ORIGINAL ARTICLE

Aminoacid-enriched sodium hyaluronate enhances keratinocyte scattering, chemotaxis and wound healing through integrin β1-dependent mechanisms

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Epithelialisation is a major component of wound healing and relies on migration and spreading of epidermal keratinocytes. An understanding of the mechanisms underlying this process has salient clinical application, as wound healing may be pharmacologically modulated to enhance repair of tissue injury. Whilst the efficacy of commercially available hyaluronic acid (HA) formulations in skin tissue repair as well as its action on mesenchymal cells (e.g. fibroblasts and condrocytes) has been well documented, the role of HA in the process of keratinocyte epithelialisation still needs to be addressed in detail. Here, we investigated the efficacy of Aminogam, a compound containing a pool of collagen precursor synthetic aminoacids (l-proline, l-leucine, l-lysine and glycine) combined with sodium hyaluronate (SH), in keratinocyte cytokinesis and epithelial wound repair in vitro. Our data show that after wounding, Aminogam-treated cells had higher rates of wound closure than untreated keratinocytes. Cell scattering of three-dimensional multicellular aggregates was 40% increased by Aminogam whereas cell spreading was not affected. Chemokinesis (random migration) and chemotaxis (directional migration) were enhanced by Aminogam at an early and later stage of migration, respectively. Silencing of β1 integrin in HaCat keratinocytes dramatically impaired epithelial cleft repair: wound healing, spreading, scattering and random/directional migration were all decreased 40 to 60% compared to controls. Aminogam could not improve ($p > 0.05$) any of these features in the absence of β1 integrin. Our data provide the first evidence that HA, namely Aminogam, exerts direct effects on keratinocyte motility via integrin β1.

***Key words*:** hyaluronic acid, aminoacids, wound healing, migration, keratinocytes, β1 integrin

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Introduction

The successful repair of tissue injury requires a well-coordinated host response to limit the extent of tissue damage [1]. In epithelial tissues, including skin and mucous membranes, this process may be enhanced by drugs that promote wound healing. In this context, the efficacy of commercially available hyaluronic acid (HA) formulations in tissue repair has been well documented [2-4].
The unique viscoelastic nature of HA along with its biocompatibility and non-immunogenicity has led to its use in a number of clinical applications, which include: the supplementation of joint fluid in arthritis; as a surgical aid in eye surgery; and to facilitate the healing and regeneration of surgical wounds. More recently, HA has been investigated as a drug delivery agent for various routes of administration, including ophthalmic, nasal, pulmonary, parenteral and topical [5-13].

HA is present in almost every tissue of all vertebrates but is most abundant in the extra cellular matrix (ECM) of soft connective tissues [14]. HA performs three basic molecular functions. First, it interacts in an autocrine manner with cell surface HA receptors on the same cell. Second, it interacts in a paracrine manner with a variety of ECM molecules on neighboring cells. Third, newly

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synthesized HA may be secreted and subsequently interact with several cell surface receptors, including cluster determinant 44 (CD44), receptor for hyaluronate-mediated motolity (RHAMM), lymphatic vessel endothelial HA receptor (LYVE-1), hyaluronan receptor for endocytosis (HARE), and TLR-4. These interactions mediate three important physiological processes: signal transduction, the formation of pericellular coats and receptor-mediated internalization [15-17].

Since HA is part of ECM of connective tissues, much research on its mechanism of action has been focused on mesenchymal cells, including fibroblasts and condrocytes. However, little is known about the putative effects of HA on keratinocytes during epithelial tissue repair.

Epithelialisation – a major component of wound healing – relies not only on proliferation, but also on migration and spreading of epidermal keratinocytes. One of key molecular players in re-epithelialisation are integrins, which is a family of major adhesive component of hemidesmosomes and focal contacts of the basal keratinocytes. Integrin β1 engagement is supposed to be a key mechanism for keratinocyte-ECM interactions, since restricted ablation of integrin β1 to the basal epidermal keratinocytes results in skin blistering. However, in vitro evidence of the role of α3β1 integrin in keratinocyte migration is controversial; although some studies have suggested that integrin α3β1 promotes keratinocyte migration [18, 19], the opposite has also been reported [20-22].
In the present study, we attempt to address the role of β1 integrin in wound healing and whether it participates to the effects of HA on keratinocyte migration and wound repair. To this aim, a novel aminoacid-enriched sodium hyaluronate formulation, Aminogam ®, will be tested.

