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Compression Therapy Affects Collagen Type Balance in Hypertrophic Scar

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Abstract

Background—The effects of pressure on hypertrophic scar are poorly understood. Decreased extracellular matrix deposition is hypothesized to contribute to changes observed after pressure therapy. To examine this further, collagen composition was analyzed in a model of pressure therapy in hypertrophic scar.

Materials and Methods—Hypertrophic scars created on red Duroc swine (n=8) received pressure treatment (pressure device mounting and delivery at 30 mm Hg), sham treatment (device mounting and no delivery), or no treatment for two weeks. Scars were assessed weekly and biopsied for histology, hydroxyproline quantification, and gene expression analysis. Transcription levels of collagen precursors COL1A2 and COL3A1 were quantified using RT-PCR. Masson's trichrome was used for general collagen quantification while immunofluorescence was used for collagen types I and III specific quantification.

Results—Total collagen quantification using hydroxyproline assay showed a 51.9% decrease after pressure initiation. Masson's trichrome staining showed less collagen after one (p<0.03) and two (p<0.002) weeks of pressure application compared to sham and untreated scars. Collagen 1A2 and 3A1 transcript decreased by 41.9 and 42.3 fold, respectively, compared to uninjured skin after pressure treatment while a 2.3 and 1.3 fold increase was seen in untreated scars. This decrease was seen in immunofluorescence staining for collagen types I (p<0.001) and III (p<0.04) compared to

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pretreated levels. Pressure treated scars also had lower levels of collagen I and III after pressure treatment ($p<0.05$) compared to sham and untreated scars.

Conclusion—These results demonstrate the modulation of collagen following pressure therapy and further characterize its role in scar formation and therapy.

Keywords

Collagen; Pressure Therapy; Compression Therapy; Hypertrophic Scar; Burn Scar

1. Introduction

Hypertrophic scars result from an abnormal wound healing response after burn or traumatic injury, surgery, or inflammation [1]. These lesions are characterized by an erythematous, raised appearance with concomitant symptoms including pruritis and pain [2, 3]. Scarring can increase morbidity through debilitating contractures that impair activities of daily living or lead to psychosocial issues such as depression or anxiety [4–6]. Hypertrophic scars are present in up to 16% of the population and particularly affect individuals with darker skin pigmentation [7, 8]. The treatment of hypertrophic scar is an area of great interest. Management strategies include silicone gels and sheeting, topical steroids, intralesional injections, radiation, laser therapy, and surgical scar correction [7, 9, 10]. Pressure therapy has emerged as a noninvasive and cost effective method of hypertrophic scar treatment [11]. However, lack of standardized protocols or validated animal models has hindered the full understanding of its mechanism of action on hypertrophic scar [12, 13].

At least 28 types of collagen have been defined [14–16] with collagen types I and III identified at higher proportions relative to other collagen types in normal human skin [17]. Type I collagen is the major component of the extracellular matrix (ECM) while type III collagen is the predominant collagen type in the healing wound [18]. Excessive collagen deposition has been implicated in the pathogenesis of hypertrophic scar. Compared to normal skin, collagen synthesis is as much as three times higher in hypertrophic scar and 20 times higher in keloids [19]. It has been shown that the regulation of type I collagen synthesis is inefficient in hypertrophic scars, resulting in a higher ratio of type I to type III collagen [20, 21]. This is further worsened by the increased activity and disturbed apoptosis mechanisms of hypertrophic scar fibroblasts compared to normal fibroblasts from uninjured skin [22, 23].

Pressure therapy is thought to work by decreasing blood flow to scar, resulting in collagenase-mediated collagen breakdown, hypoxia-induced fibroblast and collagen degradation, and decreased scar hydration [1, 24]. While studies on pressure treatment of scar have examined modulation of myofibroblasts [25], matrix metalloproteinases [26], and tumor necrosis factor- α [27], studies examining its direct effect on collagen are limited. The aim of this work was to determine if the application of pressure results in a measurable decrease in collagen composition and subtypes using a previously defined porcine model of hypertrophic scar.

2. Materials and Methods

2.1 Animal Model

All described animal work was reviewed and approved by the MedStar Health Research Institute's Institutional Animal Care and Use Committee (IACUC). Juvenile castrated male Duroc swine were received and handled according to standard facility operating procedures under the animal care and use program accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) and Animal Welfare Assurance through the Public Health Service (PHS).

