Amniotic membrane induces epithelialization in massive posttraumatic wounds

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ABSTRACT

Large-surface or deep wounds often become senescent in the inflammatory or proliferation stages and cannot progress to reepithelialization. This failure makes intervention necessary to provide the final sealing epithelial layer. The best current treatment is autologous skin graft, although there are other choices such as allogenic or autologous skin substitutes and synthetic dressings. Amniotic membrane (AM) is a tissue of interest as a biological dressing due to its biological properties and immunologic characteristics. It has low immunogenicity and beneficial reepithelialization effects, with antiinflammatory, antifibrotic, antimicrobial, and nontumorigenic properties. These properties are related to its capacity to synthesize and release cytokines and growth factors. We report the use of AM as a wound dressing in two patients with large and deep traumatic wounds. Negative pressure wound therapy followed by AM application was capable of restoring skin integrity avoiding the need for skin graft reconstruction. AM induced the formation of a well-structured epidermis. To understand this effect, we designed some assays on human keratinocyte-derived HaCaT cells. AM treatment of HaCaT induced ERK1/2 and SAP/JNK kinases phosphorylation and *c-jun* expression, a gene critical for keratinocytes migration; however, it did not affect cell cycle distribution. These data suggest that AM substantially modifies the behavior of keratinocytes in chronic wounds, thereby allowing effective reepithelialization.

Wound healing is the body's natural biological process for regenerating dermal and epidermal tissue. This process involves time and a balanced activity of inflammatory, vascular, connective tissue, and epithelial cells leading to the restoration of skin integrity.[1]

In clinical practice, the best way to heal a wound is to close it according to surgical standards as quickly as possible after injury. However, this procedure is limited to those wounds located in anatomical regions that permit adaptation of wound borders allowing wound closure by primary intention. This means closing it by suturing and restructuring of the skin continuity.[2]

In large-surface and deep wounds in which primary wound closure is neither possible nor practicable, the most important issue is to dress the wound with appropriate materials in order to keep it free from infection, to reduce or eliminate pain and all potential factors inhibiting natural healing, and to replace or substitute the missing tissue as much as possible. In these cases, autologous skin grafting in the form of split- or full-thickness skin is a standard therapeutic criterion. In some patients, however, alternative therapies are needed because of the existence of comorbidity.[2]

In the last few years, negative pressure wound therapy (NPWT) has been considered a good treatment strategy for a wide variety of acute and chronic wounds. The mechanisms involved in NPWT include increased dermal perfusion, stimulation of granulation tissue formation, reduction in edema and interstitial tissue fluid, reverse tissue expansion, reduced bacterial colonization, and acting as an effective skin graft splint over irregular surfaces.[3,4]

Acute wounds heal rapidly and proceed through the inflammatory, proliferation, and remodeling phases of healing. Reepithelialization is the final and very important phase that occurs through the migration of keratinocytes from the edge toward the wound center. Large-surface or deep wounds, with important loss of soft tissues, often become senescent in the inflammatory or proliferation stages and cannot progress to reepithelialization.[1,2] This failure in the reepithelialization process requires the need for intervention in order to provide the epithelial layer for the final sealing of the skin. The best current invasive treatment is an autologous skin graft, but usually it requires hospitalization for several days and is not free of morbidity.[1,2] Other available treatments with good results are synthetic dressings of several types and allo- or auto-skin substitutes, although its cost remains too high for routine clinical use.

The amniotic membrane (AM) is a tissue of particular interest as a biological dressing due to its special structure, biological properties, and immunologic characteristics. AM has already been applied in medicine in the treatment of burn lesions and for surgical wound covering to avoid collusion. Additionally, AM has been used as a

bandage or a substrate for epithelial growth in the management of various ocular surface conditions and in diseases like epidermolysis bullosa.[5–7] Despite all these applications, uncertainty remains regarding the mechanism of its effects.

The AM or amnion is the most internal layer of fetal membranes. It consists of a thin epithelium, a basal membrane, and a stroma of avascular connective tissue. The epithelium makes a simple, continuous, uninterrupted line of columnar, cuboid, or flat cells, in contact with the amniotic fluid. It sits over a well-defined resistant basal lamina connected with a layer of amniotic mesoderm where three structures can be identified: an acellular compact layer made up of collagens I, III, and fibronectin; a netting of mesenchymal cells called the fibroblastic layer; and an intermediate layer or sponge zone, rich in proteoglycans, glycoproteins, and a nonfibrillar collagen type III, which is loosely connected to the chorion.[8] There are no nerves, muscles, or lymphatic vessels in the amnion. AM can easily be separated from the underlying chorion, as they never truly fuse at the cellular level. Both epithelial and mesenchymal amniotic cells possess characteristics of stem cells with, at least, multipotent differentiation ability, which makes AM a good candidate for use in cellular therapy and regenerative medicine.[9,10]

The AM has low immunogenicity, and well-documented reepithelialization effects, antiinflammatory, antifibrotic, antimicrobial, and nontumorigenic properties. These pleiotropic functions are related in part to its capacity to synthesize and release biological active substances including cytokines and signaling molecules such as the tumor necrosis factor, interferon, transforming growth factor (TGF)-α, TGF-β, basic fibroblastic growth factor, epidermal growth factor, keratinocyte growth factor, hepatic growth factor, interleukin-4 (IL-4), IL-6, IL-8, natural inhibitors of metalloproteases, β-defensins, prostaglandins, etc.[8,10,11]

Recently, there has been a resurgence of interest in AM and amnion transplantation because of its ability to enhance wound healing by promoting reepithelialization and reducing scarring and inflammation; additionally it works as a scaffold where cells can proliferate and differentiate. Moreover, AM is a biomaterial that can be easily obtained, processed, and transported.[5] AM has been used in ophthalmology[5,12–14] and in the treatment of nonhealing ulcers of different etiologies[15] with satisfactory results.