Materials and methods

Cells and treatments. A non-tumorigenic human keratinocyte cell line (HaCaT), which exhibits normal differentiation both in vitro and in vivo [23], was used in this study. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (50 U ml^{-1}), streptomycin (50 lg ml^{-1}) and fungizone (2.5 lg ml^{-1}) in an atmosphere humidified with 5% CO_2.
Aminogam was diluted in the culture medium to a final concentration of 1% (v/v) and used for wound healing and chemokinesis experiments. For chemotaxis assay, 50µl undiluted Aminogam was gently placed in the 2mm well on one side of the AKGOS plate.

Wound production. At the time of the experiment, cells were seeded and grown to confluence in KAD (KGM and DMEM) medium [24] in six-well dishes. Scratches were made with a sterile blue 1 ml pipettor tip perpendicular to the bottom of the dish. This allowed identification of three sites for each well at which migration was determined. Afterward, cells were rinsed, and the wounded area was examined microscopically to ensure that cellular debris was removed. The wells then received fresh KAD medium with or without hyaluronic acid, as reported in the previous paragraph. The culture was photographed at each line / scratch intersection at zero time and again after 6, 12, and 24 hours in at least two independent experiments.

Transfection with Small Interference RNAs (siRNAs). The integrin β1-directed siRNA pool (sc-35674) and the negative control pool (sc-37007) were transfected at a final concentration of 80 nM, according to the manufacturer's instructions. Cultured HaCaT cells were incubated overnight with the siRNAs in serum-free transfection medium (sc-36868) and then for an additional 24 h in complete medium with 10% FBS. Cells were then either scratched or collected for migration assays. The efficiency of transfection was monitored by western blot analysis of integrin β1 expression.

Preparation of multicellular aggregates (MCAs). Multicellular aggregates (MCAs) were prepared as described previously [25]. Briefly, monolayers were briefly treated with EDTA to prepare single cells. To generate MCAs, cells were then plated on polyhydroxylethyl-methacrylate (poly-HEMA)-coated 60-mm dishes (6 x 10^5 cells/dish) in the presence of serum-free DMEM. To produce single cell cultures in suspension (not forming MCAs), the cells were suspended in semisolid medium consisting DMEM containing 1.5% methylcellulose (Sigma) at 6 x 105 cells/10 cm poly-HEMA-coated dishes.

Three-dimensional cell scattering assay. MCAs were plated in 6 well plates coated with collagen I. After 6 hours, the cells were visualized using a Zeiss Axiophot microscope (Carl Zeiss Imaging, Thornwood, NY) and pictures of 10 MCAs per well were taken at random using an Evolution VF fast digital camera (MediaCybernetics, Wokingham Berkshire, UK). Cells that fell under either of the

following two categories were designated as scattered cells: (1) peripheral cells of a colony that appeared to be moving away (appeared elongated radially) from the colony; or (2) isolated cells that appeared to spread less and were highly reflective under the microscope. The total number of scattered cells was divided by total number of cells and the ratio was plotted as % scattered cells under the indicated conditions.

Cell spreading assay. Cells were seeded as at low confluence and incubated at 37 °C. At given times, unattached cells were removed by rinsing the wells with warm PBS. Attached cells were fixed in 4% paraformaldehyde. Spread cells were counted in five representative high power fields at different time points. Non-spread cells were defined as small round cells with little or no membrane protrusions, whereas spread cells were defined as large cells with extensive visible lamellipodia [26]. Results represent the percentage of spread cells in five high power fields ± S.D.

AGKOS assays. HaCat keratinocytes were suspended in KGM, counted in a hemocytometer, loaded at a high density (1×10^4 cells per 10 µl) into each 3-mm well in an agarose gel, as detailed elsewhere [27].

In the chemokinesis AGKOS assay, HaCat were fed with KGM containing various concentrations of test compounds *vs* no treatment (control) and incubated for 10 days in a humid CO_2 incubator with daily changes of medium. The migration of keratinocytes was stopped by fixing the cells in 0.25% glutaraldehyde and staining them with Wright's stain. To measure the effects of HA on the random migration distance (RMD) (i.e. the distance outward from the original 3-mm well to the leading edge), the image of each megacolony was projected to the screen and the blueprint obtained. To standardize measurements, three segments were drawn through the center of each megacolony at 60° intervals. The RMD was computed in µm using the following formula:

$$RMD = (B_1B_4 - A_1A_4) + (B_2B_5 - A_2A_5) + (B_3B_6 - A_3A_6) / 6 .$$

In the chemotaxis AGKOS assay, HaCat in KGM were loaded into a 3-mm well in agarose gel, as described above, incubated overnight (to allow cells to settle), after which HA (50 µl) was inoculated in a 2-mm well cut on one side of the 3-mm well. The incubation was continued for 10 days with daily

