G1M1D12, respectively.

#### *Effect of G1G1M1D12 on wound healing in hairless mice*

The cell-proliferating activity of G1G1M1D12 was investigated by examining whether it enhances wound healing in hairless mice. Daily application of

the aloe glycoprotein fraction induced an increase in new epithelial area, and the treated lesion healed faster than the controls (Fig. 5). Healing was accompanied by enhanced granulation and increased epithelialization, starting from the periphery of the wound. At days 4, 6 and 8 the wound area recovered by, respectively, 5.9, 84.2 and 98.9% of the original size in the G1G1M1D12-treated group, compared with 4.4, 49.5 and 69.5%, respectively, in the control group. Histological sections of the recovered area at day 8 showed one basal layer in controls, with one to two layers of spinous and granular cells. However, in G1G1M1D12-treated mice three basal layers were observed with an increased number of nucleus-containing cells, and more spinous and granular cell layers.

#### **Discussion**

Aloe has long been known for its various pharmacological effects. Because the relationship between these effects and individual aloe components has not been well elucidated, this study was designed to single out the effective component, to examine its effect *in vitro*, and to confirm its effect *in vivo* in experimental animals. A glycoprotein fraction of 5.5 kDa was isolated from aloe vera. It was identified as a glycoprotein by SDS-PAGE and periodic acid-Schiff staining, TLC using carbohydrate-specific plates, spectroscopy, HPLC and amino acid analysis. It significantly stimulated cell proliferation, accelerated the recovery of an artificial wound on a monolayer of normal keratinocytes, and enhanced thickening of the epidermal covering with the overall appearance of a proliferating phenotype as

well as an increase in the expression of EGF receptor, fibronectin, fibronectin receptor, keratin 5/14 and keratin 1/10. The cell proliferation-stimulatory activity was further confirmed by enhanced wound healing in hairless mice.

As well as the whole extract of aloe vera, its components aloesin, mannose-6-phosphate, glycoprotein and aloe-emodin have been reported to stimulate cell proliferation. Yagi *et al.*<sup>5</sup> recently reported that a glycoprotein fraction of 29 kDa isolated from *Aloe barbadensis* Miller has proliferation-promoting activity. It is composed of two subunits each of 14 kDa. However, smaller proteins were neither observed nor sought during this study. At present, it is not possible to compare the homologies of the 29-kDa protein and our 5.5-kDa protein, due to lack of information on the 29-kDa protein. It is possible that aloe vera contains many cell proliferation-stimulatory components with varying activities. In our study G1G1M1D12 was probably not the only active component of aloe vera, because other fractions also exhibited cell-proliferation activity.

Various factors are known to affect cell migration, such as EGF,<sup>19</sup> interleukin (IL)-1 $\beta$ ,<sup>20</sup> nitric oxide,<sup>21</sup> platelet-derived growth factor<sup>22</sup> and short-chain fatty acids.<sup>23</sup> Transforming growth factor (TGF)- $\beta$  may either enhance or inhibit migration.<sup>24,25</sup> Machiro *et al.*<sup>26</sup> reported that epithelial restoration by EGF is done by stimulating both migration and proliferation, while Nilsson *et al.*<sup>19</sup> showed that growth factor-induced migration of thyrocytes is not strictly coupled to the mitogenic activity. It is likely that the growth factors stimulate cell migration depending on the cell type and tissue. In our study the glycoprotein fraction G1G1M1D12 accelerated cell migration. It is likely that cell migration was concomitant with cell proliferation, which was enhanced by the aloe fraction. A few cells survived in the absence of serum and aloe fraction G1G1M1D12 in the raft culture. However, in the presence of G1G1M1D12, keratinocytes survived and multiplied, even in the serum-free media, and formed epidermis with an overall appearance of a proliferating phenotype. This indicates that the aloe fraction G1G1M1D12 stimulates cell proliferation.

EGF receptor mediates paracrine and autocrine growth regulation of normal and malignant cells.<sup>27</sup> Stoscheck *et al.*<sup>28</sup> demonstrated that an increase in the number of EGF receptors preceded the hypertrophic response. The aloe fraction G1G1M1D12 enhanced DNA synthesis as well as EGF receptor expression. This suggests that EGF receptors transmitted the cell proliferation signal from the G1G1M1D12. Another

possibility is that G1G1M1D12 may activate the general metabolism, enhancing metabolic activities with a concomitant increase in EGF receptor expression.

