Dehydrated Human Umbilical Cord Modulates the Effects of TGFβ-Mediated Fibrosis in Support of Wound Management

Ariana Renrick PhD, Sarah Moreno, Michelle Massee, and John R. Harper PhD

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INTRODUCTION

Uncontrolled fibrosis can lead to major complications with the quality of healing and the process of scarring. These complications are caused by the accumulation of excess extracellular matrix (ECM) components, leading to altered tissue architecture and inhibition of function. The use of human umbilical cord allografts has exhibited promising clinical outcomes in terms of wound management [1]. Therefore, this study employs an *in vitro* macromolecular crowding (MMC) model to imitate an *in vivo* ECM-rich environment to further understand how a dehydrated human umbilical cord allograft (DHUC*) affects the pathobiological mechanisms contributing to fibrosis [2].

MATERIALS AND METHODS

Eluate Preparation: Human umbilical cord tissue (derived from the Wharton's jelly of the umbilical cord) was processed using the Proprietary PURION®‡ process, including cleansing followed by lyophilization and terminal sterilization to produce DHUC. Soluble factors from DHUC were extracted in assay-appropriate complete media at 4°C for 16 hours.

Cell Culture: Human dermal fibroblasts (HDFs) between passages 5-9 were maintained in DMEM supplemented with 10% fetal bovine serum, 1% penicillin streptomycin, and 1% sodium pyruvate. Under MMC conditions, HDFs were initially cultured in complete DMEM and after 4 hours adherent cells were washed with basal DMEM and cultured overnight in basal DMEM containing 0.4% fetal bovine serum. After overnight incubation, 0.4% basal media was replaced with 0.4 % basal DMEM containing a mixture of 37.5 mg/mL Ficoll 70 kDa and 25 mg/mL Ficoll 400 kDa, and 1 mM L-ascorbic acid 2-phosphate. Treatments were carried out with DHUC (20 mg/mL, 10 mg/mL, and 1 mg/mL) in the presence of 20 ng/mL TGFβ1. Culture media and treatments were replaced on day 3.

Quantitative polymerase chain reaction: Post treatment, RNA and complimentary DNA were prepared using the Cells-2-Ct Kit. qPCR for each target gene was performed on a QuantStudio™ 7 Flex Real-Time PCR System using predesigned TaqMan Gene Expression Assays for ACTA2, COL1A1, CTGF, LOX, BMP1, TGM2, and eukaryotic 18. Each replicate sample was analyzed in duplicate. Relative mRNA concentrations of the genes of interest were normalized to the relative mRNA of the housekeeping gene 18s. Differences were calculated with the comparative Ct method for each target gene with the results expressed as a fold increase over the control. For graphical representation, the technical replicate values for each sample were combined.

Western Blotting: Protein extracts from MMC culture treatments were resolved by SDS-PAGE and subsequently transferred onto a nitrocellulose membrane. Following membrane blocking to prevent nonspecific binding, primary antibodies specific to the target proteins were incubated, allowing for the formation of antigen-antibody complexes. After thorough washing, membranes were probed with fluorescently labelled secondary antibodies. Membranes were imaged and signal was quantified (Odyssey M, LI-COR Biosciences).

Immunofluorescence: HDF cells cultured under MMC and DHUC treatment conditions on 4-well slides, were fixed with 4% paraformaldehyde (Electron Microscopy Science) at room temperature for 30 minutes. For Intracellular analysis, cellular membranes were permeabilized with 0.1% Triton-X-100 for 2 min. Cells were blocked in serum free protein block for 1 h at room temperature. Incubation with primary antibody against collagen type I diluted in Antibody Diluent was carried out overnight at 4C. For visualization, cells were incubated with Goat anti-Mouse IgG (H+L) Alexa Fluor 488 and DAPI 4'6'-diamidino-2-phenylinodole (H-1500, Vector Laboratories) to identify nuclei. Images were acquired on a Leica microscope fitted with 20x objective using Leica Application Suite Advance Fluorescence software and the Thunder Imager from Leica (Leica Microsystems).

Matrix deposition analysis: At day 6, the matrix deposited at the bottom of the wells together with the cells were washed with PBS and stored at -80C. Prior to digestion, the matrix was thawed and dissolved with 0.5 M acetic acid overnight followed by digestion with 0.5 mg/mL Pepsin (Millipore Sigma), in 0.01 M HCl. Samples were neutralized with 1x LDS sample buffer and analyzed by SDS-PAGE under reducing conditions. Protein bands were stained with the Silver Stain Kit according to the manufacturer's instructions. Gel images were obtained with Amersham Imager 600 (GE Healthcare Systems).