Duroc swine were prepared and used as previously described [28]. Animals were brought to an operating suite and anesthetized using a combination of ketamine and xylazine delivered intramuscularly. Wounds were created on four red Duroc pigs using a Zimmer dermatome (Zimmer, Ltd, Swindon, UK). On each flank, a 4 inch $\times$ 4 inch (10.16 cm $\times$ 10.16 cm) wound was excised over the rib cage to a depth of 0.09 inches (0.03 in $\times$ 3 passes). A total of eight wounds (two flank wounds per pig) were created. Wounds were dressed with Mepilex[®] Ag (Monlylke, Gothenburg, Sweden) and changed weekly.

Wounds were observed for re-epithelialization and hypertrophic scar formation. By day 70 post-wound creation, all wounds were confirmed to have formed hypertrophic scars. At this point, an automatic pressure delivery system (APDS) for scar compression therapy [29] was mounted onto hypertrophic scars for treatment as previously described [28]. This device consisted of a polycarbonate enclosure that housed a wireless communication device (XBee 2.4 GHz RF modem, Digi International, Inc., Minnetonka, MN) and a compression plate for pressure delivery onto hypertrophic scar. A force sensor positioned on the compression plate allowed real time assessment of pressure delivery and was part of a feedback mechanism to ensure pressure delivery at a preset dose. A polycarbonate base was secured to surrounding skin using MYO/WIRE II Sternotomy Suture (A&E Medical Corporation, Durham, NC) followed by attachment of the APDS to this base. Protective padding, casting material, and a custom-fitted 5 mm thick neoprene vest [30] was then placed to ensure further protection of the animal and device.

2.2 Experimental Design

Developed scars received pressure treatment (pressure device mounting and pressure delivery, n=4), sham treatment (pressure device mounting and no pressure delivery, n=2), or no treatment at all (no pressure device mounting or pressure delivery, n=2). Pressure therapy was set at 30 mm Hg of constant pressure for two weeks.

Scar assessments were performed just prior to pressure initiation at week 0 (day 70 post-wound creation), after one and two weeks of pressure therapy, and one week after treatment removal at week 3. The APDS remained mounted for the entirety of the two weeks except when it was removed briefly for the week 1 scar assessment. Assessments included evaluation of scar maturation, biopsy procurement, and digital photography. Punch biopsies (3 mm) were taken from pretreated scars and at weekly assessments. Biopsies were then placed in formalin for histology or AllProtect Tissue Reagent (Qiagen, Valencia, CA) for RNA isolation.

2.3 Histology

Punch biopsies were fixed in 10% formalin, embedded in paraffin, and sectioned to a thickness of 6 μ m on a microtome (Leica, Germany). Slides were deparaffinized using xylene and rehydrated through an ethanol gradient. Staining was then performed by Masson's trichrome or immunofluorescence using previously described protocols [28, 31]. For immunofluorescence, primary antibodies included monoclonal anti-mouse collagen type I (Abcam, Cambridge, UK) and monoclonal anti-mouse collagen type III (Abcam, Cambridge, UK) at a 1:500 dilution. A polyclonal goat anti-mouse IgG-Cy3 (Abcam, Cambridge, UK) conjugated secondary antibody at a dilution of 1:100 was also used.

Image J (v1.48, NIH) imaging software was used to analyze Masson's trichrome digital images and quantify the amount of collagen per high powered field. A Zeiss Axioimager microscope equipped with both a color and monochrome camera with fluorescent filters was used to view slides (Carl Zeiss, Oberkochen, Germany). Zeiss Zen Pro 2012 software (Carl Zeiss, Oberkochen, Germany) was then used to capture digital images and quantify staining intensity of collagen types I and III.

2.4 Hydroxyproline Assay

Punch biopsies procured at weekly wound assessments were used to quantify hydroxyproline as a surrogate for total collagen quantity. Samples were homogenized in 4 N HCl and hydrolyzed for 18 hours at 100°C. They were then washed in buffer and concentrated using spin concentrator tubes as described previously [32]. A standard curve was generated and compared to measurable color changes at 560 nm. Three measurements were recorded using the cuvette function of a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE). The average absorbance was intercalated to a standard curve to yield a concentration of hydroxyproline in μ g/mL. The concentration per mg of tissue was calculated based on the initial biopsy weight.

2.5 RNA Quantification

RNA was isolated from punch biopsies using the RNEasy Fibrous Tissue Kit (Qiagen, Valencia, Ca) and quantified using a Bioanalyzer RNA 6000 NanoKit (Agilent Technologies, Santa Clara, CA). The transcript of collagen precursors COL1A2 and COL3A1 were quantified from scar samples using a multiplex real time RT-PCR system (SABiosciences, Qiagen, Valencia, CA). First strand cDNA synthesis was carried out using 100 ng of total RNA in an RT2 first strand kit (SABiosciences, Qiagen, Valencia, CA). Plates with wells containing gene specific primers and RT2 real-time SYBR Green/ROX PCR mix (SABiosciences, Qiagen, Valencia, CA) were used on an ABI Prism 7500Fast PCR system (Applied Biosystems, Foster City, CA).