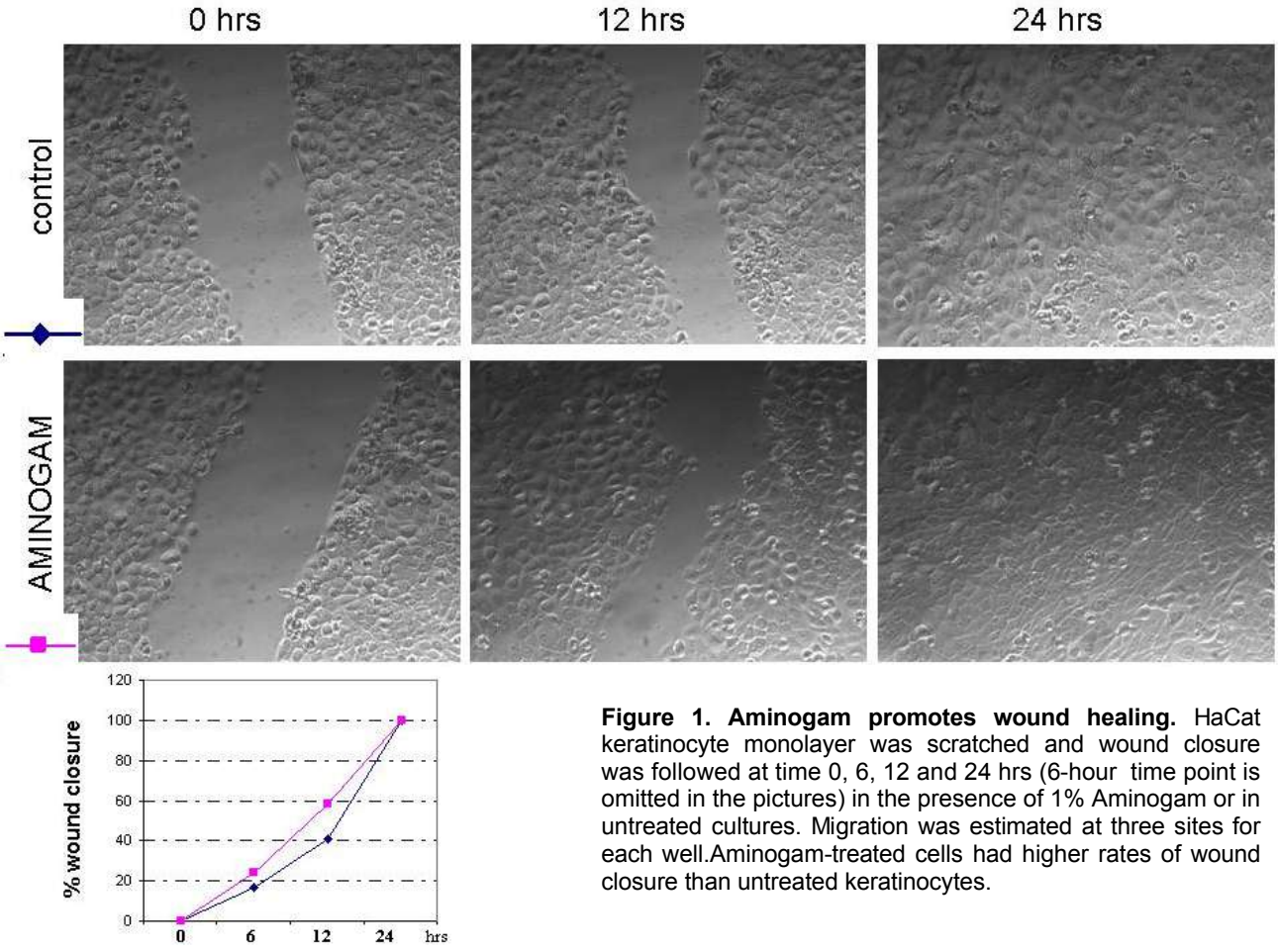


Figure 1. Aminogam promotes wound healing. HaCat keratinocyte monolayer was scratched and wound closure was followed at time 0, 6, 12 and 24 hrs (6-hour time point is omitted in the pictures) in the presence of 1% Aminogam or in untreated cultures. Migration was estimated at three sites for each well.Aminogam-treated cells had higher rates of wound closure than untreated keratinocytes.

