Retinoic Acid Engineered Amniotic Membrane Used as Graft or Homogenate: Positive Effects on Corneal Alkali Burns

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PURPOSE. Alkali burns are the most common, severe chemical ocular injuries, their functional prognosis depending on corneal wound healing efficiency. The purpose of our study was to compare the benefits of amniotic membrane (AM) grafts and homogenates for wound healing in the presence or absence of previous all-trans retinoic acid (atRA) treatment.

METHODS. Fifty male CD1 mice with reproducible corneal chemical burn were divided into five groups, as follows: group 1 was treated with saline solution; groups 2 and 3 received untreated AM grafts or grafts treated with atRA, respectively; and groups 4 and 5 received untreated AM homogenates or homogenates treated with atRA, respectively. After 7 days of treatment, ulcer area and depth were measured, and vascular endothelial growth factor (VEGF) and matrix metalloproteinase 9 (MMP-9) were quantified.

RESULTS. AM induction by atRA was confirmed via quantification of retinoic acid receptor β (RARβ), a well-established retinoic acid–induced gene. Significant improvements of corneal wound healing in terms of ulcer area and depth were obtained with both strategies. No major differences were found between the efficiency of AM homogenates and grafts. This positive action was increased when AM was pretreated with atRA. Furthermore, AM induced a decrease in VEGF and MMP-9 levels during the wound healing process. The atRA treatment led to an even greater decrease in the expression of both proteins.

CONCLUSIONS. Amnion homogenate is as effective as AM grafts in promoting corneal wound healing in a mouse model. A higher positive effect was obtained with atRA treatment.

Keywords: vitamin A, epithelial wound healing, corneal repair, amniotic membrane, graft, homogenates

Chemical ocular burns represent 10% of all ocular traumas.1 Most victims are young, and their visual acuity is reduced to less than 6/60 in 10% of cases.2 Among chemical burns, alkali burns are the most common2,3; most of these injuries occur in the workplace.2 These alkali burns are the most serious chemical eye lesions because of their capacity to damage deep structures of the cornea. Indeed, they cause severe lesions by intraocular penetration through saponification of the membranes and denaturation of the stromal collagenous matrix.4 Amniotic membrane (AM) grafts are used for many eye surface disorders.5–8 In recent years, some studies using homogenates of AMs have also been developed for the treatment of ocular surface disease.5,6,8–12 AMs may improve corneal healing through their anti-inflammatory and antiangiogenic properties.9,12,13 However, transplantation of AMs involves a substantial risk of the following complications: subconjunctival hemorrhage, early degeneration of the membrane, surface infection, and calcification.5 Recently, Guo et al.10 compared the efficacy of AM homogenates and transplanted AM for the treatment of alkali burns in rabbits and found that amnion homogenates are as effective as transplanted AM in promoting corneal healing.

To further improve the pro-healing properties of both forms of AM, a pretreatment by a well-known healing molecule, vitamin A, could be proposed. In fact, vitamin A and its active derivatives (retinoids) are necessary for normal epithelial growth and differentiation. Furthermore, a lack of nutritional vitamin A causes serious eye surface disorders, such as the disappearance of goblet cells and keratinization of the corneal and conjunctival epithelium. Recent data based on a mouse knockout model lacking corneal retinoic acid (RA) synthesis demonstrated its essentiality for corneal maintenance.14 The molecular and metabolic retinoid pathways have already been described to be present and functional in both the human ocular surface and AMs.15,16 Finally, several studies have shown the efficacy of such molecules when used to treat some corneal disorders, such as keratoconjunctivitis sicca, Steven-Johnson syndrome, and posttrauma ulcers.17–19 Visual acuity is closely linked with corneal transparency, which may involve better control of inflammation and neoangiogenesis. This can be illustrated by the regulation of vascular endothelial growth factor A (VEGF-A) production, which promotes the proliferation and division of endothelial
cells, as well as enhancing blood permeability.\textsuperscript{20,21} It is also the case for matrix metalloproteinase 9 (MMP-9) accumulation, which is known to be involved in extracellular matrix degradation.\textsuperscript{22,23} Thus, both proteins and their expressional regulation have been described to be involved in the recovery of normal corneal transparency.\textsuperscript{19} Taking all this into consideration, the use of retinoid properties to pretreat AM may potentiate their wound healing effect. This hypothesis constitutes the purpose of our study, which was conducted using a classical corneal alkali burn mouse model.

