Tri-Layer Amniotic Membrane Allografts Retain Biological Properties Supportive of Surgical Recovery

Heather Bara PhD, Lisa Godwin, Sarah Moreno, Michelle Massee, Thomas J Koob PhD, and John R. Harper PhD

SAWC Fall, October 2022

INTRODUCTION

In utero, the amniotic membrane provides the barrier in which fetal development occurs during gestation. The biological properties of native amniotic membranes may be preserved through gentle processing techniques to produce allografts used in a variety of acute and chronic wound settings. Techniques vary and grafts may be comprised of a single amnion layer as well as amnion/chorion bilayers and other configurations. Recently, a novel method was developed for producing a tri-layer lyophilized human amnion chorion membrane (LHACM*), containing amnion, intermediate and chorion layers. This study evaluates the proteomic composition and *in vitro* biological properties of LHACM allografts, and the *in vivo* response to surgical implantation.

MATERIALS AND METHODS

Graft Preparation: LHACM was prepared using the PURION process, consisting of a series of gentle rinses in non-denaturing reagents.

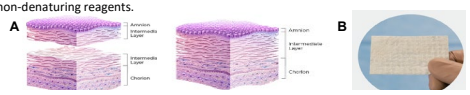

Figure 1. (A) Amniotic membrane layers are separated during the gentle cleansing process and relaminated prior to lyophilization and terminal sterilization. (B) Photograph of LHACM graft.

Bioactivity: LHACM extracts were made by incubating minced grafts (20 mg/mL) in basal medium for 24 hours at 4˚C and filtering. **Proliferation:** Human dermal fibroblasts (HDFs) were treated with basal media supplemented with LHACM extract at a final concentrations of 10, 5, or 1 mg/mL. Following a 72 hour incubation at 37˚C, cellular proliferation was determined by CyQuant Assay. **Migration:** HDFs were plated at confluence on ImageLock plates (Sartorius) and incubated overnight at 37˚C. Monolayers were scratched using the WoundMaker (Sartorius) and treatments applied at final concentrations of 20, 10, 5, and 1 mg/ml LHACM extract. Basal (DMEM with 0%FBS) and complete (DMEM with 10%FBS) media served as controls. Cellular migration was determined by live cell imaging for 120 hours with automated image processing to determine % Wound Confluence at each time point (S3 IncuCyte, Sartorius).

Proteomic Characterization: LHACM allografts were characterized by fractionated liquid chromatography and tandem mass spectrometry. Three fractions for each of two samples were analyzed. Raw data was analyzed using Label Free Quantification in MaxQuant. Processing of the analyzed data files was performed using Perseus. Identifications from <1 unique peptide and with MaxQuant intensity scores <10 were filtered out. Geneontology (GO) analysis was performed with the webgestalt web tool.

***In vivo* mouse model:** The host response to LHACM was assessed in athymic nude mice. LHACM (0.5 cm x 0.5 cm) grafts were implanted subcutaneously in 18 mice. The implant sites were evaluated at 1, 2, and 4 weeks post surgical implantation. At each time point mice were euthanized, and the implant sites were harvested, fixed in neutral buffered formalin, paraffin-embedded and stained with Hematoxylin and Eosin (H&E). Independent histopathologist assessment performed using tables shown below.

Implant Reorganization		Collagen Deposition	
0	Not present	0	Not present
1	Minimal; <25% implant reorganization/bioresorption	1	Minimal peripheral band and/or minimal deposition within implant material
2	Mild; <50% implant reorganization/bioresorption	2	Mild peripheral band and/or mild deposition within implant material
3	Moderate; ≥50% to <75% of implant reorganization/bioresorption	3	Moderate peripheral band and/or moderate deposition within implant material
4	Marked; ≥75% of implant reorganization/bioresorption	4	Marked peripheral band and/or marked deposition within implant material

Cellular Infiltration/Ingrowth	
0	Not present
1	Minimal infiltration, cells present in <25% of implant mass or material layers
2	Mild infiltration, cells present in 25% to <50% of implant mass or material layers
3	Moderate infiltration, cells present in 50% to <75% of implant mass or material layers
4	Marked infiltration, cells present in ≥75% of implant mass or material layers

RESULTS

LHACM promotes proliferation and migration of HDFs in vitro

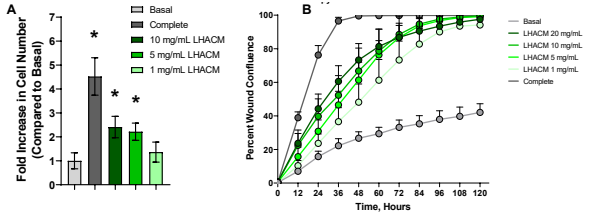

Figure 2. (A) Proliferative response to LHACM (N=5 LHACM lots) when HDFs were incubated with LHACM extract for 72 hours. Data normalized to the basal group as average fold change ± the standard deviation. *p<0.05 relative to the basal group. (B) Migration of HDFs in a 2D scratch assay. Live cell label-free imaging of HDF over 5 days under exposure of LHACM extracts or media controls. Data presented as % coverage of the initial scratch area at each time point ± standard error.

