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Comparison of cryopreserved and air-dried human amniotic membrane for ophthalmologic applications

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Abstract
Background Cryopreserved amniotic membrane (Cryo-AM) is widely used in ocular surface surgery because of its positive effect on wound healing and its anti-inflammatory properties. A new peracetic acid/ethanol sterilized air-dried amniotic membrane (AD-AM) recently became available which might be an alternative to Cryo-AM. Our aim was to compare AM preserved with both methods with regard to the release of wound-healing modulating proteins, the preservation of basement membrane components, and the ability to serve as a substrate for the cultivation of human limbal epithelial cells (HLECs).
Methods Pieces of Cryo-AM and AD-AM from three different donors were incubated in DMEM for five days. The culture supernatant was collected after an incubation period of 0.1, 24, 48, 72 and 120 h; in the case of AD-AM, this period was extended up to 14 days. TIMP-1, IL-1ra, CTGF and TGF-β1 were detected in the culture supernatant using Western blotting. Twenty human limbal epithelial cultures were initiated on both AD- and Cryo-AM. The cultures were analyzed morphologically, and the outgrowth area was measured in 3-day intervals. Cryosections of Cryo- and AD-AM from three different donors were analyzed histochemically to detect the basement membrane components collagen IV, collagen VII, laminin, laminin 5 and fibronectin.
Results The release of TIMP-1, IL-1ra and TGF-β1 from Cryo-AM was constant for the studied period. CTGF showed a stronger signal after 120 h. None of the analyzed proteins, except for a small amount of IL-1ra, could be detected in the supernatant of AD-AM. An outgrowth of HLEC was observed in all cultures on Cryo-AM, but in only 30% of cultures on AD-AM. The outgrowth area on Cryo-AM was at all time points significantly higher than on AD-AM ($p<0.0001$). Collagen IV, -VII, laminins and fibronectin were detectable in the basement membrane of Cryo-AM, but only collagen IV and fibronectin in AD-AM.
Conclusions Cryo-AM is a more suitable substrate for the cultivation of HLECs than AD-AM. The higher outgrowth rate of cultured limbal epithelium, release of intact soluble wound-healing modulating factors and a better preservation of basement membrane components suggest the superiority of Cryo-AM for use in ophthalmology in comparison to AD-AM.

Keywords Amniotic membrane · Cryo-preservation · Air-drying · Growth factors · Basement membrane · Cell cultivation

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Conflict of interest None of the authors has any financial interests to disclose. The authors have full control of all primary data, and agree to allow Graefe's Archive for Clinical and Experimental Ophthalmology to review our data upon request

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Introduction

Amniotic membrane (AM) is the innermost layer of the placenta. It is composed of three layers: an epithelial monolayer, a thick basement membrane and stroma. The latter can be further subdivided into a compact layer, a fibroblast layer containing a loose network of fibroblasts, and a spongy layer [1]. The transplantation of AM for conjunctival reconstruction was first introduced by de Rotth in 1940 [2], but gained popularity since its re-introduction by Kim and Tseng in 1995 [3]. Since then, AM has been used in ophthalmology for several indications because of its beneficial effects. The AM facilitates reepithelialization in case of persistent corneal epithelial defects with stromal ulceration, and reduces inflammation, vascularization and scarring [4–9]. The tissue lacks most of the major histocompatibility antigens, therefore only a small immunologic response is expected, and an allogenic transplantation does not require immunosuppression [10, 11]. In addition, AM is an excellent substrate for the *ex vivo* expansion of limbal epithelial cells, and can be transplanted together with the expanded cells for the treatment of limbal stem cell deficiency [12]. Previous studies have demonstrated a similar expression of collagen IV, collagen VII, laminin 5 and fibronectin in the corneal and amniotic basement membrane [13–15]. Recent studies have demonstrated the presence of several growth factors and cytokines in AM which may influence wound healing, as well as possessing an anti-inflammatory and anti-angiogenic effect [16]. Therefore, AM transplantation is increasingly used for the treatment of various ocular surface conditions [9, 17]. AM can be used as a graft (inlay) to replace the damaged basement membrane, as a patch protecting and supporting the ocular surface until reepithelization has occurred (onlay), or as a combination of both methods ("sandwich method") [18].