**Materials and Methods**

**In Vivo Mouse Model of Alkali Burn**

All experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the protocol was approved by the Regional Ethics Committee on Animal Experimentation in Auvergne (CEMEA Auvergne, No. 65.113.15). Animals were placed and monitored in the School of Medicine Facility of Auvergne University.

In detail, 50 CD1 male mice were divided into five groups of 10 animals (Table). At day 0, we performed a standardized reproducible corneal chemical burn on the right eye of each animal. This was done under general anesthesia administered through an intraperitoneal injection of pentobarbital (0.82 mg/mL; CEVA Laboratories, Libourne, France) and local anesthesia (oxybuprocaine 0.4%: MSD CHIBRET, Riom, France). The cornea was injured by placing a 3.0-mm-diameter disc of filter paper (Duriex, Paris, France) saturated with NaOH (1 N) that straddled the limbus for 15 seconds. The wound surface was then washed with balanced salt solution (BSS), and antibiotic (norfloxacin, Chibroxine, 0.3% eye drops; Laboratoire THEA, Clermont-Ferrand, France) was applied three times per day. The graft surgical procedure was based on overlay strategy. An hour after the injury, two mouse groups were treated with transplanted AM that was untreated or pretreated with all-trans retinoic acid (atRA); the other groups were treated with eye drops six times daily, and these comprised homogenates of AM that were untreated or pretreated with atRA. In detail, the first group (G1) was the control group and was treated only with saline solution eye drops six times daily. The second (G2) and fourth (G4) groups were treated with 24-hour-cultivated AM over 7 days (transplant or homogenate, respectively), and the third (G3) and fifth (G5) groups were treated with 24-hour-cultivated AM pretreated with atRA over 7 days (transplant or homogenate, respectively; Table). All animals were euthanized under general anesthesia on day 7.

**Pharmacologic Products**

All-trans RA and dimethyl sulfoxide (DMSO) were supplied by Sigma-Aldrich (Saint-Quentin-Fallavier, France). Phosphate-buffered saline (PBS) was distributed by PAA Laboratories GmbH (Pasching, Austria). The Dulbecco’s modified Eagle’s medium (DMEM) culture medium and streptomycin-penicillin-amphotericin B mix were acquired from Fisher Scientific (Cergy-Pontoise, France). Pentobarbital was distributed by CEVA Laboratories, and meloxicam oral suspension was obtained from Boehringer Ingelheim (Ingelheim am Rhein, Germany). Oxybuprocaine 0.4%, BSS, and norfloxacin were obtained from Laboratoire THEA.

**Amniotic Membrane Treatment**

One fetal membrane was collected in accordance with the tenets of the Declaration of Helsinki from a woman with a healthy pregnancy after caesarean birth (Centre Hospitalier Universitaire Estaing, Clermont-Ferrand, France); this individual provided informed consent. The membrane was checked to ensure the absence of structural abnormalities, inflammation, or infection. The amnion was dissociated from the chorion under aseptic conditions. The membrane was washed several times with PBS and cut into 1-cm² patch explants. All patches were immersed in well plates containing 2 mL DMEM–Nutrient Mixture F-12 (DMEM-F12), 10% of delipidated and decomplemented fetal bovine serum, and a mix of antibiotics and antifungal agents—streptomycin, penicillin, and amphotericin B (Invitrogen, Carlsbad, CA, USA). After 24 hours of culturing in an incubator at 37°C, 5% CO₂, the medium was changed according to the following conditions: 2 mL delipidated medium for the cultivated groups (i.e., control group), 2 mL delipidated medium plus DMSO (DMSO/atRA vehicle), and 2 mL delipidated medium with atRA (atRA group). All plates were then stored in an incubator and wrapped in aluminum foil because of atRA’s photosensitiveness. The used concentration of atRA was 1 μM.