changes of KGM and HA. After migration was terminated, a blueprint of the outgrowth was obtained and used to compute the directional migration distance (DMD). To standardize measurements, two segments in addition to the median segment B_1B_4 were drawn through the center of megacolony at the 30° intervals in the direction of the chemoattractant well. The DMD was computed in μm using the following formula:

$$DMD = (A_1B_1 + A_2B_2 + A_6B_6) - (A_3B_3 + A_4B_4 + A_5B_5) / 3 .$$

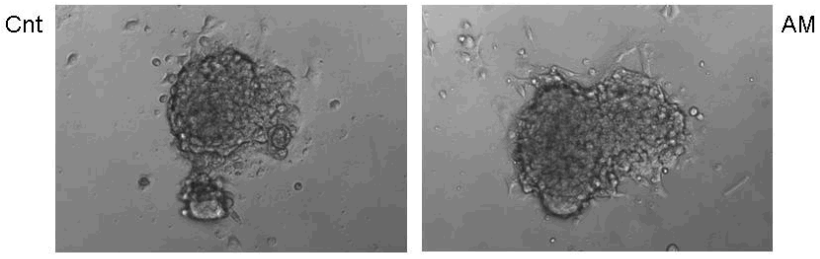
To control for possible changes of cell cycle progression speed that could affect measurements of migration distances, the effect of Aminogam on keratinocyte proliferation were assessed separately and no differences ($p<0.05$) were found.

Statistical analysis. Data are given as the average ±SD of independent experiments, as detailed in figure legends. Differences were assessed by T-test and a P value less than 0.05 was considered to be significant.

Results

Aminogam improves wound healing. First, we determined if this newly formulated compound, Aminogam, was able to improve wound healing by acting directly on keratinocytes. Six and twelve hours after wounding, aminogam-treated cells had higher rates of wound closure than untreated keratinocytes (**Fig.1**). To control for possible changes in the rate of keratinocyte proliferation that could affect migration and/or wound closure, we investigated wound healing in the presence of the growth-arresting agent mitomycin C at 10 μg ml^{-1}. Although migration was slower in mitomycin-C-treated cultures, there was no difference in the relative differences between treated and controls ($p < 0.05$). These data demonstrate that aminoacid-enriched sodium hyaluronate can enhance wound healing by acting directly on keratinocytes.

Aminogam increases three-dimensional cell scattering of multicellular aggregates without affecting cell spreading. Cell scattering is a measure of motility and migration properties of cells. However, the tendency of keratinocytes to spread and move away from small colonies is not representative of a physiological condition: keratinocytes are tightly assembled in human skin and therefore scattering is influenced by the establishment of intercellular contacts that hold the cells together. For this reason, we used three-dimensional aggregated of cells called MCAs. When MCAs are confronted with a substrate, they gradually disrupt. Keratinocytes down-regulate cell-cell adhesion whereas integrin expression promotes cell-ECM interactions and migration [28]. For our experiments, HaCat cells were grown as MCAs for 24 hours and then left to confront with a collagen I substrate. Optimal conditions were set in pilot experiments and cell scattering was recorded 6 hours after adhesion to collagen. In the presence of culture medium containing Aminogam 1% (v/v), cell scattering increased of about 50% compared with MCAs incubated control medium (**Fig.2**). Conversely cell spreading, a morphologic feature of migratory phenotype, was found not to be affected by HA 6 hours after treatment (**Fig.3**). Taken together, these data suggest that Aminogam enhances wound healing, al least in part, by promoting cell scattering of keratinocytes.



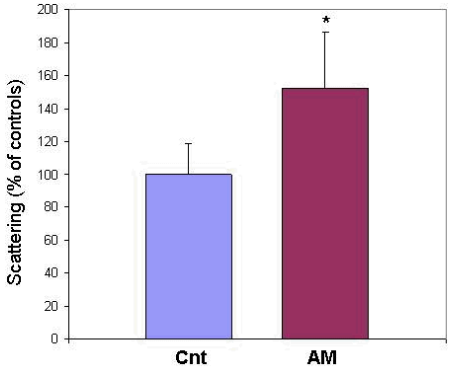


Figure 2. Cell scattering from three-dimensional multicellular aggregates (MCAs) is increased by treatment with Aminogam. Twenty-four hours MCAs were generated as described in the text and then subjected to the assay. Cell scattering was recorded 6 hours after adhesion to collagen. Culture medium containing 1% medium significantly stimulated cell scattering by 50% compared with controls.
*, $p < 0.05$.