There was a significant increase in EGF receptor expression in the artificial epidermis raised in raft culture. It has been suggested that overexpression of EGF receptors may be in part responsible for the tumorigenic potential of cutaneous epithelial malignancies.<sup>29</sup> Furthermore, it was reported that there were twice as many EGF receptors in SCCs of the head, neck, lung, cervix and skin.<sup>30</sup> Contrary to this, Tomatis<sup>31</sup> suggested that the cell-proliferation effect is not necessarily correlated with metastasis in keratinocyte proliferation. Jacobberger *et al.*<sup>32</sup> showed that the proliferation rate was decreased by TGF- $\beta$ 1 in cervical epithelial cells, while EGF receptor levels were increased. Ottensmeier *et al.*<sup>33</sup> suggested that EGF receptor activation through autocrine pathways is not a major mechanism for the growth of many ovarian cancer cell lines. In our study, although EGF receptor expression was significantly increased by G1G1M1D12 in the artificial tissue raised in the raft culture, keratinocytes did not invade into the matrix, but entered terminal differentiation as *in vivo* when the stratified epidermal cells were exposed to air, as suggested by Tomatis.<sup>31</sup> G1G1M1D12 did not give rise to uncontrolled cell proliferation.

Human keratinocytes express several receptors of the integrin family. Expression is normally confined to the basal layer of keratinocytes, both in mature epidermis and during development.<sup>34</sup> Ohashi *et al.*<sup>35</sup> reported that increased attachment of corneal epithelial cells to fibronectin and enhanced migration on corneal stroma by IL-6 is due to temporal upregulation of  $\alpha$ 5 $\beta$ 1 expression in corneal epithelial cells. Nista *et al.*<sup>36</sup> reported that fibronectin delivers a mitogenic signal to human mammary carcinoma cells. The enhanced expression of fibronectin receptor is also observed in wound healing and cellular activation.<sup>37</sup> In our study, the fibroblasts seeded in the collagen matrix of the raft culture produced fibronectin in proportion to the concentration of G1G1M1D12. In addition, the keratinocytes in the basal layer of the raft culture expressed fibronectin receptor in a dose-dependent manner. This shows that G1G1M1D12 stimulated both the fibroblasts and the keratinocytes in producing fibronectin and its receptor.

A keratin pair represents the most sensitive marker for the state of the epidermis.<sup>38,39</sup> Significant changes were observed in the raft culture: the expression of keratin 5/14 was enhanced proportionally with respect

to the G1G1M1D12 concentration, but the synthesis of keratin 1/10 was slightly decreased at higher G1G1M1D12 concentrations. This indicates that G1G1M1D12 affects cell proliferation rather than differentiation. Moreover, in the control no immunostaining of keratin 1/10 was observed, which might be due to undetectable levels of expression of the keratin in the surviving cells or to the death of most basal cells in the absence of serum or of glycoprotein fraction G1G1M1D12. Keratin 6/16 is a marker of regeneration-associated differentiation and is induced in epidermal diseases associated with hyperproliferation.<sup>40</sup> The expression of keratin 6/16 was not examined in this study, because we studied the effect of G1G1M1D12 in artificial epidermis raised in raft culture, not in regenerated tissue or in the diseased epidermal tissue associated with hyperproliferation. Furthermore, it was our intention to observe the dose-dependent effect of G1G1M1D12 on cell proliferation. Therefore, the dose-dependent expression of keratin 5/14 was studied in comparison with that of keratin 1/10. However, we do not exclude the possibility that keratin 6/16 is increased in artificial tissue, because the keratinocytes in raft culture bear some similarity with those in wounds.<sup>10</sup>

The hairless mouse was selected as a wound model for epidermal injury because it offers several advantages. First, the epidermis does not have a fur coat, which interferes with the separation of epidermis from dermis; second, the size and economy of a hairless mouse makes it an ideal model to evaluate the effects of pharmacological agents on the wound-healing process. In the wounded hairless mouse, G1G1M1D12 treatment stimulated keratinocyte proliferation and thus shortened the healing time, which was demonstrated by several basal and suprabasal layers with well proliferating cells. G1G1M1D12 was used for the wound healing experiment. Other fractions (G1, G1G1 and G1G1M1) were not examined because we were apprehensive that inhibitory components possibly residing in the other crude fractions may lower the wound-healing effect of G1G1M1D12.<sup>5</sup> It is possible that the conflicting effects of G1G1M1D12 and of the other inhibitory components cause variability in the results of therapeutic experiments.

Although there have been discrepant observations on the wound-healing effect of whole extracts of aloe, this report clearly demonstrates that the glycoprotein fraction G1G1M1D12 enhances keratinocyte multiplication and migration, expression of proliferation-related factors, and epidermis formation, thereby leading to wound healing.

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