**Retinoic-Induction Confirmation for Amniotic Membrane Treated by atRA**

Induction of AMs was checked by the quantification of expression of an atRA-induced gene, namely, RA receptor β (RARβ), following a previously described procedure\textsuperscript{24} after using the RNaysy Mini Kit (Qiagen, Venlo, Holland) for extraction. Total RNA isolation was performed and quantified with a NanoDrop ND-100 spectrophotometer (Thermo Fisher, Waltham, MA, USA). The RNA quality was controlled with an Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Synthesis of cDNA was achieved from 3 μg total RNA according to the RTSuperScript III protocol (Invitrogen). Real-time polymerase chain reaction (PCR) was performed with a LightCycler 480 (Roche Diagnostics, Saint Quentin Fallavier, France). For a total reaction volume of 15 μL, we added 2 μL cDNA diluted to 1/10 in the following reaction mixture: 6 μL H₂O, 0.75 μL forward primer (10 μM), 0.25 μL reverse primer (10 μM), and 7.5 μL MasterMix SYBR GREEN I (Roche Diagnostics). The PCR program used was 10 minutes of denaturation at 95°C, then 40 cycles with 10 seconds of denaturation at 95°C, 10 seconds of hybridization at 61°C, and 15 seconds of elongation at 72°C. Results were normalized to the housekeeping genes (acidic ribosomal phosphoprotein P0, RPLP0, and ribosomal protein S17, RPS17). All the steps followed the MIQE guidelines.\textsuperscript{25} Three independent experiments were run, each in duplicate.

**Storage of Transplants and Homogenate Preparation**

The classical membranes for transplants were placed over a nitrocellulose patch, epithelial face up, and then stored in a 57.5% glycerol solution at −80°C. For homogenates, and after incubation, patches were placed in Eppendorf tubes and stored at −80°C. They were prepared with a homogenizer in 600 μL saline solution (Precellys; Bertin Technology, Montigny
le Bretonneux, France) for three cycles of 23 seconds at 6300 r/min, with 1-minute breaks between cycles. Homogenates were then collected and placed in centrifuge tubes for 5 minutes at 20,800 g at 4°C. The supernatant was separated and aliquoted. The protein content of the homogenate was determined using the Bradford method (Bio-Rad Protein Assay; Bio-Rad, Hercules, CA, USA) and adjusted to the classically used concentration of 1.8 g/L. Eye drops were then stored at –80°C until use and kept in a refrigerator at 4°C thereafter. For the treatment, one drop of 5 µL was inoculated six times per day in the alkali-burned eyes.

Quantification of Corneal Ulcer Surface

The epithelial defect size was determined by slit-lamp examination in a blind trial with 1% fluorescein staining. Photographs were taken at day 1 and day 7 (Hawk Eye handheld microscope, Dioptix [Toulouse, France]; Camera 6.0 Megapixels Optio S60 Pentax [Tokyo, Japan]). The exact measurement of the ulcer areas was carried out with a computer-assisted image analyzer using ImageJ V.1.50d software (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA). The corneal wound healing was calculated using the following formula: [(ulcer area before treatment – ulcer area after treatment) / (ulcer area before treatment)].

Quantification of Ulcer Depth

At day 7, after euthanasia, all eyes were collected and placed in 4% paraformaldehyde for 24 hours. Tissues were dehydrated in ethanol baths. Then, paraffin embedding was performed to create 5-µm sections with a microkeratome. Slides for histologic analysis were stained with hematoxylin and eosin before examination with an Axiovert microscope 200M (Zeiss, SAS, Le Pecq, France). Ulcer depth was measured in a blind trial with the Axiosvision LE 4.5 software (Zeiss, SAS).

Immunohistochemistry Analysis of the MMP-9 and VEGF Expression

A 35-minute dewaxing procedure was carried out on the slide followed by an antigen retrieval method with 10 mmol citrate pH = 6 on a heating plate for 25 minutes. After cooling for 30 minutes, slides were washed twice for 5 minutes with distilled water and assembled in PBS. Slides were then placed in an Intavis AG ResPep SL robot (Saint-Marcel, France) for analysis. Primary rabbit antibody anti-VEGF antibody (ab46154; Abcam, Cambridge, UK) diluted at 1/300, and anti-MMP-9 antibody (ab38898, Abcam), diluted at 1/1000, were used. Then, secondary antibody AlexaFluor 488 F(ab’2) goat anti-rabbit immunoglobulin G (IgG) antibody fragment (H+L; A-11070; Invitrogen) was used. The negative control was generated without primary antibody incubation. Slide analysis was performed using an Axiovert 200M confocal microscope (Zeiss SAS) with ×100 magnification. Image adaptation was performed with ImageJ V.1.50d software. Quantification of the fluorescence signal of the secondary antibody was carried out in a blind trial with the same software. Contrast parameters were preserved to permit comparison and interpretation between groups.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 5.04 (GraphPad Software, San Diego, CA, USA). The normality of data was checked using the Shapiro-Wilk test. As the results followed a nonparametric representation, nonparametric tests (Kruskal-Wallis and Mann-Whitney U test) were used for analyses and presented as mean values (± standard error of the mean [SEM]). Values were considered significantly different at P < 0.05.