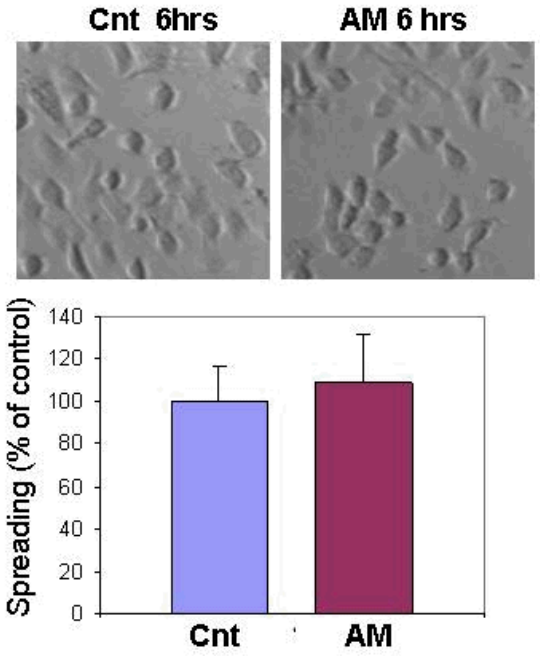


Figure 3. Keratinocyte spreading is not affected by Aminogam. Cells were seeded at low confluence and assessed for cell spreading after 6 hours (see text for details)

Aminogam promotes directional migration of keratinocytes. The physiological relevance of HA effects on keratinocyte crawling locomotion was investigated in chemokinesis assays. HaCat were loaded into the chemokinesis AGKOS plates, incubated overnight to allow cells to settle, after which Aminogam was added at a concentration of 1% (v/v). Random migration appeared to be stimulated at day 2 and day 3, whereas no significant changes were found afterwards (**Fig.4**).
To determine whether aminoacid-enriched HA is chemoattractive for human keratinocytes, we measured directional migration toward Aminogam using a chemotaxis AGKOS assay. A statistically significant (p<0.05) increase in the directional migration distance (DMD) was observed starting at day 4 to day 7 (**Fig.4**). These results show that Aminogam stimulates cellular motility and acts as a chemoattractant for keratinocytes.

The effects of Aminogam on keratinocytes depend on integrin β1 expression. To gain a mechanistic insight into HA control of keratinocyte motility, we silenced one of the crucial molecules known to mediate cell-ECM interaction and migration. Expression of β1 integrin in keratinocytes seeded in 6-well plates was abrogated by siRNAs, after which scratches were performed as described and epithelial cleft closure analysed microscopically at several time points in the presence of 1% (v/v) Aminogam or control medium. In these conditions, wound healing was heavily affected (**Fig.5**). Aminogam failed to improve wound closure in keratinocytes lacking β1 integrin, suggesting that this aminoacid-enriched HA enhances keratinocyte migration through β1 integrin engagement.
Next, we wanted to investigate whether the defects

