Tri-Layer Amniotic Membrane Allografts Support Cell In-Growth and Promote Angiogenesis: Therapeutic Potential for Acute and Chronic Wounds

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INTRODUCTION

Restoration of vasculature via specific angiogenic mechanisms, is essential for adequate healing of acute and chronic wounds, whereby, oxygen and nutrients are supplied to the wound and waste products are removed.[1] Treatment of acute and chronic wounds with amniotic membranes resets the wound healing cascade, leading to improved clinical outcomes, and recent research has uncovered its role in regulating angiogenesis.[2-5] This study evaluates the angiogenic properties of a novel tri-layer lyophilized human amnion chorion membrane (LHACM®), containing the amnion, intermediate and chorion layers. The effect of LHACM on angiogenesis was evaluated in both *in vitro* and *in vivo* systems.

MATERIALS AND METHODS

LHACM Extract Preparation: Human amniotic tissue (amnion, intermediate, and chorion layers) was processed using a proprietary and patent-pending cleansing process followed by lyophilization and terminal sterilization‡. Soluble factors from LHACM were extracted in assay-appropriate Basal media at 4°C for 24 hours.

Identification of angiogenic factors: The presence of angiogenic factors was evaluated in LHACM extract (n = 5 LHACM donors). High Performance Luminex assays (R&D Systems) were used for identification of angiogenic factors in LHACM extracts, according to the manufacturer's instructions. Each sample was tested in duplicate.

***In vitro* cell invasion:** Endothelial cell invasion was evaluated using the Incucyte® Chemotaxis Cell Invasion Assay (Sartorius). Human microvascular endothelial cells (HMEC1) were combined with Reduced Growth Factor Matrigel (Corning) and added to the Incucyte® Clearview insert. The Matrigel was allowed to polymerize at 37°C for 45 minutes. LHACM extract was used as the chemoattractant and added to the wells of a Incucyte® Clearview reservoir plate (n=3 LHACM donors). Basal media (MCDB 131 medium containing 1% Gluta-gro, and 1% penicillin streptomycin) and complete media (MCDB 131 medium containing 1% Gluta-gro, 1% penicillin streptomycin, 10% fetal bovine serum, 10 ng/mL ECF, and 1 µg/mL hydrocortisone) were used as the negative and positive controls respectively. Assay was conducted at 37°C 5% CO_2 for 24 hours. Invasion was assessed by quantifying total area of 'objects' (cells) located on the top surface and the bottom surface of the Clearview membrane. Automated image processing was performed with the Chemotaxis Analysis module (Sartorius, version 2019B REV2). The metric of "Total Phase Object Area Normalized to Initial Top Value" was calculated at each time point by dividing the total area of cells on the bottom surface of the membrane by the initial cell area of the top surface of the membrane.

***In vivo* mouse model:** Female and male NU/J athymic nude mice were implanted with a 1 cm x 1 cm piece of LHACM into a surgical pocket. Mice were euthanized at 1, 2, and 4 weeks post implantation. The implant sites were harvested *en bloc* with >10 mm tissue margins to include epidermis, dermis, muscle, and other surrounding soft tissues. Samples were fixed in 10% neutral buffered formalin for at least 12-24 hours, then transferred into 70% ethanol. Samples were paraffin-embedded and sections stained for Hematoxylin and Eosin (H&E). H&E slides were reviewed and scored by a histopathologist at StageBio.

Implant Reorganization	
0	Not present
1	Minimal; <25% implant reorganization/bioresorption
2	Mild; <50% implant reorganization/bioresorption
3	Moderate; ≥50% to <75% of implant reorganization/bioresorption
4	Marked; ≥75% of implant reorganization/bioresorption

Collagen Deposition	
0	Not present
1	Minimal peripheral band and/or minimal deposition within implant material
2	Mild peripheral band and/or mild deposition within implant material
3	Moderate peripheral band and/or moderate deposition within implant material
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

Immunofluorescence: Immunofluorescence was performed on formalin-fixed paraffin-embedded sections. Briefly, sections were deparaffinized, subjected to antigen retrieval followed by blocking in Serum-Free Protein Block (Agilent Dako) for 1 hour at room temperature. Incubation with primary antibody against human-specific collagen type IV, mouse-specific collagen type I, and CD31 in Antibody Diluent (Agilent Dako) was carried out overnight at 4°C. For visualization, cells were incubated with Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 488 and Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 647 (Thermo Fisher) and DAPI (Vector Laboratories) to identify the nuclei. Images were acquired on a Leica microscope fitted with 10x and 40x objectives, using Leica Application Suite Advance Fluorescence software and the THUNDER Imager (Leica Microsystems).

REFERENCES

1. Honnegowda, T. M. et al. Role of angiogenesis and angiogenic factors in acute and chronic wound healing. Plastic and Aesthetic Research 2, 243-249 (2015).
2. Duan-Arnold, Y., Uveges, T. E., Gyurdieva, A., Johnson, A. & Danilkovitch, A. Angiogenic Potential of Cryopreserved Amniotic Membrane Is Enhanced Through Retention of All Tissue Components in Their Native State. Adv Wound Care (New Rochelle) 4, 513-522, doi:10.1089/wound.2015.0638 (2015).
3. Wu, M. et al. Human Amniotic Membrane Promotes Angiogenesis in an Oxidative Stress Chronic Diabetic Murine Wound Model. Adv Wound Care (New Rochelle), doi:10.1089/wound.2022.0005 (2022).
4. Koob, T. J. et al. Angiogenic properties of dehydrated human amnion/chorion allografts: therapeutic potential for soft tissue repair and regeneration. Vasc Cell 6, 10, doi:10.1186/2045-824X-6-10 (2014).
5. Niknejad, H., Paeini-Vayghan, G., Tehrani, F. A., Khayat-Khoei, M. & Peirovi, H. Side dependent effects of the human amnion on angiogenesis. Placenta 34, 340-345, doi:10.1016/j.placenta.2013.02.001 (2013).