RESULTS

Retinoic Induction of Amniotic Membranes by atRA

To ensure that the atRA treatment of AM was effective (before using it as transplant or homogenate), quantification of RARβ by RT-qPCR was performed. A 5-fold induction of RARβ quantification was obtained between AMs treated by atRA compared to cultivated untreated membranes. Such results validate the use of this membrane (as atRA was well engineered) for the subsequent experiments.

Effect of Amniotic Membrane and atRA Treatment on Ulcer Area

The first parameter tested in this study concerned the residual ulcer area after each treatment (Fig. 1). For all groups compared to the first one, the use of AM led to better corneal healing. Specifically, after 7 days and if the percentage of healing was calculated compared to group 1 (considered as basic wound healing, representing 12% of wound but put back to a reference of the ulcer area [i.e., 1]), the wound healing improvement was 81 ± 21% for group 2 (P = 0.018), 87 ± 12% in group 3 (P = 0.035), 77 ± 25% (P = 0.024) in group 4, and 97 ± 5% in group 5 (P = 0.012; Supplementary Table). Furthermore, concerning the ulcer area, a significant improvement in healing was observed in the transplanted AM group and in the homogenate group when this was treated by atRA (comparison of group 2 with group 3 and group 4 with group 5). Finally, to compare the biological and mechanical effects of the membrane, a significant difference was also found between groups 2 and 4 (P = 0.048), with better healing in the transplant group, whereas between groups 3 and 5 (P = 0.045), better results were observed in the AM homogenate group.

Effects of Amniotic Membrane and atRA Treatment on Ulcer Depth

Concerning ulcer depth, another important parameter for corneal wound healing and eye functional recovery, compar-
ison of the nontreated group with all the others again demonstrated a statistical difference for each tested condition (Fig. 2). If the ulcer depth in group 1 was considered as a reference (i.e., 1), the thickness of the ulcer at day 7 showed a reduction of depth of 52 ± 22% (P = 0.0056) in group 2, 74 ± 12% (P = 0.0056) in group 3, 54 ± 4% (P = 0.0004) in group 4, and 86 ± 8% (P = 0.0001) in group 5. Furthermore, there were no significant differences in the thickness recovery between groups 2 and 3 (nontreated transplanted membrane compared to atRA-treated membrane, P = 0.67), but atRA treatment in the homogenate group improved thickness recovery significantly between group 4 (34 ± 4%) and group 5 (86 ± 8%; P = 0.008).

**Effects of Amniotic Membrane atRA Treatment on VEGF and MMP-9 Expression**

In light of the better overall corneal wound healing with AM treated with atRA, the expressions of MMP-9 and VEGF were characterized and quantified. Representative photographs are presented for both proteins tested in Figure 3a (for VEGF) and Figure 4a (for MMP-9). The signal quantification was expressed relative to group 1 (corneas treated with saline solution). For VEGF (Fig. 3b), all relative signal quantifications were carried out compared to group 1 (100.0 ± 0.2%). For all groups, the signal was decreased by 33.8 ± 1.4% in group 2, 66.3 ± 0.7% in group 3, 34.6 ± 5.4% in group 4, and 52.0 ± 6.8% in group 5. The statistical analysis underlined a significant difference between group 1 and all other groups (P < 0.05). Furthermore, a difference was also found between groups 2 and 3 (P = 0.038) and groups 4 and 5 (P = 0.043). For MMP-9 (Fig. 4b), the relative signal was also decreased between group 1 and all other groups. Specifically, decreases of 9.7 ± 0.6% for group 2, 60.7 ± 1.3% for group 3, 45.2 ± 12.9% for group 4, and 18.8 ± 14.2% for group 5 were obtained. The statistical analysis found a significant difference only between groups 1 and 3 (P = 0.0052) and between groups 2 and 3 (P = 0.0032). No significant difference was found between group 1 and groups 2, 4, and 5 (P = 0.071, P = 0.064, and P = 0.068), between groups 2 and 4 (P = 0.37), between groups 3 and 5 (P = 0.23) and between groups 4 and 5 (P = 0.23).