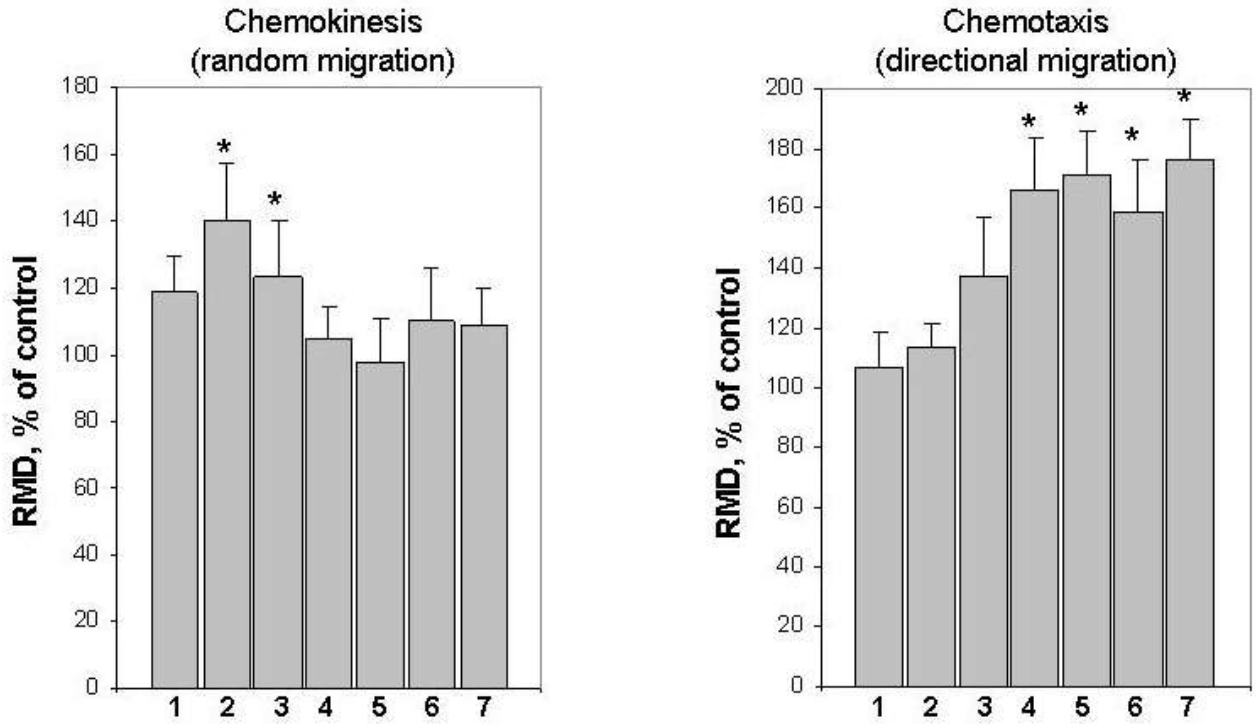


Figure 4. Aminogam affect keratinocyte migratory properties. Random migration (left panel) and directional migration (right panel) were assessed with the AGKOS chemokinesis and chemotaxis assays, respectively. Aminogam stimulated chemokinesis at an early stage (day 2 and 3), whereas chemotaxis was enhanced at later stages (day 4 to 7). *, p < 0.05