This prompted us to use AM as a wound dressing in two patients with deep and extensive traumatic wounds. In both cases, AM application was capable of restoring skin integrity avoiding the need for skin graft reconstruction. To understand the possible effect of AM on wound reepithelialization, we designed some cell assays on HaCaT cells, a human spontaneously immortalized keratinocyte cell line.

MATERIALS AND METHODS

This study was approved by the local ethics committee (University Hospital Vírgen de la Arrixaca, Murcia, Spain), the Spanish Agency for Drugs (AEMPS), and was conducted after appropriate written informed consent was obtained from the AM donors and patients.

AM processing and preparation

Term placenta from healthy donor mothers was obtained from uncomplicated cesarean sections. The fetal membranes were washed in 1,000 mL of physiological saline solution (PSS) supplemented with amphotericin (50 mg), clotrimazole (48 mg), tobramycin (50 mg), and vancomycin (50 mg) and rapidly transferred to laboratory under sterile conditions. Under a laminar flow cabinet, the amnion was mechanically peeled from the chorion, washed three to four times with 200 mL of phosphate-buffered saline (PBS), and flattened onto sterile nitrocellulose paper with the basement membrane surface up. The paper with the adherent membrane was then cut into 5 cm × 5 cm segments. AM was placed in a sterile vial containing a freezing solution made of 10% dimethyl sulfoxide, TC-199 medium, and human albumin and then frozen at −80 °C until its use. The day of wound application, AM was thawed at 37 °C, then segments were washed three times with PBS and placed onto sterile petrolatum gauze (Tulgrasum) with the epithelial side contacting it. AM processed this way was taken to the surgical room and applied onto the selected patients. Bacteriology tests were performed to the AM-containing medium at different stages of the process, including thawing, melting, and further washes. Other quality controls including testing maternal blood samples for infectious markers such as syphilis, hepatitis B virus, hepatitis C virus, HIV, and HTLV were carried out on the day of birth and after a quarantine period of at least 90 days.

Patients and usage of AM for massive wound healing

Two patients with large-surface and deep traumatic wounds with patent retard in epithelialization received AM as a biological dressing. Their clinical characteristics are shown in Table 1. The surgical protocol was as follows: the wound was thoroughly debrided under anesthesia, and extensively washed with a local antiseptic solution. Then, wounds were treated by NPWT as follows: clean wound cavity was packed with polyvinyl foam and subjected to vacuum using V.A.C.™ (Kinetic Concepts Inc., San Antonio, TX). Once a significant granulation tissue was observed, the wound bed was very carefully washed with PSS. Several fragments of AM on sterile petrolatum gauze with the basal membrane facing down were placed over the whole wound area and NPWT was reapplied. Wound evaluation was performed at the time of AM transplantation and every 3–4 days until healing was complete. In Patient 2, a second AM application was considered. To better follow the wound healing process, a planimetry was taken along the treatment course. Wound area was calculated using Visitrak wound measurement system (Smith & Nephew Plc., London, UK).

Histopathological studies

To evaluate the effect of AM in wound epithelialization, biopsies were obtained from newly formed and from normal surrounding skin in both patients. The samples were prepared following standard procedures for paraffin-embedded sections that were stained with hematoxylin and eosin and evaluated under the microscope by a pathologist.

Immunohistochemistry on paraffin-embedded sections was performed following standard procedures. Briefly,

Table 1. Clinical features of the patient wounds

Patient characteristics					
Age/gender	Etiology	Wound size (cm)	Time to AM transplant (days)	Comorbidity	End point results (days)
49/female	Traumatic	17×15	60	No	272
76/female	Traumatic	18×10	48	No	667

AM, amniotic membrane.

sections were incubated with a blocking buffer (3% bovine serum albumin, 0.1% Tween-20 in PBS) at room temperature for 1 hour. Subsequently, the sections were incubated with the primary antibodies in the blocking buffer at room temperature for 2 hours, washed three times in PBS/0.1% Tween-20 for 10 minutes, and then incubated at room temperature for 1 hour with secondary antibodies and bisbenzimide 1.2 μg/mL final concentration (Fluka Chemie AG, Buchs, Switzerland). After several washings with PBS, the sections were mounted and examined by confocal microscopy (LSM 510 META, Zeiss, Jena, Thüringen, Germany).

Cell culture and AM assay

Cell line HaCaT, human spontaneously immortalized keratinocytes, was grown in Dulbecco's modified Eagle's medium (DMEM) medium supplemented with 50 U/mL penicillin, 50 μg/mL streptomycin, and 10% fetal bovine serum. To perform the AM assay, five to six pieces of 4 cm^2 of AM were placed in 6 cm diameter dishes containing 60–70% confluent HaCaT cells. HaCaT cells stimulated for several periods of time were lysed using the appropriate buffers, and then either protein or RNA was extracted. In the case of protein studies, cells were lysed with 50 mM HEPES pH 7.2, 150 mM NaCl, 1 mM EDTA, 1.2 mM $MgCl_2$, 1% Triton X-100, and 10% glycerol, 1 mM DTT, 25 mM NaF, and 25 mM β-glycerophosphate supplemented with phosphatase (I and II), and protease inhibitors (Sigma, St. Louis, MO). Protein extracts were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by Western blot. For gene expression assay, RNA was extracted using RNeasy-mini (Qiagen, Hilden, Germany). Typically, 1 μg of RNA was retrotranscribed by GeneAmp (Applied Biosystems, Foster City, CA), and the resulting cDNA was used for qPCR following the SYBR premix ex Taq kit (Takara, Otsu, Shiga, Japan) according to the manufacturer's instructions. Primers used for gene amplification were GGAAACGACCTTCTATGACGATGCCC cJun-F; GG CGCGCACGAAGCCCTCGGCGAACC cJun-R; ACCA CAGTCCATGCCATCAC GAPDH-F; TCCACCACCC TGTTGCTGTA GAPDH-R.[16] All gene expression was referred to as a ratio to the GAPDH expression. For cell cycle analysis, cells were stimulated with AM, as described above. Additionally, cells were trypsinized, fixed in 70% ethanol, and stained for cell cycle with propidium iodide using a standard method. Cells were analyzed by flow cytometry using an FACS Calibur 1 (Becton Dickinson, Franklin Lakes, NJ). For conditional medium assays, AM was maintained in DMEM medium supplemented with 50 U/mL penicillin, 50 μg/mL streptomycin, and 10% fetal bovine serum for 48 hours. The resulting medium was filtered through a 0.45 μm membrane and applied to HaCaT cells in culture that were assayed in a similar way as described above.