The amniotic membrane has to be preserved before it can be released for clinical use, for safety reasons. Serological testing of the donor and sterility controls are performed during the preservation time, and the AM is released only if the possibility of contamination or transfer of infections is ruled out. Cryo-preservation is the most common preservation method. The amniotic membrane is isolated under sterile conditions from the placenta obtained by an elective caesarean section, washed several times with a sterile buffer containing antibiotics, and stored at −80°C. Different freezing media have been used, but most of them contain glycerol, culture media and antibiotics. The main advantages of this method are a relatively large number of published experimental and clinical studies, confirming the safety and effectiveness of this method, and a relatively cheap preparation process. Although available from commercial companies in some regions, it is prepared in the local clinic in most cases, having the disadvantage of a need to learn the preparation method and possible variations from the preparation process.

Freeze-dried and air-dried AM (AD-AM) are available from different companies, and have the advantage that the preparation process is standardized and the AM is delivered as a ready-to-use product. Their main disadvantage is the limited amount of published data describing its effect and clinical efficiency. Every step of the handling procedures, including preparation, preservation and sterilization, may affect the biological, chemical and physical properties of a biological material. Microscopic studies have revealed that different sterilization and preservation procedures influence the histological and biophysical properties of amniotic membrane [19, 20]. Unfortunately, available data concerning the effect of each sterilization/preservation method on the biological function is rather limited.

The purpose of this study was to compare the biological properties relevant for the ophthalmic application of peracetic acid/ethanol sterilized air-dried AM and cryo-preserved AM (Cryo-AM). We examined the release of soluble wound-healing modulating proteins, the preservation of basement membrane components, and the suitability of both AM types to serve as a matrix for the cultivation of limbal epithelial cells.

Material and methods

Preparation of AM

Human tissue was handled according to the tenets of the Declaration of Helsinki. The Cryo-AM was prepared according to a method reported elsewhere [21]. All donors were tested for the infection with human immunodeficiency virus, hepatitis virus type B and C, and syphilis by serological tests. After obtaining proper informed consent, the placentas were obtained directly after elective caesarean sections. AM was separated from chorion under sterile conditions. The membrane was rinsed several times with sterile phosphate buffered saline (PBS, Invitrogen, Grand Island, NY, USA) containing penicillin–streptomycin–neomycin and amphodericin B (both from Sigma Chemie GmbH, Steinheim Germany) to remove blood residues and placed epithelial side up on a nylon membrane (RotiNylon, Carl Roth GmbH, Karlsruhe, Germany) before dividing into slices of appropriate size. The pieces were stored into cryo-medium consisting of 1:1 vol Dulbecco´s modified Eagles medium (DMEM, Invitrogen) and sterile glycerol and stored at −80°C for at least 1 month before use. The ready-to-use sterilized AD-AM (#GT1100, #GT1080) was obtained from the Deutsche Institut für Zell- und Gewebeersatz GmbH (DIZG Berlin, Germany). The preparation method of AD-AM used by DIZG has been described elsewhere [19].

Collection and isolation of soluble proteins released by AM

Cryo-AM from three different donors and AD-AM from three different batches was cut into 9 cm^2 slices, and placed into a 6-well plate containing 4.5 ml of serum-free DMEM. The Cryo-AM slices were incubated for up to 120 h and slices of AD-AM up to 336 h at 37°C and 5% CO_2. The culture supernatant was collected after an incubation period of 0.1, 24, 48, 72 and 120 h before it was snap frozen in liquid nitrogen and stored at −80°C until use. In the case of AD-AM this period was extended up to 14 days, to ensure that soluble proteins in the dried tissue are completely rehydrated and released into the culture medium.

Trichloracetic acid (TCA) was added to the supernatant to an end concentration of 10%. Samples were incubated on ice for 30 minutes following by a 10-minute centrifugation at 16000 g and 4°C. The supernatant was removed and pellet resuspended in 2× Laemmli buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue, 0.125 M Tris/HCl). The pH value was adjusted using 0.25 M NaOH until the Laemmli buffer regained its original blue colour.

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

The samples in Laemmli buffer were heated at 75°C for 5 minutes before loading onto the gel. Lysates of placenta served as a positive control. The proteins were separated on a 15% separation gel and either silver-stained or transferred onto nitrocellulose membrane (BioRad Laboratories, Hercules, CA, USA). The membranes were blocked overnight at 4°C or for 2 h at room temperature with TN-buffer (75 mM Tris/HCl, 150 mM NaCl, pH 7.5) containing 10% low-fat milk powder and 0.1% Tween-20. Proteins were detected by incubation with primary antibodies at appropriate dilutions (Table 1) in blocking buffer either at room temperature for 2 h or at 4°C overnight. The membrane was washed with TN-buffer containing 0.1% Tween-20 (TN-T), followed by an incubation with the appropriate horseradish peroxidase-conjugated secondary antibodies (Table 2) at room temperature for an hour. The reaction was visualized using the ECL Plus chemoluminescence kit (GE Healthcare UK Limited, Buckinghamshire, UK). For silver staining the gels were incubated in fixation solution (v/v: 45% methanol (MetOH), 5% acetic acid, 45% dH_2O, all from Sigma-Aldrich) for about 20 minutes, followed by washing in dH_2O. Subsequently, the samples were incubated for 10 minutes in 50% (v/v) MetOH and washed three times for 20 seconds in dH_2O followed by treatment with 0.02% (w/v) sodium thiosulfat solution for 10 minutes. The next step was incubation in 0.1% silvernitrate at 4°C for 10 minutes. Finally, the staining reaction was performed with 2% (w/v) Na_2CO_3, 0.04% (v/v) formaldehyde until protein bands were clearly visible. At this point, the reaction was stopped using 1% (v/v) acetic acid.