**DISCUSSION**

Several studies have already demonstrated the benefit of AMs in treating many ocular surface disorders.7,8,11-13,26 Such positive actions may be explained by growth factors released in contact with the wound. For example, epithelial growth factor (EGF)27 and keratinocyte growth factor (KGF)26 promote epithelial and keratinocyte cell migration and proliferation after injury. Our results confirmed a highly significant improvement of corneal wound healing after AM...
treatment in the presence or absence of transplantation, as shown previously. AM transplants present some logistic difficulties in relation to access to an operating room environment. Furthermore, because of their quite rapid destruction, grafts may need to be repeated several times. In light of these facts, comparison between this traditional method and homogenate eye drops has been raised. Some studies revealed interesting results concerning homogenates. Based on a mouse model, our study presented similar results when comparing the mechanical effect to the biological effect.

The proposed hypothesis of the benefits of vitamin A was confirmed by our results. In fact, the induction of AM with atRA significantly improved epithelial wound healing in both arms of the study. In the transplanted AM arm, the ulcer area decreased by 6%, and in the homogenate arm, the ulcer area decreased by 20%. Concerning the ulcer depth, a significant improvement of the corneal thickness recovery was also observed for the homogenate arm (P = 0.008), and the same trend was observed, although this was nonsignificant, in the transplanted arm (P = 0.067). Therefore, benefits of induction by atRA was observed in terms of both epithelial healing and corneal thickness recovery. Several hypotheses may explain this benefit related to ulcer depth. Contrary to the simple method of healing the epithelium, which consists of the movement and differentiation of limbal stem cells, the healing mechanism of the stromal layer consists of a complex transformation of stromal keratocytes to fibroblasts and myofibroblasts. The subtle balance between stromal regeneration and fibrosis depends on the activity of the myofibroblasts. Vitamin A can inhibit the production of some proteinases, such as MMP-9, an enzyme implicated in extracellular matrix degradation. Several studies have revealed a high level of this enzyme after various corneal injuries, including alkali burn. As MMP-9 seems to be expressed at the leading edge of the migrating epithelium, an excessive level of this protein in the corneal tissue seems to be associated with an inhibition in corneal healing due to the degradation of the extracellular matrix. In dry eye disease, overproduction of MMP-9 has been associated with the disruption of corneal epithelial barrier integrity. Because the level of MMP-9 seems to be correlated with the level of inflammation in the cornea in these surface disorders, and because the level of inflammation is directly correlated with long-term decreased corneal transparency, the staining intensity of this enzyme was quantified in all groups. The MMP-9 amount decreased in each cornea treated with AM (homogenate or transplant) but with significant values only for transplanted conditions combined with atRA. Adjunction of atRA decreased MMP levels even more. These results are in the line with previous findings showing that atRA downregulates MMP-9 expression via the upregulation of TIMP-1 and E-cadherin and the downregulation of EGFR, NFKB, and FAK.

Besides the extracellular matrix reorganization, the excessive inflammation induced by chemical burns is responsible for the destruction of limbal stem cells, the occurrence of neovascularization, and stroma necrosis via keratoacytis apoptosis. Neovascularization is a major cause of visual loss after severe ocular burns. Maintaining corneal lack of vascularity as a guarantor of tissue transparency is essential for the wound healing process, and it is the result of active regulation. Subtle regulation exists between pro- and antiangiogenic factors. Proangiogenic factors include VEGF, FGF, platelet-derived growth factor (PDGF), and antiangiogenic factors, including endostatin, angiotatin, and so on. The first factor, VEGF, appears to be particularly important. Indeed, Edelman et al. showed that an increase of VEGF expression is found near a cautery-induced lesion on rat corneas and overexpression is correlated with limbal and corneal vessel proliferation. Thus, VEGF appears to be an interesting marker for neovascularization risk, and its expression must be decreased during the wound healing process. Our results confirm the antiangiogenic properties of the AM that have already been described. Indeed, all the corneas treated with AM in the presence or absence of homogenate exhibited decreased VEGF levels. Furthermore, when treated with atRA, the VEGF level decreased even more, with significant values in both situations (graft or homogenate), suggesting an underlying synergic effect.

In conclusion, to our knowledge, this study is the first to compare the effectiveness between AM homogenates and transplanted AM combined with atRA pretreatment. Our results confirmed the biological efficiency of AM homogenate. This homogenate is less traumatic than a transplanted amniotic graft, and it is not associated with the complications or surgical constraints of transplantation. Furthermore, it can be instilled for a long period. The results also suggested a synergic effect of AM combined with atRA on ulcer surface wound healing and corneal thickness recovery. Furthermore, this joint action gives better results for neovascularization markers. This may pave the way for better efficacy in functional ocular prognosis, which is highly dependent on corneal transparency.

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