Antibodies

The following commercial antibodies were used: anti-phospho-ERK1/2, anti-phospho-p38, anti-phospho-SAP/JNK, anti-phospho-MKK3/6, anti-Grb2 (all Cell Signaling Technology, Danvers, MA) and anti-c-Jun and anti-ZO-1 (all Santa Cruz Biotechnology, Santa Cruz, CA).

RESULTS

Clinical results

Case report 1

A 49-year-old woman with poly-trauma and a 234 cm^2 deep incised contused wound in the right thigh was admitted to the hospital (Table 1). After wound conditioning, NPWT treatment was applied; 60 days later, a complete restoration of the soft tissue was observed with negative microbiological culture, and the wound had reduced to 104 cm^2 without signs of epithelialization (Figure 1A, day 0). At this time, AM dressing was applied (Figure 1A, day 0). Nine days after treatment with AM, petrolatum gauze was removed, and a complete adhesion of AM to the wound bed was observed. Most importantly, an incipient reepithelialization from the margin of the wound had started (Figure 1A, day +16). Such epithelialization increased over the course of the AM application (Figure 1C, Patient 1). Wound size was gradually reduced and total wound reepithelialization was successfully established by day +272 (Figure 1A).

Case report 2

A 76-year-old woman with a crush trauma and massive wound in the lateral exterior area of the right leg developed a necrotic dermis lesion of approximately 81.3 cm^2 with destruction of soft and muscle tissues (Figure 1B, day −48). The wound was cured according to standard surgical procedures and microbiological cultures were negative. However, it developed inflammatory local signs, edema, hemathoma, and flictens with serohematic fluid content. Overall, the clinical outcome was not satisfactory. For 20 days, a local cure was applied to the wound, after which the wound bed was conditioned, and finally ready to be treated with NPWT (Figure 1B, day −28). Twenty-eight days later, the granulation tissue appeared satisfactory and the wound had reduced to 58.1 cm^2 without signs of epithelialization. Then, AM dressing was applied (Figure 1B, day 0). Petrolatum gauze

