Effect of Transforming Growth Factor –beta1 (TGF-β1) on human vocal fold fibroblasts in 3-Dimensional (3-D) hyaluronan hydrogel environment

Xia Chen and Susan Thibeault

Division of Otolaryngology-Head and Neck Surgery, Department of Surgery, University of Wisconsin-Madison, Madison, WI 53705

Statement of Purpose: Abnormal vocal fold wound healing is characterized by overexpressed extracellular matrix (ECM) and cells, causing a voice disorder decreasing patient's quality of life. TGF-β1 is one of the most important cytokine expressed during wound healing. It controls cellular proliferation and differentiation, contributes to the fibrotic process by recruiting fibroblasts, stimulating their synthesis of ECM and concurrently inhibiting proteases while enhancing protease inhibitors, favoring matrix accumulation [1]. Previous studies have used primary or cell lines in 2-dimensional (2-D) monolayer *in vitro* cultures to elucidate such mechanisms. Unfortunately, 2-D culture systems do not represent the complex architecture of vocal fold lamina propria *in vivo*. Therefore, we set out to investigate the effect of TGF-β1 exposure on the immortalized human vocal fold fibroblasts (hVFFs) in 3-D hyaluronan hydrogel culture. This study is expected to exucidate mechanisms by which hyaluronan hydrogel replaces tissue improving wound healing, and preventing scar formation.

Methods: hVFFs were isolated from 21-year old donor and steadily immortalized with a retroviral vector plasmid DNA encoding human telomerase reverse transcriptase (hTERT) [2]. Injectable chemically modified hyaluronan-gelatin hydrogel (Carbylan-GSX) was synthesized using a biocompatible, thiol-modified semisynthetic glycosaminoglycan analogous (HA-DTPH), thio-modified gelatin (gelatin-DTPH) and crosslinked by PEGDA [3]. For 3-D culture, 500µl Carbylan-GSX with $2X10^6$ cells/ml were placed onto each transwell permeable support in 6-well plate and cultured. To characterize the cell response to TGF-β1and myofibroblast differentiation of hVFF, we treated hVFF with various dosages of TGF-β1. The morphological features and phenotypic conversion of hVFF were detected by immunocytochemistry for the cell markers, prolyl-4-hydroxylase (hPH, Millipore) and α-SMA (myofibroblast marker, Sigma), imaged by Nikon A1R high speed spectral confocal microscope. Total cellular α-SMA protein levels were analyzed versus GAPDH using western blot. In order to study ECM regulation and remodeling, matrix metalloproteinase 1 and 2 (MMP1 & MMP2) and tissue inhibitor of metalloproteinase 3 (TIMP3) gene expressions were analyzed versus β-Actin housekeeping gene using quantitative Real-time PCR (standard curve method). MMPs to TIPM3 ratios were investigated.

Results: hVFFs clearly adapt their morphology to the characteristics of their environment. On polystyrene surfaces, hVFFs were larger, spindle shape; while in 3-D Carbylan-GSX, cells were smaller, rounded (Fig. 1A and 1B). On polystyrene, strong α-SMA staining was found in hVFFs cytoplasmic myofilaments along the cell axis after stimulated with TGF-β1 (5ng/ml x 48h); however, in 3-D Carbylan-GSX less α-SMA was detected (Fig. 1C and 1D).

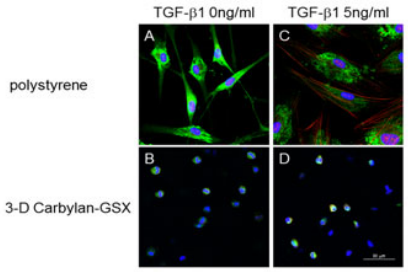

Figure 1. Representative images of hVFFs cultured in polystyrene and 3-D Carbylan-GSX. The cells were labeled with hPH (green), α-SMA (red) and nucleus (blue). Scale bar, 30µm.

Further, on polystyrene, α-SMA protein expression was significantly up-regulated by TGF-β1 in a dose-dependent manner; however, in 3D Carbylan-GSX TGF-β1 did not induce α-SMA expression (Fig. 2).

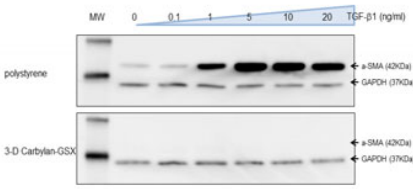

Figure 2. α-SMA protein expression levels on polystyrene and 3-D Carbylan-GSX after 48hours varying concentrations of TGF-β1 treatment.

MMP1 and MMP2 gene expression levels in 3-D Carbylan-GSX were significantly up-regulated compared to polystyrene. This was not inhibited by TGF-β1, as MMP1 and MMP2 to TIMP3 ratios increased in 3D versus polystyrene control (Fig. 3).

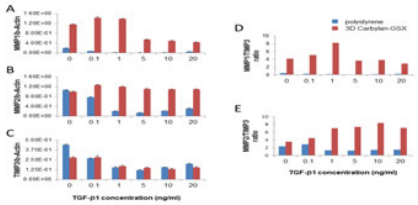

Figure 3. MMP1, MMP2, TIMP3 gene expressions and MMPs to TIMP3 ratios in response to TGF-β1 in 3-D Carbylan-GSX

Conclusions: In order to understand how hyaluronan hydrogel promotes vocal fold wound healing and how biological activity affects subsequent cell behavior, we have investigated hVFFs in 3-D Carbylan-GSX in response to TGF-β1. Carbylan-GSX inhibited TGF-β1-induced myofibroblast differentiation and up-regulated MMPs expression, increased MMPs to TIMP3 ratios, (indicate) ECM degradation and remodeling. This study suggests that after vocal fold injury, locally injected Carbylan-GSX can promote the structure and function recovery of the vocal fold. Further work will focus on the mechanism of Carbylan-GSX affects cell behaviors, including MSCs differentiation and signal transduction.

References: 1. Branton MH, et al., Microbes Infect. 1999;1:1349-65. 2.Chen X. and Thibeault SL. Tissue Eng. Part C. 2009;15:201-212. 3. Shu XZ, et al., Biomaccromolecules 2002;3:1304-1311

Funding: NIH-NIDCD RO1DC4336