Immunohistological staining

Pieces from three different patches of AD-AM and Cryo-AM from three donors were embedded in Tissue-Tek OCT compound (Sakura Finetek Europe, Zoeterwoude, NL), cut into 12-µm sections and mounted on SuperFrost plus object slides (Langenbrinck, Emmendingen, Germany). The slices were fixated in ice-cold MetOH (Sigma-Aldrich) for 10 minutes. Subsequently, the slides were washed two times in dH_2O and treated with permeabilizing buffer (0.1% Triton X-100 (Sigma–Aldrich) in PBS) for 10 minutes, washed three times with PBS and blocked with 5% BSA (Sigma–Aldrich) in PBS for 45 minutes. The slices were incubated with the primary antibodies to collagen IV, collagen VII, laminin-γ1 chain, laminin 5 and fibronectin at room temperature for 1 h in indicated dilutions (Table 1), followed by four 10-minute washing steps in PBS. Staining was visualized by 1 h incubation at room temperature with

Table 1 Primary antibodies for Western blotting and Immunohistochemistry

Antibody	Clone	Company	Dilution factor	Reference
Western blot				
TIMP-1	7-6C1	Chemicon Inc. Temecula, CA, USA	1: 1000	[22]
CTGF	L-20	Santa Cruz Biotech Inc. Santa Cruz, CA, USA	1: 1000	[23]
TGF-β1	TB21	Santa Cruz Biotech Inc. Santa Cruz, CA, USA	1: 1000	[24]
IL-1ra		R&D Systems Inc. Mineapolis, MN, USA	1: 1000	[25]
Immunohistochemistry				
Collagen IV		Abcam Plc, Cambridge, UK	1: 200	[26]
Collagen VII	LH 7.2	Millipore GmbH 65824 Schwalbach/Ts,Germany	1: 100	[27]
Laminin γ1	2 E 8	Millipore GmbH 65824 Schwalbach/Ts, Germany	1: 500	[28]
Laminin 5		Abcam Plc, Cambridge, UK	1: 500	[29]
Fibronectin		Abcam Plc, Cambridge, UK	1: 200	[30]

Table 2 Secondary antibodies for Western blotting and Immunohistochemistry

Antibody	Conjugate	Company	Dilution factor
Western blot			
Anti-mouse	HRP	GE-Healthcare Ltd., UK	1: 2000
Anti-rabbit	HRP	GE-Healthcare Ltd., UK	1: 2000
Anti-goat	HRP	Santa-Cruz Biotech Inc., Santa Cruz, CA, USA	1: 2000
Immunohistochemistry			
Anti-mouse	ALEXA Flour 488	Invitrogen Corp. Eugene, OR, USA	1: 500
Anti-rabbit	ALEXA Flour 488	Invitrogen Corp. Eugene, OR, USA	1: 500

Alexa-488 conjugated anti-mouse or anti-rabbit antibodies (Invitrogen) diluted 1:500 in PBS (Table 2). The cell nuclei were counterstained with Hoechst 33342 diluted 1:500 in PBS, and the slices embedded in Prolong anti-fade mounting medium (both from Invitrogen). Slices treated after the same protocol but with applying PBS instead of primary antibodies served as negative control. The slices were examined and documented using a fluorescence microscope (IX-51, Olympus Tokyo, Japan).

Cultivation of human limbal epithelial cells (HLEC)

Limbal tissue was obtained after informed consent from four eyes enucleated because of isolated tumours in the posterior section from patients with the average age of 47 years (range 39–56). Patients who had received radiation therapy because of the tumour had previous operations on the cornea, known corneal disease or known systemic inflammatory disease were excluded. The patients were carefully examined before enucleation to exclude eyes with any signs of ocular surface disease.