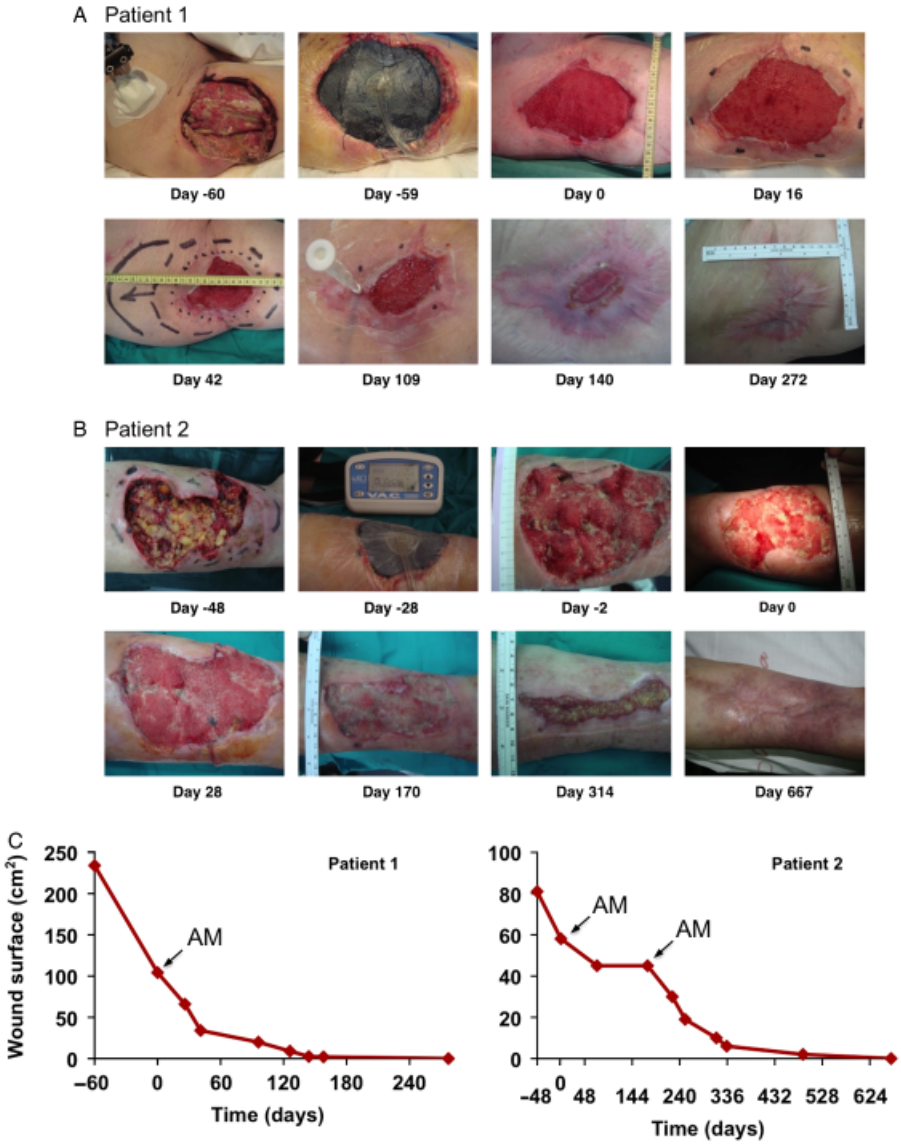

Figure 1. Amniotic membrane (AM) induces epithelialization in deep extensive wounds. (A) Clinical evolution of Patient 1. Wound morphology before AM application. AM was applied at day 0. Several stages in the evolution of the wound after application of AM until complete healing at day 272 are shown. (B) Clinical evolution of Patient 2. Wound morphology before AM application. AM was applied at day 0. Several stages in the evolution of the wound after application of AM are shown. (C) Size evolution of wounds of Patients 1 and 2. As in pictures, negative days represent negative pressure wound therapy before AM application, day 0 represents the day AM was applied. In Patient 2, a second arrow is drawn when AM was reapplied. Measurements of the wound bed are represented as a function of time.

removal, 8 days after the first AM application, revealed minimal epithelialization from the wound edges that stay still for about 3 months (Figure 1C). Then, a second AM application was decided at day +170 (Figure 1B, day +170, Figure 1C, Patient 2) together with NPWT. Wound size was gradually reduced (Figure 1B, day +314) and total wound re-epithelialization was successfully established by day +667 (Figure 1B, day +667).

Neither of the two cases presented adverse events. AM was well tolerated and the surrounding skin did not show any irritative dermatitis in these patients. No wound infection was observed after AM transplantation.

Histopathological results: structure of AM-induced newly formed skin

Paraffin-embedded section from Patient 1 AM-induced newly formed skin showed evidence of fibrotic scarring tissue with large and abundant blood vessels both in deep and superficial dermis (Figure 2D and E). In contrast to control skin samples, annexes were not present (Figure 2, compare [A] and [D]). A close observation of the edge of the wound (Figure 2F) showed the enrichment in inflammatory cells with fewer blood vessels and a more immature epidermis characterized by the presence of parakeratin in the incipient stratum corneum (Figure 2F and G). In contrast, tissue at the far end of the wound edge showed a more consolidated epidermis with orthokeratotic stratum corneum and more organized cell layers beneath it (Figure 2F and H). Moreover, on this side, the dermis had no inflammatory cells and was abundantly vascularized (Figure 2, compare [H] to [G] and [B]).

Paraffin-embedded section of normal surrounding skin from Patient 2 showed a typical structure with papillary and reticular dermis that contains annexes and normal vascularization, and an epidermis with the usual strata organization (Figure 3A and B). The histopathology of

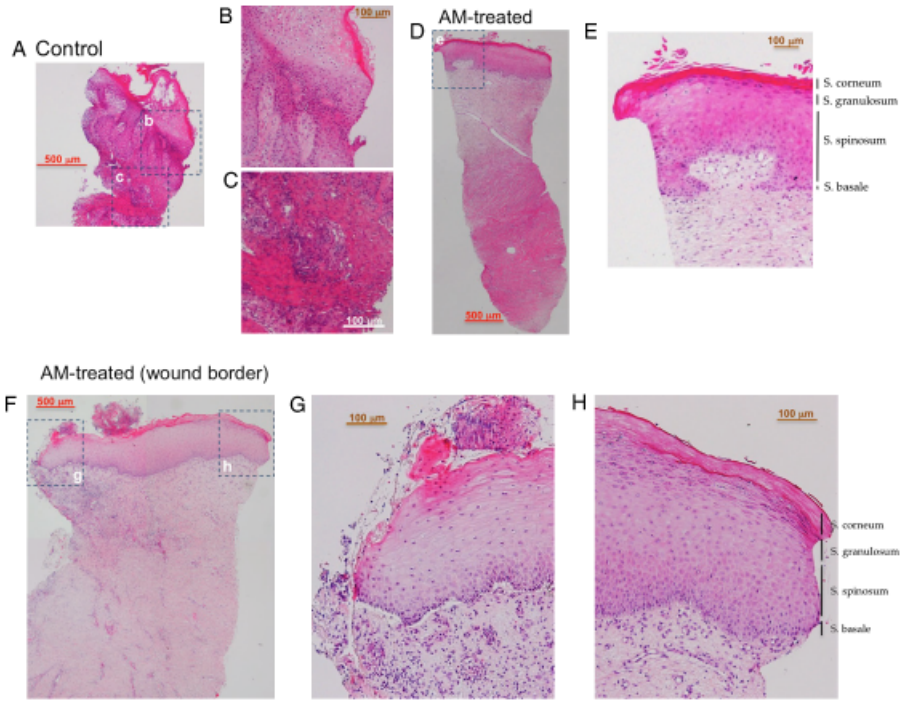

Figure 2. Histopathological study of amniotic membrane (AM)-induced epithelialization; Patient 1. (A) Microscopic section from normal skin. (B) Higher magnification microphotograph, taken from (A), shows the normal structure of papillae at epidermis. (C) Higher magnification microphotograph, taken from (A), shows skin annexes. (D) Skin section from AM-induced newly formed skin. Dermis shows a high degree of cicatricial fibrosis with an abundance of large blood vessels. Note the absence of dermal papillae and annexes. (E) Higher magnification microphotograph, taken from (D), shows the layers of the newly formed skin. (F) Microscopic section of the edge of the wound where AM was applied. It shows from left to right the increasing maturation of the epidermis. (G) Higher magnification microphotograph taken from (F); note parakeratosis. (H) Higher magnification microphotograph taken from (F); note orthokeratosis. In each picture, bar indicates scale. S., stratum.