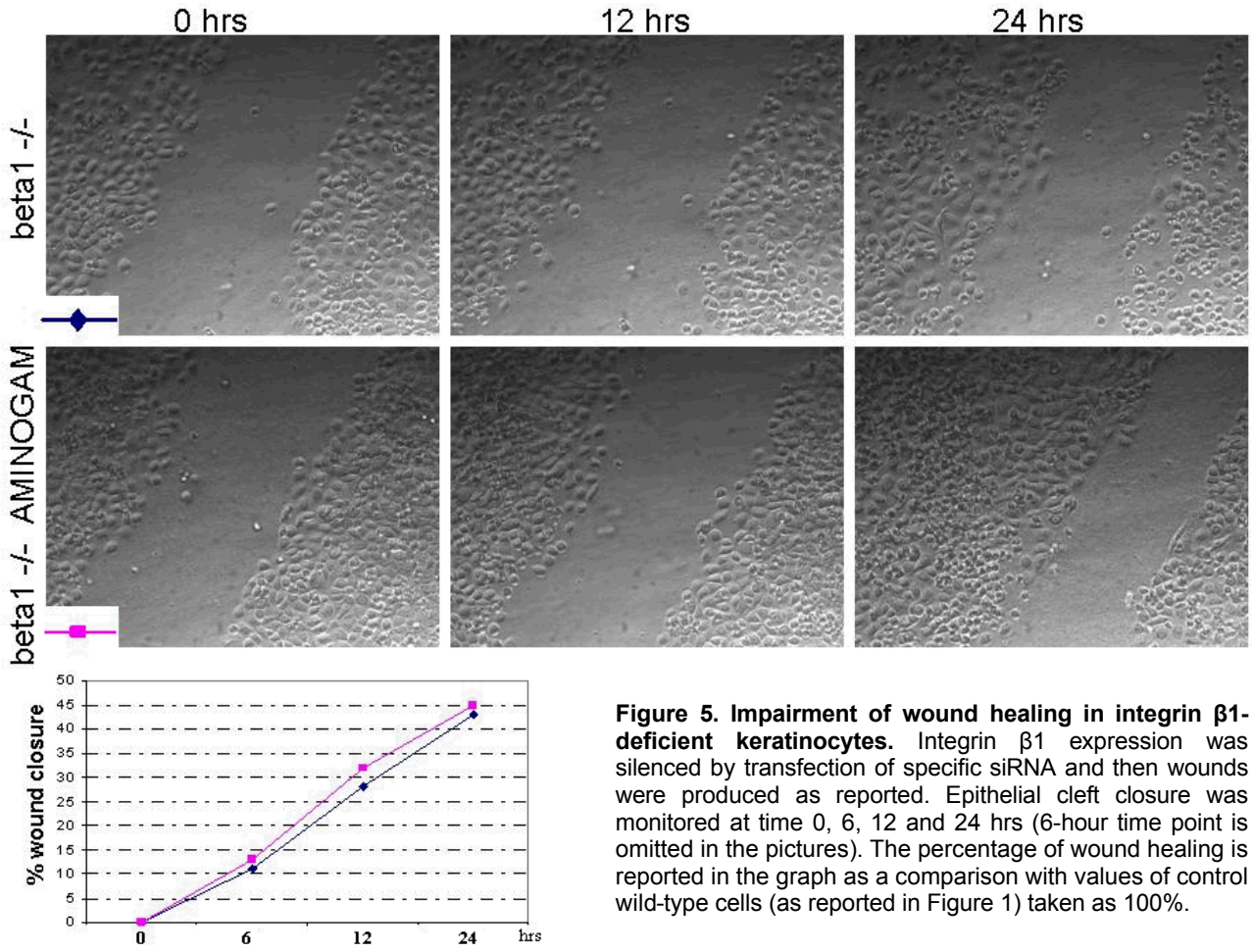


Figure 5. Impairment of wound healing in integrin β1-deficient keratinocytes. Integrin β1 expression was silenced by transfection of specific siRNA and then wounds were produced as reported. Epithelial cleft closure was monitored at time 0, 6, 12 and 24 hrs (6-hour time point is omitted in the pictures). The percentage of wound healing is reported in the graph as a comparison with values of control wild-type cells (as reported in Figure 1) taken as 100%.

in would closure reflected an impairment in keratinocyte motility. Cell scattering, spreading, and directional migration were massively reduced by treatment with β1 integrin siRNAs compared to control values (**Fig.6**), whereas random siRNAs had no effect (not shown). These functional features were not significantly (p>0.05) improved by Aminogam in β1 integrin-deficient keratinocytes.
Collectively, our data demonstrate that β1 integrin is required to mediate the effects of Aminogam on keratinocyte wound regeneration.

Discussion

HA is a potent stimulator of tissue repair and is widely used in dermatology and oral medicine for epidermal injuries. In the present study, we shed light on the molecular and functional mechanisms underlying the effectiveness of Aminogam ®, an aminoacid-enriched sodium hyaluronate formulation, in promoting keratinocyte wound healing. We report that Aminogam can stimulate keratinocyte motility in a fibroblast-free environment leading to enhanced epithelial wound closure and that this process is dependent on β1 integrin.

The biological function of HA has been extensively studied in tissue injury, inflammation, and fibrosis. For this reason, and given the observation that hyaluronan is found mainly in connective tissues, most studies have investigated the effects of HA on mesenchymal cells, including chondrocytes, osteoblasts, and fibroblasts. Changes of HA turn-over as well as altered expression and activity of hyaluronidases have long been noticed in diseases such as rheumatoid arthritis [29] and periodontal disease [30]. Patients with advanced scleroderma have decreased serum Hyal-1 activity and elevated circulating levels of HA [31]. In one study, six patients with bone or connective tissue abnormalities had lower levels of Hyal-1 activity than did healthy donors [32].
Recent study by Favia and colleagues reported that Aminogam accelerated the wound healing process of oral mucosa and promoted angiogenesis in vivo via induction of fibroblast proliferation, collagen biosynthesis and secretion of angiogenic cytokines, respectively [33, 34]. In clinical practice, these properties translate into enhanced epithelial repair in patients with various types of injuries of oral mucosa [33, 35]. Aminogam was able to reduce the healing time of oral soft tissues after three different