Nearly 2000 unique proteins identified in LHACM by Mass Spectrometry

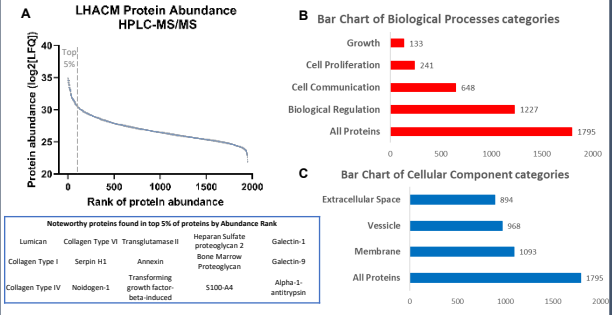

Figure 3. Proteomic characterization by LC-MSMS of LHACM samples, prepared by trypsin digestion and fractionation, identified 1,949 unique proteins. (A) Protein abundance (Log2[LFQ]) vs. rank of protein abundance illustrates that many of the abundant proteins found are ECM molecules or proteins associated with the ECM. Noteworthy proteins from top 5% of identifications shown in table. (B) Categorization of the proteins identified by GO analysis included Biological Process terms consistent with known activities of amniotic membranes observed *in vitro*. Number to the right of each bar is the number of proteins associated with the GO term. (C) GO analysis (Cellular Component GO terms) demonstrated that a majority of proteins found in LHACM are found in locations where they may still exert potential biological effect upon transplantation.

RESULTS

Host cells grow into and around LHACM in vivo

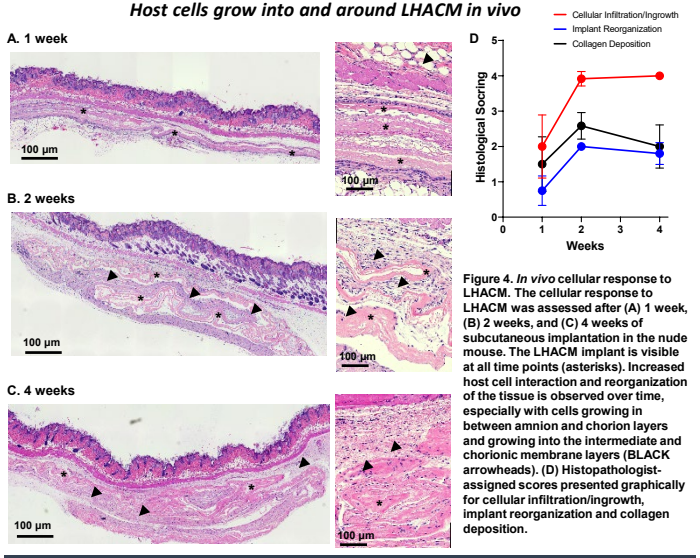

Figure 4. *In vivo* cellular response to LHACM. The cellular response to LHACM was assessed after (A) 1 week, (B) 2 weeks, and (C) 4 weeks of subcutaneous implantation in the nude mouse. The LHACM implant is visible at all time points (asterisks). Increased host cell interaction and reorganization of the tissue is observed over time, especially with cells growing in between amnion and chorion layers and growing into the intermediate and chorionic membrane layers (BLACK arrowheads). (D) Histopathologist-assigned scores presented graphically for cellular infiltration/ingrowth, implant reorganization and collagen deposition.

CONCLUSION

Amniotic membranes contain numerous ECM components and regulatory proteins that are retained by PURION‡ processing techniques and thought to contribute to the observed biological activity of the allografts. The methods described for producing LHACM allografts resulted in a thick allograft, with retained amniotic proteins capable of promoting *in vitro* cellular bioactivities relevant to wound management. *In vivo* responses to LHACM demonstrated direct cellular interaction and neocollagen deposition, consistent with the allograft's intended use. LHACM represents a novel product that enhances the versatility of amniotic membrane allografts in applications where a thicker amniotic membrane is desired or improved handling of the allograft is required.

ACKNOWLEDGEMENTS

In vivo study was conducted at Global Center for Medical Innovations (Atlanta, GA). Histological processing and assessment of the *in vivo* study was conducted by StageBio (Mt. Jackson, VA). Proteomics sample processing was performed by Creative Proteomics (Shirley, NY). Matthew Giedd (MIMEDX Group Inc.) assisted with proteomics data analysis.

All authors are employees of MIMEDX Group, Inc.

*AMNIOEFFECT, MIMEDX Group Inc. Marietta, GA ‡PURION® Process, MIMEDX Group, Inc., Marietta GA