2.6 Data Analysis

Data was stored in Microsoft Excel (Microsoft, Redmond, WA) and analyzed using GraphPad Prism 6 (GraphPad Software, Inc, La Jolla, CA). All data are represented as mean values with standard error of the mean. Statistical comparisons were made using student's t-test and ANOVA with a statistical significance set at $p < 0.05$.

3. Results

3.1 Pressure Treated Scars Exhibit Decreased Hydroxyproline Concentrations

Mean hydroxyproline quantity decreased by 51.9% in pressure treated scars after one week of therapy (Figure 1). This was followed by a 20% and 13.6% decrease at two and three weeks following pressure treatment initiation, respectively. In contrast, mean hydroxyproline quantity increased by 61.1% in sham treated scars at two weeks and 70.7% at three weeks following treatment initiation. Using hydroxyproline as a surrogate for collagen quantity, the results suggest major remodeling following the initiation of pressure.

3.2 Pressure Therapy Results in Decreased Collagen

Masson's trichrome was subsequently used to visualize and quantify total collagen amounts among treatment groups. Pressure treated scars demonstrated decreased staining density compared to other treatment groups (Figure 2A). When images were quantified for total collagen, pressure treated scars had significantly less collagen at one week of treatment compared to sham and untreated scars ($p<0.03$; Figure 2B). This effect was still present at two weeks of pressure therapy ($p<0.002$), but resolved after pressure therapy removal. Pressure treated scars also had decreased collagen quantity after one and two weeks of pressure treatment compared to pretreated scar at week zero ($p<0.05$).

3.3 Pressure Therapy Alters Collagen Gene Expression

Samples were next examined for gene expression behavior during and after pressure therapy. In pressure treated scars, collagen I precursor COL1A2 was downregulated 41.9 fold after pressure therapy compared to a 2.3 fold increase from uninjured skin in untreated scars at week 3 (Figure 3). A similar effect was seen in collagen III precursor COL3A1 where pressure treatment resulted in a 42.3 fold decrease while untreated scars exhibited a 1.3 fold increase relative to uninjured skin at week 3. These results illustrate a downregulation of collagen precursor transcripts following pressure therapy compared to untreated scar.

3.4 Immunofluorescence Staining Confirms Gene Expression Findings

Immunofluorescence staining was then performed to correlate transcript level findings. Pressure treated scars demonstrated a significant decrease in collagen type I intensity compared to sham and untreated scars at two and three weeks following pressure initiation ($p<0.001$; Figure 4B). Pressure treated scars additionally showed significantly decreased collagen type I intensity at two weeks of pressure treatment and the week following compared to pretreated scar at week 0 ($p<0.05$). Stains for collagen type III had a significant decrease in intensity in pressure treated scars compared to sham treated scars at one and two weeks of pressure treatment ($p<0.001$; Figure 5B) as well as the week following APDS removal ($p<0.01$). Pressure treated scars additionally had significantly decreased collagen type III intensity at one and two weeks of pressure treatment and the week following compared to pretreated scar at week 0 ($p<0.05$).

4. Discussion

Collagen is the most abundant protein of the extracellular matrix and excessive deposition has been implicated in the formation of hypertrophic scar. Collagen types I and III have both been shown to increase in hypertrophic scar compared to other subtypes [33] with a concomitant decrease in collagenase production [34]. Studies examining the use of pressure therapy in hypertrophic scar are limited and even more so on its effect on collagen. There has not to date been a comprehensive study examining the modulation of collagen composition by pressure therapy in hypertrophic scar. This work represents a longitudinal study that directly examines the effect of mechanical pressure on hypertrophic scar collagen composition and its subtypes in a controlled environment.

The use of pressure therapy appears to decrease the amount of collagen in scar relative to pretreated scar as well as other treatment groups. Collagen analysis of Masson's trichrome stains by Image J analysis showed decreased collagen quantities following the initiation of pressure therapy. This was supported by hydroxyproline quantification that similarly exhibited sustained decreases following the initiation of pressure. Collagen type specific quantification echoed those results. Collagen types I and III both had significantly decreased intensities relative to other treatment groups. As these are the two most common collagen types seen in skin, these results could explain the significant differences seen in pressure treated scar and suggest major ECM remodeling following pressure therapy. These findings are in line with previously published reports examining in-vitro collagen behavior in pressure therapy. For example, Huang et al. reported a significant decrease in collagen type I expression following pressure therapy using harvested hypertrophic scar fibroblasts [35].