AM-induced newly formed skin showed a dermis with abundant blood vessels, scarring fibrosis, and a low degree of inflammation (Figure 3C). All the epidermis strata looked normal including the presence of an orthokeratotic stratum corneum. The section of the AM-treated wound edge clearly revealed the migrating epithelial tongue or epidermal leading edge (Figure 3D), typically showing keratinocytes that are regenerating the epidermis.[17] Different degrees of maturation of the epidermis can be observed from the far (left) side toward the center of the wound, where there is inflammatory granulation tissue and a formatting crust (Figure 3E). The dermis underneath showed a high degree of fibrosis, abundant new vessels, and the absence of annexes.

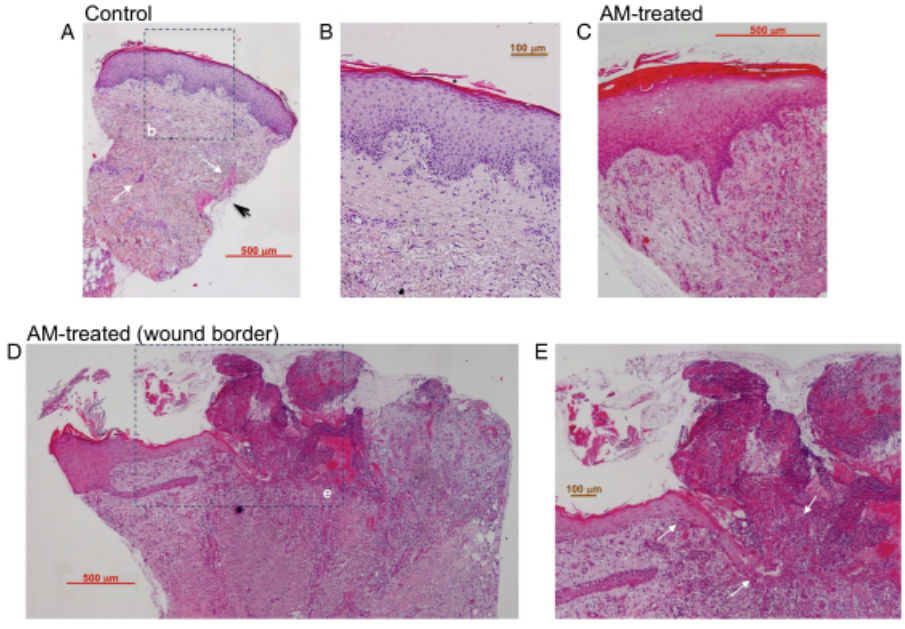

Figure 3. Histopathological study of amniotic membrane (AM)-induced epithelialization; Patient 2. (A) Microscopic section from normal skin. Arrows point at skin annexes. (B) Higher magnification microphotograph taken from (A) shows the normal structure of skin papillae. (C) Skin section from AM newly formed skin. Dermis shows a high degree of cicatricial fibrosis with an abundance of large blood vessels. Note the absence of dermal papillae and annexes. (D) Microscopic section of the edge of the wound where AM was applied. Picture shows the increasing maturation of the epidermis from center to left. (E) Higher magnification microphotograph taken from (D) shows the epidermal leading edge (arrows). In each picture, bar indicates scale.

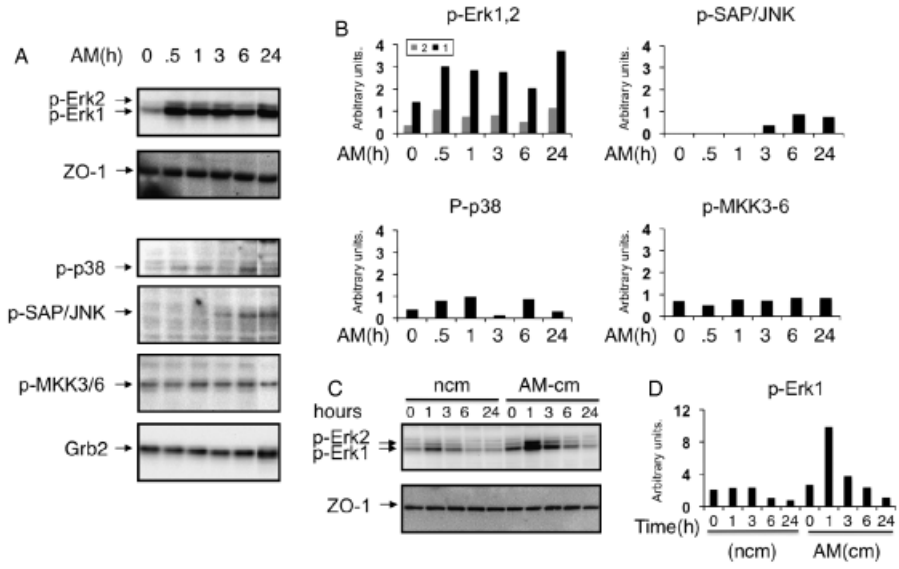

Figure 4. Amniotic membrane (AM) induces the phosphorylation of SAP/JNK and ERK1 and 2 in HaCaT cells. (A) HaCaT cells were stimulated with AM for the indicated times, protein was extracted, and analyzed by Western blot. Antibodies for detection were used as indicated. (B) Signal obtained from Western blot in (A) was quantified. Bars represent the signal obtained in each lane of the Western blot as a ratio to ZO-1 protein, in the case of phospho-ERK1 and 2, and Grb-2 protein for phospho-p38, phospho-SAP/JNK, and phospho-MKK3/6 proteins. (C) HaCaT cells were stimulated with AM-conditioned medium (AM-cm) or nonconditioned medium (ncm) for the indicated times, protein was extracted and later analyzed by Western blot. Signal obtained from Western blot in (C) was quantified. Bars represent the signal obtained in each lane of the Western blot as a ratio to ZO-1 protein. These are a representative sample of three independent experiments.