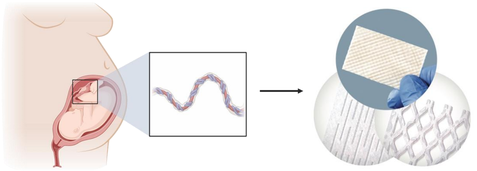

Figure 1. DHUC is a human umbilical cord allograft. DHUC consists of an extracellular matrix of hyaluronic acid (HA) and collagen. Umbilical cord tissue was prepared via the PURION®‡ process generating terminally sterilized, lyophilized tissue product. Created using BioRender.

RESULTS

DHUC Modulates the Expression of Profibrotic Effectors In Vitro

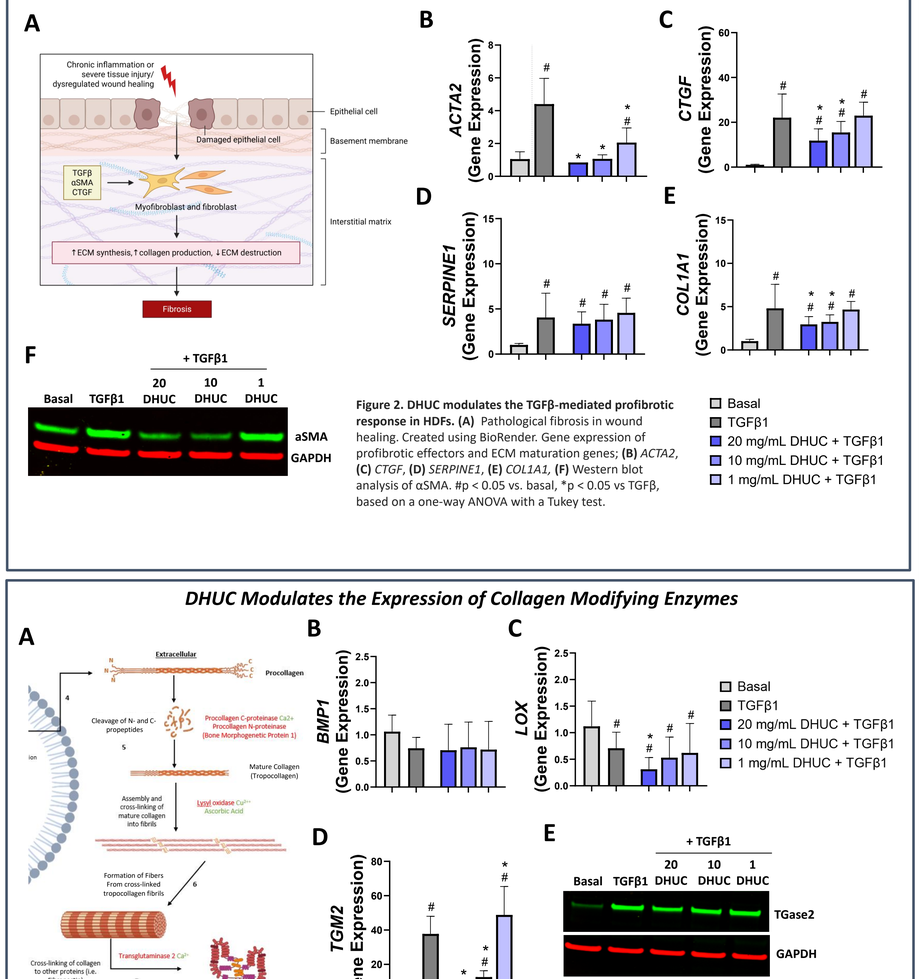

Figure 2. DHUC modulates the TGFβ-mediated profibrotic response in HDFs. (A) Pathological fibrosis in wound healing. Created using BioRender. Gene expression of profibrotic effectors and ECM maturation genes; **(B)** *ACTA2*, **(C)** *CTGF*, **(D)** *SERPINE1*, **(E)** *COL1A1,* **(F)** Western blot analysis of αSMA. #p < 0.05 vs. basal, *p < 0.05 vs TGFβ, based on a one-way ANOVA with a Tukey test.

DHUC Modulates the Expression of Collagen Modifying Enzymes

Figure 3. Effect of DHUC on TGFβ1 induced fibrotic response & collagen modifying enzymes. **(A)** Collagen I biosynthesis and assembly. Created using BioRender. Gene expression of collagen modifying enzymes; **(B)** *BMP1,* **(C)** *LOX,* **(D)** *TGM2* ***(E)*** Western blot analysis of collagen modifying enzymes TGase2, LOX proenzyme, and LOX Mature Enzyme. #p < 0.05 vs. basal, *p < 0.05 vs TGFβ, based on a one-way ANOVA with a Tukey test.