RT-PCR analysis showed major mRNA downregulation of collagen types I and III precursors following pressure therapy. The lowered transcript expression of collagen precursors following pressure therapy may result from a decrease in the number and biologic function of collagen producing cells. This can lead to a decrease in overall collagen production that manifests as the phenotypic changes seen following pressure treatment. Additionally, these transcripts were most downregulated following APDS removal, suggesting a potentially sustained response following pressure therapy.

It is interesting to note that in some instances, collagen content and gene expression did not necessarily correlate. Collagen quantities decreased while precursor gene expression did not experience decreased fold changes until the week following pressure therapy. In some instances, collagen quantity normalized at the point that gene expression did not. This is likely attributable to the role of post-transcriptional, post-translational, and degradation regulation that follows gene expression [36]. This study provides preliminary information detailing collagen quantity following pressure intervention. Pressure therapy may affect post-transcriptional modifications, rates of protein production and turnover and are an area of interest in future studies.

Pressure therapy is thought to modulate changes in scar through a variety of means, such as myofibroblasts [25, 37, 38], matrix metalloproteinases [26], tumor necrosis factor- α [27], and transforming growth factor- β 1 (TGF- β 1) [27, 39]. Despite these studies, no unifying

mechanism has been described that explains the effect of pressure therapy on hypertrophic scar and, more specifically, collagen behavior. Furthermore, these studies mostly rely on in-vitro testing that vary when used in established animal models. This study identifies clear collagen behavioral patterns following pressure intervention in a novel porcine model and, as a result, allow further exploration of the aforementioned mechanisms in future studies.

This study utilized a red Duroc swine model to create reproducible hypertrophic scars. The use of the APDS device in this model has been previously described [29], but its use remains novel and limited. The results of this study add more validity to this combined hypertrophic scar and APDS model in studying the effect of pressure on hypertrophic scars.

There are several limitations of this study. This study examined the modulation of collagen under controlled conditions. As a result, specific set times and pressures were mandated to study collagen behavior in a controlled manner. Future studies examining collagen behavior would need a higher powered number of scars, alternate treatment lengths, and varying pressure doses to further characterize the effect of pressure therapy on collagen composition. This study also did not look at the effect of pressure therapy on fully matured scars or uninjured skin. Answering these questions could provide further insight and understanding of collagen behavior in response to pressure therapy and may further validate the animal model used.

5. Conclusion

This study provided a comprehensive examination of collagen and associated subtype behavior in hypertrophic scar undergoing pressure treatment at the gross, histopathologic, and transcript level. Using an established and well controlled pressure device and swine model, significant decreases in collagen quantity were noted in each component of the study. This data shows that pressure therapy results in a decrease in overall collagen quantity as well as its most common subtypes, collagen types I and III. These findings help further characterize extracellular matrix collagen remodeling in response to the therapeutic application of pressure.

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Figure 1.

Temporal changes in collagen amounts from scar biopsies in pressure or sham treated scars using hydroxyproline quantification as a surrogate.

Figure 2.

Changes in total collagen quantity assessed from biopsies obtained from pressure treated, sham treated, or untreated scars.

(A) Representative photomicrographs at 40x magnification of biopsied sections stained with Masson's trichrome at pressure initiation and at weeks 1 and 2 afterwards.

(B) Image-based quantification of collagen using Masson's trichrome stained sections. * denotes significant differences between pressure treated scars and other treatment groups (p

< 0.05). ** denotes significant differences between pressure treated scars and pretreated scar ($p < 0.05$).

Figure 3.
Assessment of collagen precursor transcript fold change in pressure treated and untreated scars using RT-PCR.

Figure 4.

Assessment of collagen type I by immunofluorescence.

(A) Representative photomicrographs at 40x magnification of biopsied sections stained for collagen type I immunofluorescence between pressure treated, sham treated, and untreated scars at pressure initiation and at weeks 1 and 2 afterwards.

(B) Image-based quantification of collagen type I sections. * denotes significant differences between pressure treated scars and other treatment groups ($p < 0.05$). ** denotes significant differences between pressure treated scars and pretreated scar ($p < 0.05$).

Figure 5.

Assessment of collagen type III by immunofluorescence.

(A) Representative photomicrographs at 40x magnification of biopsied sections stained for collagen type III immunofluorescence between pressure treated, sham treated, and untreated scars at pressure initiation and at weeks 1 and 2 afterwards.

(B) Image-based quantification of collagen type III sections. * denotes significant differences between pressure treated scars and other treatment groups ($p < 0.05$). ** denotes significant differences between pressure treated scars and pretreated scar ($p < 0.05$). At week 3, differences are noted only between pressure treated and untreated scars.