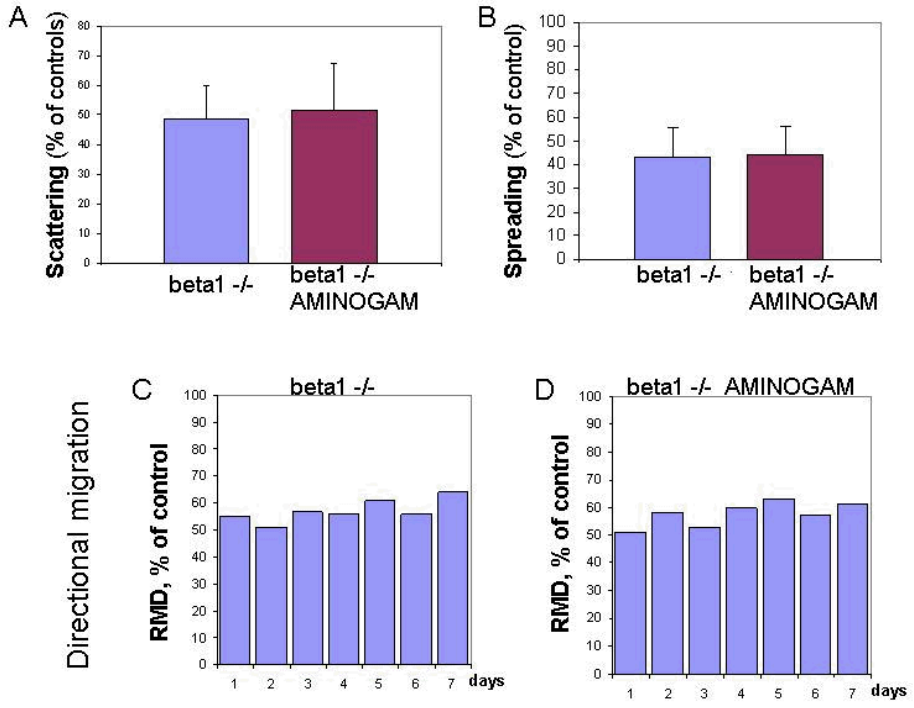


Figure 6. Lack of integrin β1 delays wound healing. After transfection with integrin β1 specific siRNA pools, keratinocytes were assessed for cell scattering, spreading and directional migration in the presence or absence of Aminogam. Cells incubated with medium containing 1% Aminogam did not differ from controls in terms of migratory features. Scattering and spreading were reduced in the order of 50-60%; chemotaxis was decreased of 40-50%. The percentage reported in the graph represent a comparison with values of control wild-type cells (as reported in Figures 2 to 4) taken as 100%. Values are given as an average of three independent experiments.

kinds of surgery and also the absence of post-operative infections [33]. Data from other groups also suggest that an Aminogam-based spray accelerates lesion healing and above all helps manage mucositis-related pain, especially in terms of immediate pain relief (after 2 hours from application) [35].

HA appears to promote corneal, diabetic foot, tendon, bone, nasal, mucosal, and venous leg ulcer wound healing. In addition, HA is used as an anti-adhesion and anti-scar drug in general surgery [7, 8, 13, 33, 35-37]. It acts as an antioxidant for inflamed body tissues, protecting body tissue from free radical damage and supporting immune function by inhibiting germs and viruses from moving between cells and getting into cells [17]. HA's viscoelastic matrix can act as a strong biocompatible support material and therefore is commonly used as growth scaffold in surgery. In addition, administration of purified high molecular weight HA into orthopaedic joints can restore the desirable rheological properties and alleviate some of the symptoms of osteoarthritis [38]. The success of the medical applications of HA has led to the production of several successful commercial products, which have been extensively reviewed previously [5-12]. However, the exact molecular mechanism of exogenous HA activity has yet to be identified. In this study we reported for the first time that one of the mechanisms enhancing epidermal repair by HA involves β1 integrin.

The integrin repertoire in basal keratinocytes is restricted to integrins α2β1, α3β1, α9β1 and α6β4, whereas de novo expression of additional integrins, for example, integrins α5β1, αvβ1, αvβ5 and αvβ6 is induced upon wounding (reviewed in ref. 39). β1 integrin is a key player in epidermal biology and may couple with various α subunits, which comprise a distinct heterodimeric receptor whose ligands are arginine-glycine-aspartic acid-containing ECM molecules [40]. Deletion of the gene encoding the integrin β1 subunit causes early embryonic lethality [41]. Evidence for the function of β1 integrin in skin stems from conditional knockout mouse models in which the ablation of integrin β1 is restricted to the basal epidermal keratinocytes, resulting in skin blistering at the dermal-epidermal junction, a reduced number of hemidesmosomes, failure of basement membrane assembly, impaired invagination of hair follicles and eventual hair loss [42, 43]. Here we have shown that lack of β1 integrin impairs keratinocyte scattering and migration leading to delayed epidermal wound closure in vitro.

Among various clinical applications, Aminogam has been proven to be particularly effective in oral mucositis after radiotherapy [35]. In this context, it is interesting to note that β1 integrin expression is aberrantly up-regulated in after ionising radiation (IR) exposure of non-tumourigenic human mammary epithelial cells [44]. In a matrix-dependent fashion, integrins also modulate the survival of various cancer cell types post-IR. For

example, IR induces the expression of functional β1 integrin and its co-localised protein kinase, integrin-linked kinase (ILK), in two lung cancer cell lines A549 and SKMES1 cells [45]. In addition, a few studies have revealed that up-regulation of β1 integrin and its signaling essentially contributes to cell survival after radiaton exposure in pancreatic, prostate, glioblastoma, melanoma, and colorectal tumour cells [46-48]. Therefore, the pronounced effectiveness of the present aminoacid-enriched sodium hyaluronate formulation on radiotherapy-induced mucositis may depend on the increased function of β1 integrin. The increased expression of β1 integrin in keratinocytes subject to radiation therapy may represent a biological event which favours the effect of Aminogam in promoting wound healing.

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