RESULTS

DHUC Reduces Intracellular & Extracellular Collagen Type I Deposition in HDFs

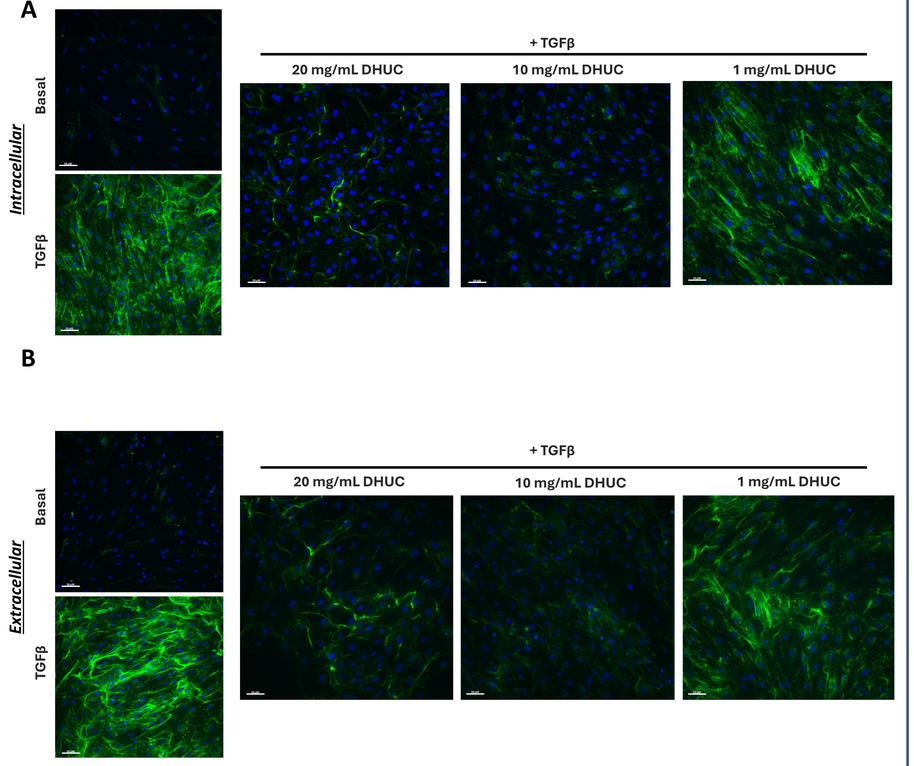

Figure 4. DHUCs influence on the regulation of collagen type I biosynthesis. Immunofluorescence staining of intracellular and extracellular collagen type I in HDFs after 6 days in the presence of TGFβ1 cultured under MMC conditions and treated with DHUC. **(A)** Intracellular collagen type I staining. Green = intracellular collagen, Blue = nuclear stain, **(B**) Extracellular collagen type I staining. Green = extracellular collagen, Blue = nuclear stain. Scale Bar = 50 μM

CONCLUSION

Under MMC conditions, fibroblasts treated with TGFβ1 increased the expression of pro-fibrotic effectors such as *ACTA2, CTGF, SERPINE1, and COL1A1.* The addition of DHUC modulates the expression of these effectors in support of an anti-fibrotic phenotype, indicating their role in the regulation of downstream collagen accumulation and increased ECM degradation. Additionally, DHUC promotes less accumulation of intracellular and extracellular collagen in the context of TGFβ-induced fibrosis. DHUC's effect on LOX and TGase2 expression also suggests reduced ECM remodeling and susceptibility to degradation. This *in vitro* data further highlights the ability of DHUC to regulate the fibrotic response. This study illustrates the capabilities of DHUC in modulating the fibrotic process and the potential for therapeutic applications that lead to better wound healing management through the regulation of ECM formation, remodeling, and inhibition of excessive fibrosis.

REFERENCES

1. Bullard, J., Lei, J. et al. Evaluation of dehydrated human umbilical cord biological properties for wound care and soft tissue healing. *Journal of Biomedical materials, (2018).* DOI: 10.1002/jbm.b.34196
2. Moreno, S., Enwerem-Lackland, I. et al. Human amniotic membrane modulates collagen production and deposition in vitro. Scientific Reports, (2024)14:15998. https://doi.org/10.1038/s41598-024-64364-2

All authors are employees of MIMEDX Group, Inc. *EPICORD®, MIMEDX Group, Inc. Marietta, GA; ‡PURION® Process, MIMEDX Group, Inc., Marietta GA