AM induces ERK1, 2, and SAP/JNK phosphorylation in human HaCaT cells

To understand the molecular mechanisms involved in AM-induced skin reepithelialization, we studied, at the molecular level, the effect of AM on HaCaT cells. The human cell line HaCaT has been widely used as a model for keratinocyte behavior. These cells, human primary spontaneously immortal keratinocytes, largely retain their capacity to reconstitute a well-structured epidermis after transplantation in vivo.[18] AM fragments placed for several hours on cultured HaCaT cells produced a stimulation of phosphorylation of ERK1 and 2 that was already evident in 30 minutes and was maintained over time (Figure 4A and B). The addition of AM-conditioned medium to HaCaT cells produced strong ERK1 and 2 phosphorylation that lasted for 3 hours (Figure 4C and D). This is in agreement with the fact that AM has the capacity to synthesize and release biologically active substances including cytokines and growth factors.[10] Additionally, phosphorylation of SAP/JNK kinases was evident 3 hours after AM stimulation. A weak p38 phosphorylation was also detected 30 minutes after AM stimulation (Figure 4A and B); however, no phosphorylation was apparent in MKK3/6, an upstream kinase activator for p38 (Figure 4A and B). Furthermore, ERK1 and 2, and SAP/JNK kinases phosphorylations were still detectable 24 hours after AM treatment, suggesting an AM-sustained stimulus on these pathways (Figure 4A and B). JNK1 is a positive regulator of c-Jun contributing to its phosphorylation and stabilization.[19,20] Members of the AP1 family had been involved in keratinocyte migration. The AP1 transcription factors regulate the expression of various genes involved in the wound healing process.[21,22] c-Jun is a central molecular effector of epidermal leading edge formation in a vertebrate model organism.[23] In order to ascertain the effect of these kinase activations on gene transcription, we measured the expression of *c-jun*. AM treatment up-regulated c-Jun protein levels at early time points (Figure 5A). Furthermore, 24 hours later, c-Jun protein levels were still high in comparison with nonstimulated cells. Study of *c-jun* mRNA levels revealed a fourfold increase of expression 24 hours after HaCaT cells were stimulated with AM (Figure 5B). In conclusion, the presence of AM on HaCaT cells stimulates several signaling pathways and the expression of transcription factors that have been involved in keratinocyte migration.

Effect of AM on cell cycle regulation in HaCaT cells

We reasoned that the AM effect on components of different signaling pathways (ERK1 and 2, SAP/JNK, and p38) could have implications for cell proliferation. Therefore, we studied the effect of AM on cell cycle regulation. Exponentially growing HaCaT cells were stimulated for 24 hours with AM and the cell cycle was analyzed. We did not detect any changes in AM-stimulated cell proliferation when compared with control nonstimulated cells. Moreover, addition of AM to confluent HaCaT cells did not affect cell cycle arrest due to cell contact inhibition (Figure 6A). A similar result was obtained when serum-deprived arrested HaCaT cells were stimulated with AM (Figure 6B).

c-jun is highly expressed at the epidermal leading edge

Our results in HaCaT cells showed a strong long-lasting induced expression of c-Jun after AM application (Figure 5A). Protein c-Jun is an essential player in the organization of keratinocyte leading edge.[23] In order to evaluate the contribution of c-Jun in the reepithelialization of our patients, we studied its expression at the wound borders. Interestingly, massive wound borders showed a strong c-Jun expression. This was particularly evident at the basal epithelium near the leading edge and at the dermal leading edge or keratinocyte tongue (Figure 7C and H, see

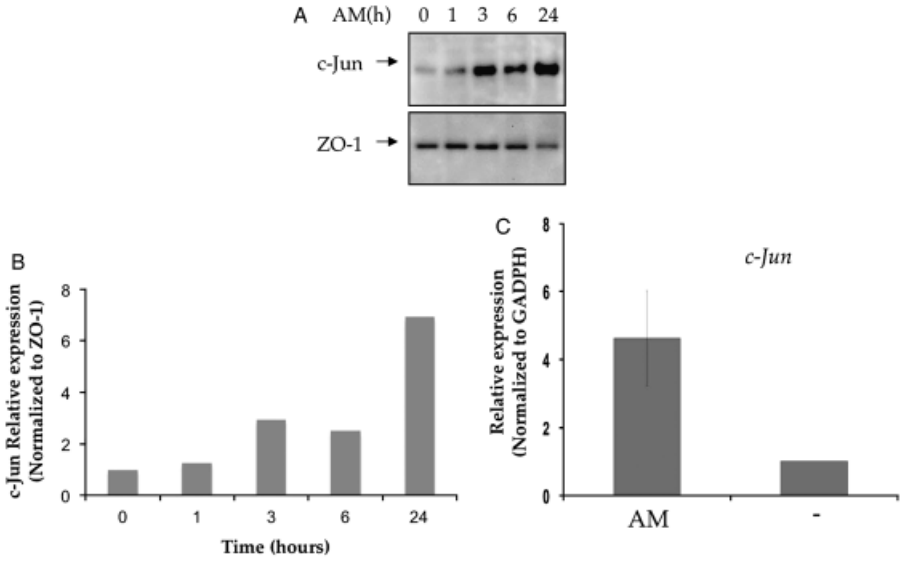

Figure 5. Amniotic membrane (AM) induces the expression of c-Jun in HaCaT cells. In all cases, HaCaT cells were stimulated with AM for the indicated times. (A) HaCaT cell protein extracts were analyzed by Western blot to study the expression of c-Jun. Quantification of the protein levels as a protein ratio to ZO-1. (B) Expression of *c-jun* 24 hours after stimulation with AM. Total RNA was extracted, converted into cDNA, and analyzed by qPCR. The *c-jun* expression is represented as a ratio to one of the housekeeping genes *GAPDH*. Error bars represent mean ± SD. This is a representative sample of three independent experiments. h, hours.

arrows). In control skin, however, c-Jun was not expressed either at the basal epithelium or at any other part of the epidermis (Figure 7A and E). All these data indicate that c-Jun expression might be an important event for epithelialization occurring at the AM-stimulated wound borders.