The HLECs were cultivated on AD-AM and Cryo-AM according to a method described elsewhere [31]. Three different patches of AD-AM and Cryo-AM from three donors were used in these experiments to exclude possible variations between donors and preparation procedures. Briefly, after careful removal of excessive sclera, iris, and conjunctiva was the limbal region of each of the four donor eyes cut into 1×2 mm pieces that were treated with 1.2 U/ml Dispase in DMEM (Invitrogen). Five randomly selected pieces of each donor limbus were placed on the epithelial side of five pieces of both intact AD- or intact Cryo-AM (resulting in 20 cultures on both AD- and Cryo-AM) and cultured in previously described SHEM medium [30]. The medium contained an equal volume of HEPES-buffered DMEM and Ham's F12, 5% fetal bovine serum (all from Invitrogen), 0.5% DMSO, 2 ng/ml mouse EGF, 5 mg/ml insulin, 5 mg/ml transferrin, 5 ng/ml selenium, 0.5 mg/ml hydrocortisone, 30 ng/ml cholera toxin A subunit, 50 mg/ml gentamicin, and 1.25 mg/ml amphotericin B (all from Sigma–Aldrich Chemie GmbH). Cultures were incubated for 18 days at 37°C under 5% carbon dioxide and 95% air. Medium was changed every 2–3 days. Air-lifting was not applied to avoid cell differentiation.

Evaluation of cell growth

The outgrowth of the limbal tissue and cell morphology of the HLECs was monitored and documented at day 9, 11, 14, 16 and 18 with a phase contrast microscope (Eclipse TE2000-S, Nikon Corporation, Tokyo, Japan). The outgrowth area was measured from digital photographs using ImageJ (National Institutes of Health, Bethesda, MD, USA). Statistical analysis of the obtained data was performed using two-sample *t*-test and StatCrunch on-line statistical software (http://www.statcrunch.com/, Pearson Education Core Technology Group, Boston, MA, USA). $P<0.05$ was considered as significant.

Results

Soluble proteins

Silver staining revealed numerous distinct protein bands in Cryo-AM conditioned medium, but only a few bands in AD-AM conditioned medium (Fig. 1). In Western blots, all examined proteins were detectable in supernatant of Cryo-AM (Fig. 2). Specific bands were visible after a 24-h incubation, the intensity of the chemoluminescece signal of TIMP-1, IL-1ra and TGF-β1 was constant for the studied period in the supernatant of Cryo-AM. CTGF showed a constantly weak signal for the first 72 h, but in two of three cases, a stronger signal after 120 h. In the conditioned medium sample of AD-AM, only a faint signal for IL-1ra could be detected after 7 to 10 days of incubation (Fig. 2), and no other studied proteins were detectable.

Immunohistological staining

The examined extra-cellular matrix proteins were detectable in all analyzed Cryo-AM samples as a continuous line beneath the epithelium, indicating that the basement membrane

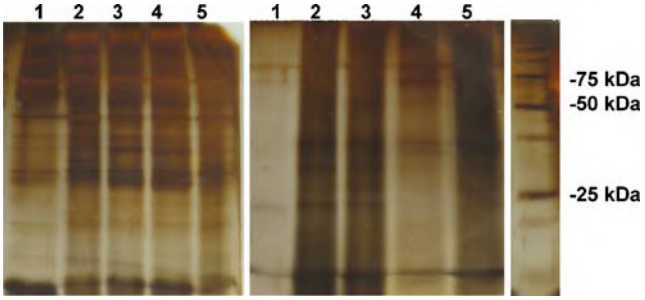

Fig. 1 Silver staining of conditioned media collected from cryopreserved amniotic membrane (Cryo-AM, *left column*) and air-dried amniotic membrane (AD-AM, *right column*) over 5 days and separated on 15% SDS gels. The numbers indicate the incubation time over which the media was conditioned: 1: 0.1 h; 2: 24 h; 3: 48 h; 4: 72 h; 5: 120 h. The marker is shown *right*

components were not influenced by the preservation method (Fig. 3). Collagen IV and fibronectin were also detected in the stroma of Cryo-AM. Negative controls from Cryo- and AD-AM displayed no specific staining (data not shown).

In AD-AM, collagen IV and fibronectin showed a distribution similar to the one observed in Cryo-AM. On the other hand, collagen VII and laminins were not detectable in the AD-AM samples and the staining did not exceed background staining (Fig. 3).