RESULTS

LHACM contains pro-angiogenic factors and promotes endothelial cell invasion

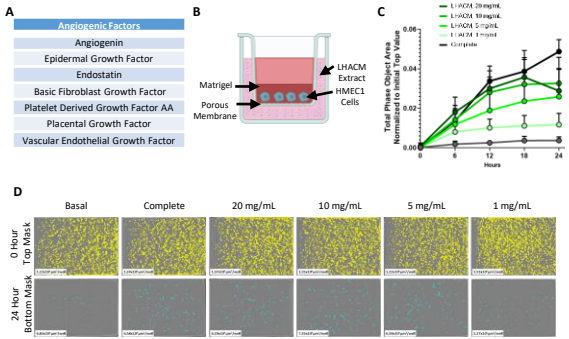

Figure 1. Angiogenic properties of LHACM. (A) Angiogenic factors identified in LHACM extract. (B) Schematic representation of the *in vitro* cell invasion assay. (C) Graphical representation of the total phase object area of bottom normalized to initial top value from 0 to 24 hours. (D) Representative images at 0 hour and 24 hour highlighting the total object area of the top (yellow) and bottom (blue) of the porous membranes used for the invasion assay.

In vivo: Progressive host cell infiltration/ingrowth of LHACM with extensive reorganization and neocollagen deposition

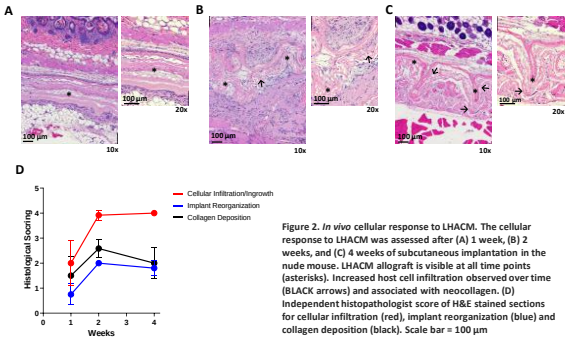

Figure 2. *In vivo* cellular response to LHACM. The cellular response to LHACM was assessed after (A) 1 week, (B) 2 weeks, and (C) 4 weeks of subcutaneous implantation in the nude mouse. LHACM allograft is visible at all time points (asterisks). Increased host cell infiltration observed over time (BLACK arrows) and associated with neocollagen. (D) Independent histopathologist score of H&E stained sections for cellular infiltration (red), implant reorganization (blue) and collagen deposition (black). Scale bar = 100 µm

RESULTS

LHACM reorganization and neocollagen formation associated with infiltrating host fibroblasts

Recruitment of endothelial cells and neovascularization within the LHACM implant

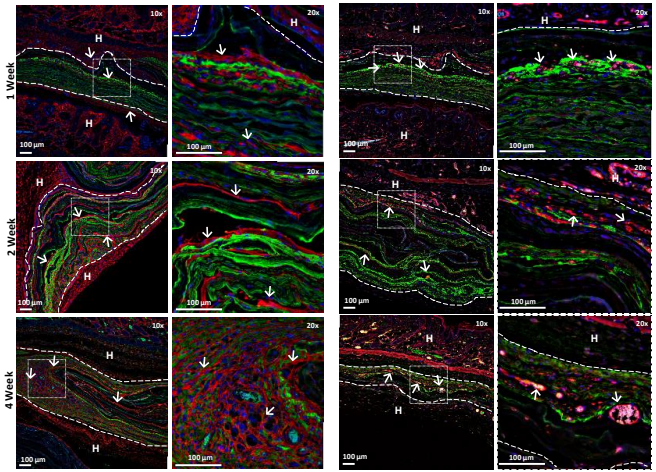

Figure 3. Host cell infiltration in response to LHACM post *in vivo* implantation. LHACM after 1 week, 2 weeks, and 4 weeks of subcutaneous implantation in the nude mouse; 10x (left) and 20x (right). (A) Reorganization and neocollagen formation associated with infiltrating host fibroblast cells (arrows). Immunofluorescence of cellular infiltration and associated neocollagen formation: human collagen type IV (green); mouse collagen type 1 (red); cell nuclei (blue). (B) Recruitment of endothelial cells and neovascularization within the LHACM implant (arrows). Immunofluorescence of endothelial cells: human collagen type IV (green); CD31 (red); cell nuclei (blue). H: host tissue; Scale bar = 100 µm

CONCLUSION

PURION-processed LHACM retains regulatory factors native to the amniotic membrane, several of which are established pro-angiogenic cytokines. LHACM promotes endothelial cell activity *in vitro* and the results of the *in vivo* experiments demonstrate that LHACM provides a scaffold into which cells migrate and establish new blood vessels. LHACM is a promising advanced treatment modality that, while providing a protective barrier, may also support the healing process through enhanced granulation tissue formation within various acute and chronic wounds.

ACKNOWLEDGEMENTS

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

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