DISCUSSION

The treatment of chronic large-surface and deep wounds of any etiology is still a challenge in the wide field of reconstructive skin surgery, and is hence the subject of great controversy. In this kind of wound, it is common to use a variety of synthetic and biological dressings in combination with NPWT.

In our two patients, we have combined the usage of NPWT and AM dressing to provide effective healing of their massive wounds. NPWT resulted in the formation of granulation tissue, but it was unsuccessful in re-epithelializating the wounds. This phase was stimulated by the application of AM. In both patients, wound conditions improved remarkably after AM treatment, resulting in reepithelialization of the wounds. At 8 days, the AM was completely adhered to the wound bed and an incipient epithelialization from the edges was evident. However, in Patient 2, a second AM application was needed. Eventually, epithelialization was consolidated finally covering the whole wound in both cases. Although the process was slow, AM application was capable of restoring skin integrity inducing the formation of a well-structured epidermis with all relevant layers. The single application of AM appears to be insufficient for explaining its therapeutic effect on massive wound epithelialization. The effect of AM is attributed to the combination of growth factors it releases.[24] The fact that the healing benefit still persists after several weeks suggests that a first burst of growth factors initiate a durable wound healing process.

The effect of AM in the healing of ulcers of different etiologies has already been reported.[6,7,15,25] Recently, Mermet et al.[5] evaluated the safety, feasibility, and the effects on healing of AM graft in 15 patients with chronic venous leg ulcers and found a significant clinical response in most of them, including complete healing in three during the 3-month follow-up period.

The mechanisms involved in AM-induced skin re-epithelialization are largely unknown. It has been shown

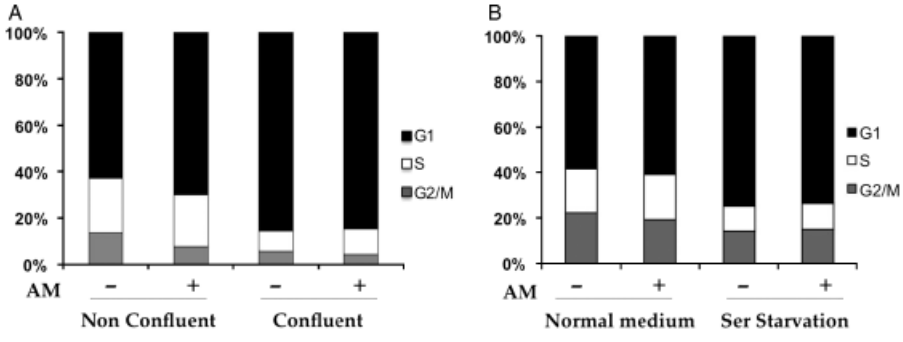

Figure 6. Effect of amniotic membrane (AM) on cell cycle. (A) Confluent and nonconfluent (40%) HaCaT cells were grown in the presence or absence of AM for 24 hours. (B) Nonconfluent (40%) HaCaT cells cultured in medium without serum were AM treated for 24 hours and compared with cells without AM. Both were compared with control cells grown in normal serum-supplemented medium. In all cases, cells were collected, processed, and analyzed by fluorescence-activated cell sorting to determine the number of cells in the G1, S, and G2/M phases. Cells in each phase were represented as a percentage of total cells. This is a representative example of two independent experiments.