Cultivation of HLEC

An outgrowth was observed in all 20 HLEC cultures on Cryo-AM but only in 30% (six of 20) of cultures on AD-AM. In addition, the few growing cultures on AD-AM failed to form a cell layer and the growth stopped immediately after cells had migrated out of the biopsy. The outgrowth area was at all analyzed time points on Cryo-AM significantly higher ($p<0.0001$) than on AD-AM (Fig. 4). Therefore, the morphological analysis or comparison of gene expression profiles of the cell cultures was not possible, as cultures on AD-AM did not yield enough material for characterization. On the other hand, cells on Cryo-AM formed a layer of relatively uniformly small cells. None of the 20 cultures on AD-AM formed a large enough cell layer to cover a human cornea, where most cultures on Cryo-AM reached a sufficient area during the observed 18 days.

Discussion

The preservation process may influence any biological property of a tissue. An optimal preservation method of AM maintains the important biological features of the tissue while ensuring clinical safety and easy handling. Fresh AM is not applied in clinical practice. It is not used in Europe, due to the risk of transferring HIV and other serious blood-borne infections. Cryopreservation of the AM enables us to test the donor directly before and 6 months after the caesarean delivery. Previous studies comparing fresh and cryopreserved AM have shown that the AM epithelial cells do not survive the cryopreservation and have no proliferative capacity. No histological differences were detected, indicating that cryopreservation does not alter the AM too much [32]. Furthermore, it does not seem to have an advantage in clinical use over Cryo-AM [33].

Previous works have demonstrated that AD-AM possesses similar biophysical properties to AM preserved by storage in glycerol at 4°C and by deep freezing at 80% [19], but to our knowledge the present- study was the first attempt to compare the biological properties of PAA-sterilized AD-AM and Cryo-AM manufactured according to the procedure applied in this study. We focused on three clinically important aspects of cryo-preserved and PAA/ethanol-sterilized air-dried AM: the release of wound-healing modulating soluble proteins, the preservation of the basement membrane and the ability to serve as a substrate for the cultivation of HLECs.

AM contains a number of growth factors and cytokines, which have beneficial effects on wound healing, inflammation and counter corneal neovascularization [12, 16, 34, 47]. In this study, we examined the presence of the factors TGF-β1, TIMP-1, IL-1ra and CTGF in conditioned medium harvested

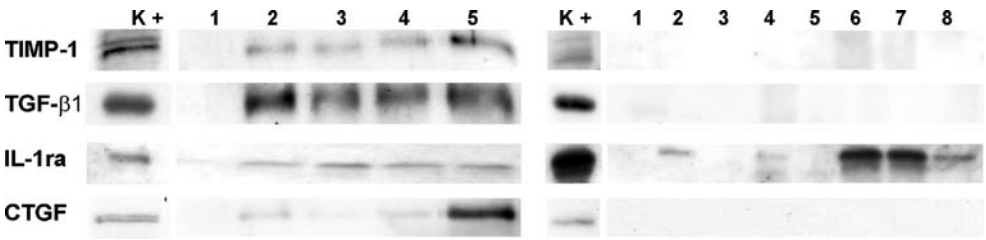

Fig. 2 Western blot analysis of conditioned media collected from cryopreserved amniotic membrane (Cryo-AM, *left column*) and air dried amniotic membrane (AD-AM, *right column*), at 5 days (Cryo-AM) and 14 days (AD-AM). K+: positive control (placenta lysate). The numbers indicate the incubation time over which the media was conditioned: 1: 0.1 h; 2: 24 h; 3: 48 h; 4: 72 h; 5: 120 h; 6: 7 days; 7: 10 days and 8: 14 days. The negative control, unconditioned media is not shown

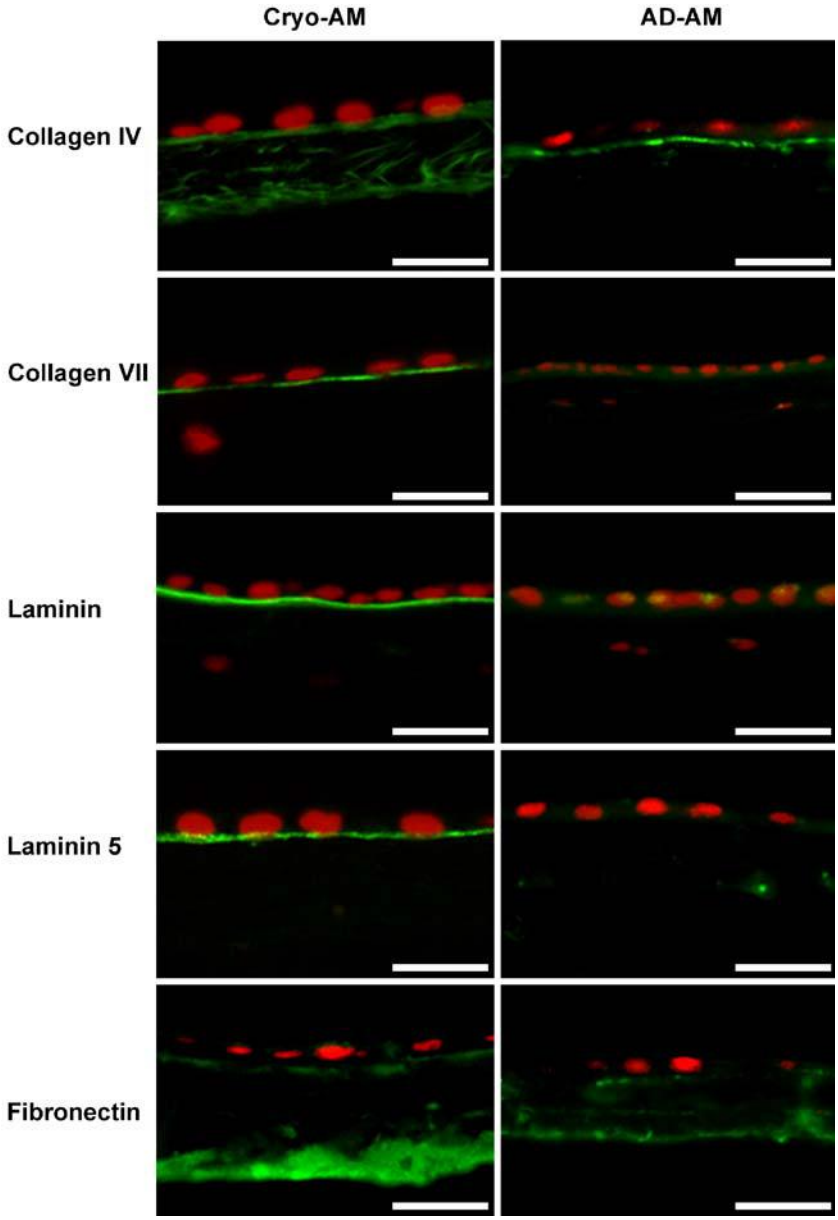

Fig. 3 Immunofluorescence analysis of collagen IV, collagen VII, laminins γ1-chain, laminin 5, and fibronectin (all green) expression in cryopreserved amniotic membrane (Cryo-AM, *left column*) and air dried amniotic membrane (AD-AM, *right column*). The cell nuclei are counterstained with Hoechst 33342 and presented in a false colour (red) for a better contrast. Size bar = 100 μm

from Cryo- and AD-AM. These proteins are known to be involved in corneal wound healing. TGF-β1 is a protein which occurs in a 12.5 kDa monomeric and 25 kDa dimeric form secreted by almost all nucleated cells. It is expressed in corneal cells, and together with other TGF-β isoforms has an important role in the regulation of corneal cell growth, proliferation and differentiation [35–37]. Tissue inhibitors of metalloproteinases (TIMPs) are the major endogenous regulators of matrix-metalloproteinase activity in tissue. The MMP/TIMP ratio assists in the regulation of the overall increase/decrease in matrix degradation, a process especially important for the migration of cells during wound healing and inflammatory response. Four homologous TIMPs have been identified to date [38, 39], which are all present in fresh as well as preserved amniotic membrane [22, 40, 41]. The Interleukin-1 receptor antagonist relieves the impact of the pro-inflammatory cytokine IL-1 by blocking its receptor. The balance between IL-1 and IL-1ra in tissues plays an important role in the severity of many diseases [42]. The protein occurs as a secreted (23 kDa) and as an intracellular form (20 kDa) [43]. Connective tissue growth factor (CTGF) is a cysteine-rich, matrix-associated, heparin-binding protein; it has a modular structure composed of four domains which resemble domains in other extracellular proteins, and orchestrate many essential biological functions. Active CTGF has a molecular weight of 38 kDa; there are also reports of low molecular weight forms of 10 to 20 kDa, as well as higher molecular mass forms. The expression of CTGF is induced by TGF-β signaling, and is involved in wound healing, scarring and tissue fibrosis [44, 45].