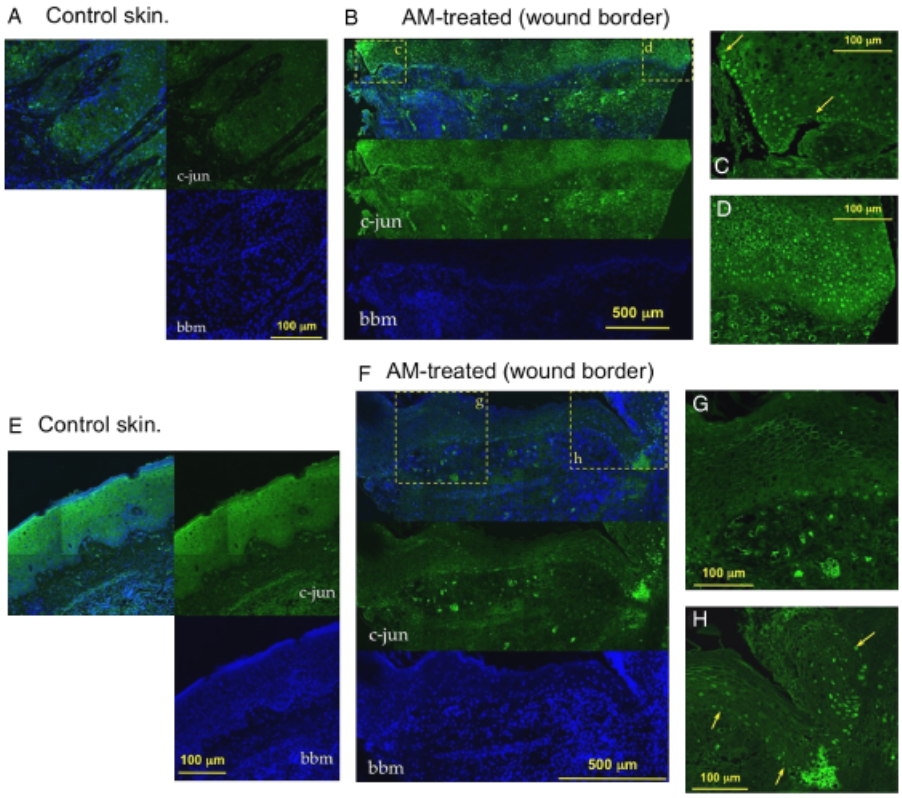

Figure 7. Expression of c-Jun at the epidermal leading edge. In all cases, microscopic sections from Patient 1 or 2 were immunostained against c-Jun. Cell nuclei were revealed by bisbezimide (bbm) staining. (A) Microscopic section of control skin from Patient 1. (B) Microscopic section of amniotic membrane (AM)-treated wound from Patient 1 showing the edge in the process of reepithelialization. (C) Higher magnification microphotograph taken from (B) shows the less mature portion of the reepithelializing area. Arrows point at the epidermal leading edge. (D) Higher magnification microphotograph taken from (B) shows the more mature section of the reepithelialized area. (E) Microscopic section of control skin from Patient 2. (F) Microscopic section of AM-treated wound from Patient 2. The section shows the edge of the wound in the process of reepithelialization. Note the epidermal leading edge or keratinocyte tongue [enclosed in box (H)]. (G) Higher magnification microphotograph taken from (F) shows the more mature section of the reepithelialized wound area. (H) Higher magnification microphotograph taken from (F) shows the less mature section of the reepithelializing wound area. Arrows point at the epidermal leading edge. In each picture, bars indicate scale.

that AM might exert its wound healing effect by accelerating keratinocyte migration from the wound edge and inducing its differentiation, thereby contributing to the generation of intact epithelium.[14] Niknejad et al.[26] considered that the stimulatory effect on epithelialization from the wound bed and/or the wound edge is mediated by growth factors and progenitor cells released by AM. In addition, it has been reported that the maintenance of the integrity of the basement membrane and stromal matrix improves the healing potency of the AM and is crucial in promoting rapid reepithelialization.[27]

In the present study, the human keratinocyte-derived cell line HaCaT showed different molecular responses upon stimulation with AM. An increased phosphorylation in ERK1 and 2, p38, and SAP/JNK was detected at early time points, most being detectable 24 hours after stimulation. These results could be interpreted as the effects of soluble AM-released factors acting over HaCaT cells. In fact, AM-conditioned medium has the ability to induce a similar response in HaCaT cells to the one obtained by the direct application of AM. It would be very interesting to measure the effect of AM on human primary keratinocytes in order to fully address the effect of AM on wound epithelialization.

HaCaT cells stimulated with AM for 24 hours showed an increased expression of *c-jun*. Members of the AP1 family had been involved in keratinocyte migration. AP1 transcription factors regulate the expression of various genes involved in the wound healing process.[21,22] Upon the wounding of organotypic cultures of E17 rat skin, a transient induction of *c-fos* and *c-jun* occurs.[28] Mice with keratinocyte-specific deletion of the *c-jun* gene had delayed wound closure owing to impaired keratinocyte migration.[23] AM induced the phosphorylation of SAP/JNK kinases in HaCaT cells. JNK1 is a positive regulator of c-Jun, and it contributes to its phosphorylation and stabilization.[19,20] A detailed study of the massive wound borders treated with AM showed a high level of c-Jun expression at the epidermal leading edge. This observation, together with the effect of AM on *c-jun* expression found in HaCaT cells, might explain the AM-induced reepithelialization that we observed in our patients. Further studies have to be performed to elucidate the contribution of AM to the expression of several members of the AP1 family at the wound edge.

Several other properties contribute to AM as an ideal dressing with advantages over other biological and synthetic dressings. It is bacteriostatic and acts as an effective barrier reducing heat, desiccation, and protein loss. Additionally, AM decreases infection incidence and local pain as observed in our patients. Moreover, AM has good adherence to the wound, it easily conforms to body contours, has good patient acceptability, and permits its early mobilization.[15]

Solomon et al.[29] reported that the AM stromal matrix markedly suppresses the expression of the potent

proinflammatory cytokines, IL-1α and IL-1β. Natural inhibitors of matrix metaloproteases have been found in AM.[30] Epithelial amniotic cells have the ability to express β-defensins, a group of peptides with antimicrobial and antiinflammatory properties.[31] These antiinflammatory effects of AM could facilitate keratinocyte migration over the wound bed. The effect of AM on inflammation in the patients' wounds is an issue that we want to address in the near future.

The low immunogenic capacity of AM[32] is another important property that allows its implantation without the immunological complications of xenogenic biomaterials.[33] In our patients, AM transplantation was safe and did not produce any adverse side effects. None of them showed evidence of acute or chronic rejection. The histological studies evidenced a normal regenerating skin without signs of teratomas. The safety of AM transplant has been supported by several studies. Although human amniotic epithelial cells (hAEC) are pluripotent, they do not form teratomas.[34–36] The lack of telomerase activity in hAECs might contribute to tumor suppression in vivo.[36] HaCaT cells did not overproliferate in the presence of AM and the treatment of HaCaT cells with AM revealed no variations in the cell distribution in the different stages of cell cycle. On the other hand, HaCaT cells arrested by serum deprivation were not proliferative in the presence of AM. Similarly, growth-inhibited confluent cells treated with AM showed the same distribution as untreated cells, suggesting that AM did not override cell-to-cell contact growth inhibition. These data support previous observations in the literature about the lack of a tumorigenic effect of AM on receptor tissues.[5]

In this study, we have clinically followed the evolution of two cases in which AM has been used for the treatment of large-surface wounds. This is an observational study that does not include control patients. As a consequence of these preliminary results, we have initiated a prospective clinical trial. So far, we believe that AM could function as a safe dressing, promoting epithelialization in massive wounds. The usage of AM dressing might be considered as a reemerging therapeutic option in the reepithelialization of large massive wounds.

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