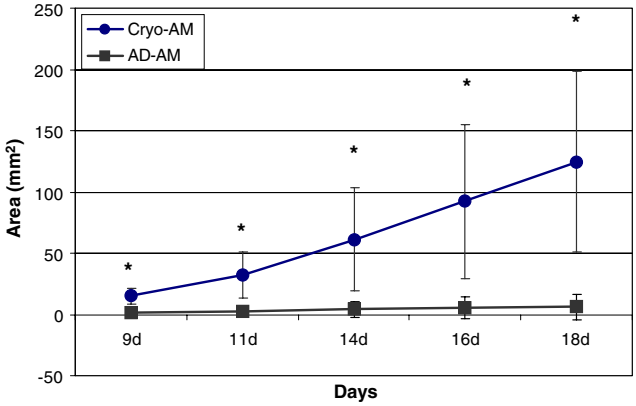

Fig. 4 Average outgrowth area of HLECs cultures on cryopreserved amniotic membrane (Cryo-AM, n=20) and air dried amniotic membrane (AD-AM, n=20). The Y-axis shows the size of the area (mm^2), the X-axis shows the time points. *Error bars* indicate the standard deviation. * = statistically significant difference ($p<0.0001$) in comparison to the cultures on AD-AM at the same time point

Western blot analysis revealed that TGF-β1, TIMP-1 and IL-1ra detach from Cryo-AM. The majority of these proteins are released into the media by Cryo-AM within 24 h of incubation. Because of this, the protein concentration of the culture supernatant harvested at the examined time points did not show a great variation, which can be seen by the relatively constant Western blot signal. Since Cryo-AM does not host any viable cells, proteins are not actively secreted but rather appear as residues washed out from the membrane.

CTGF showed a specific Western blot signal at approximately 60 kDa. This molecular size corresponds to a high molecular weight form of the protein. In two of three samples, an increase in the Western blot signal was detectable in samples incubated for 120 h. This indicates that the protein is bound to the membrane and released after a prolonged incubation time. Another possible explanation for this observation is interdonor variation. Different areas of AM vary in their protein content [46]; therefore, it is possible that the region from which the membrane pieces were taken contained different concentrations of proteins. Furthermore, slight variations in preparation as well as storage in cryo-media for a long time might affect the protein content. However, in the herein presented study, pieces were less than 3 months old. Evaluation of the effect of time storage on the protein release of AM is of high clinical relevance, and will be performed in a further study. In the AD-AM conditioned medium, only IL-1ra was detected in samples incubated for a period of at least 7 days.

In our study, we demonstrated that the analysed proteins are released from the Cryo-AM, which is essential to provoke an effect on corneal cells. Since we did not quantify the amount of protein, we cannot assess the impact of the detected proteins on wound healing and cell proliferation. Nevertheless, the results support previous studies which showed that Cryo-AM contains growth factors that might have a beneficial effect on corneal cell growth. Koizumi et al. measured the concentrations of different growth factors, including TGF-β1, in Cryo-AM, showing that the concentration of growth factors in the preserved tissue reached levels of several pg/mg [47]. Therefore, we assume that the amount of detected proteins is sufficient to have a clinical effect.

The examination of the basement membrane composition of Cryo- and AD-AM revealed further differences. The basement membrane is a continuous sheet of specialized extracellular matrix proteins, which serve as a scaffold for tissue architecture. It promotes adhesion, migration and proliferation of the overlying cells. Previous studies demonstrated similarities between the distribution of certain components in corneal and amniotic basement membrane [13–15]. Both tissues contain in addition to the major structural component collagen IV, collagen VII and several glycoproteins including laminins and fibronectin. These resemblances might support the migration of corneal cells on AM, which is important for reepithelization of the cornea, as well as expansion of HLECs.

Collagen IV, Collagen VII, laminins including laminin 5 and fibronectin were detectable as a continuous layer beneath the epithelium, indicating that the components are still preserved in the basement membrane of Cryo-AM. In AD-AM, the distribution of collagen IV and fibronectin was similar to that in Cryo-AM, but collagen VII laminin and laminin 5 were not traceable. Previous studies have also not detected collagen VII in AD-AM [19]. Since laminin 5 is connected to collagen VII [48], it is possible that the preservation process affects both proteins, leading to their destruction. The lack of laminins, especially laminin 5, is interesting with regard to the inability of AD-AM to support growth of corneal epithelial cells. Laminins are anchoring proteins composed of three disulfide bond-like chains: α, β, and γ which form a cruciform-shaped molecule and are involved in the attachment of cells to basal lamina, and affect mitogenesis, motility, and differentiation. The used MAB1920 antibody detects the γ-1 chain of laminin, which is a component of at least ten laminin isoforms [49]; the total absence of a staining signal for this subchain in AD-AM leads to the conclusion that none of these isoforms were present in their native state. HLECs are capable of rapid adhesion to laminin isoforms present in the adult corneal as well as amniotic basement membrane, especially laminin 5 (or laminin 332 in new terminology) is regarded as important for cell adhesion and migration. Laminin 5 is composed of the α3, β3 and γ2 subchains: studies revealed that exogenous added laminin 5 is able to promote corneal cell adhesion [50]. The absence of laminin 5 in AD-AM could hinder the adhesion of

HLECs to the basement membrane and prevent the outgrowth of cells. Our results indicate that the observed differences in the basement membrane composition, as well as the denaturised soluble growth factors in AD-AM, might be responsible for the reduced ability of AD-AM to support corneal cell growth.

The HLEC cultures on Cryo-AM showed a significantly higher outgrowth rate and area in comparison to cultures on AD-AM. The results demonstrate clearly that Cryo-AM is a suitable substrate for corneal epithelial cell expansion, while AD-AM has lost these properties during the preparation process. The transplantation of amniotic membrane and HLECs cultivated on AM is gaining popularity in ophthalmologic surgery [51–53]. There is a debate about the question whether intact AM or epithelium-denuded AM should be preferred for the cultivation of limbal cells. In our study, the used Cryo- as well as AD-AMs were intact. In this culture system, the corneal cells usually push aside the devitalized amniotic epithelial cells during migration, and grow in direct contact to the basement membrane. Intact AM has proven to maintain characteristic features of limbal stem cells [54, 55]. The use of denuded AM and air-lifting promote cell differentiation and the formation of a stratified epithelial layer [56, 57]. An alternative for using intact AM would be to co-cultivate HLECs with gamma-irradiated mouse 3T3 feeder cells. This method preserves stem cells on denuded AM [58, 59], but applying those feeder layers opens up the risk of transferring xenobiotic cells during transplantation.

For clinical application, the intention should be to transplant a relatively undifferentiated cell layer containing as many limbal stem cells as possible, because the essential goal of the therapy should be the restoration of the stem cell population and not the differentiated central corneal epithelium. Our studies have shown that after transplantation the relatively undifferentiated monolayer forms a differentiated stratified epithelium with a normal central corneal phenotype, and in most cases restores the limbal barier [60, 61]. Because of that, we prefer the use of intact AM for cultivation of HLEC.

In addition to the therapeutic use, the expansion of HLEC on AM may be considered a model for studying epithelial migration during wound healing, as AM is often used as a basement membrane replacement in ophthalmic surgery. However, the exact mechanism which enables the growth of HLEC on AM is to date not fully understood. It has been proposed that a combination of the amniotic basement membrane and growth factors provided by the AM epithelium creates a microenvironment similar to the limbal stem-cell niche [62]. Since these two features are clearly different in Cryo- and AD-AM, it can be concluded that the observed differences are a reason for the lack of HLEC outgrowth on AD-AM.

The differences between Cryo- and AD-AM arise probably from the different preservation and sterilization processes. The AD-AM is produced according to a standardized procedure [19]. The process involves sterilization in a mixture containing peracetic acid (PAA) and ethanol for 4 h at room temperature. Subsequently, the membrane is rinsed three times in sterile physiological saline solution to remove the PAA/ethanol residuals. For air-drying, the amnion pieces are spread under a laminar flow cabinet (epithelial side downwards, spongy layer upwards) and left there overnight. The PAA/ethanol sterilization method used in AD-AM inactivates bacteria, fungi and viruses. The agent which is used, especially applied ethanol, denatures proteins, and might be a reason for the changes in the basement membrane and the denaturation of the soluble growth factors/cytokines. Furthermore, the air-drying process might also lead to reduced protein content. In this line, it has been reported that lyophilized AM, which involves an air-drying step, contains less protein than Cryo-AM [63]. The described preparation process for AD-AM is unique to the manufacturer. Since human cell and tissue transplants are regulated as drugs in Germany, they require a manufacturing license and pharmaceutical approval by the local and a marketing authorization from the competent authorities. Therefore, AMs are commonly manufactured by the hospitals themselves for their own requirement. The AD-AM examined in this study is the only AM type that has been certified, and it is therefore the only AM that is commercially distributed in Germany.

We conclude that AD-AM might not achieve a clinical effect comparable to that of Cryo-AM because of the lack of wound-healing modulating factors and changes in the basement membrane, leading to a reduced ability to support corneal cell migration. Because there are very little data concerning the clinical efficiency of AD-AM, further studies are needed to evaluate the clinical suitability of this product. The main advantages of AD-AM are its validated processing procedure, which leads to a biologically safe product and easy applicability, but unfortunately at the cost of the favorable biological properties. In contrast, Cryo-AM is widely used around the world, and has been shown to be clinically effective and safe. It contains more proteins with a favorable clinical effect, and is a good substrate for the expansion of epithelial cells. Furthermore, if properly processed, it is as safe as AD-AM.

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