Elastin Is Differentially Regulated by Pressure Therapy in a Porcine Model of Hypertrophic Scar

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Beneficial effects of pressure therapy for hypertrophic scars have been reported, but the mechanisms of action are not fully understood. This study evaluated elastin and its contribution to scar pliability. The relationship between changes in Vancouver Scar Scale (VSS) scores of pressure-treated scars and differential regulation of elastin was assessed. Hypertrophic scars were created and assessed weekly using VSS and biopsy procurement. Pressure treatment began on day 70 postinjury. Treated scars were compared with untreated shams. Treatment lasted 2 weeks, through day 84, and scars were assessed weekly through day 126. Transcript and protein levels of elastin were quantified. Pressure treatment resulted in lower VSS scores compared with sham-treated scars. Pliability (VSS_p) was a key contributor to this difference. At day 70 pretreatment, $VSS_p = 2$. Without treatment, sham-treated scars became less pliable, while pressure-treated scars became more pliable. The percentage of elastin in scars at day 70 was higher than in uninjured skin. Following treatment, the percentage of elastin increased and continued to increase through day 126. Untreated sham scars did not show a similar increase. Quantification of Verhoeff–Van Gieson staining corroborated the findings and immunofluorescence revealed the alignment of elastin fibers. Pressure treatment results in increased protein level expression of elastin compared with sham-untreated scars. These findings further characterize the extracellular matrix's response to the application of pressure as a scar treatment, which will contribute to the refinement of rehabilitation practices and ultimately improvements in functional and psychosocial outcomes for patients. (J Burn Care Res 2017;38:28–35)

Hypertrophic scarring (HTS) is often a complication following burn injury.[1,2] Prevention and treatment of HTS in burn patients is an important area of study because of both the potential for contractures and loss of function and the risk for development of psychosocial problems directly correlated with hypertrophic scar severity.[3–6]

Pressure therapy is a widely used technique for treating HTS and has been the standard of care for almost 50 years. Acceptance of this treatment modality is based mainly on anecdotal experience as the mechanism of action is not fully understood.[7] In a recent evidence-based practice review by Sharp et al,[7] pressure therapy is recommended as a successful scar treatment, which results in improved esthetic outcomes by reducing scar height and erythema.[8–12] This review further reports that pressure therapy is not useful for treating dyspigmentation or improving scar formation. Patient compliance and duration of treatment have also been outlined as important factors in the success of pressure therapy.[13]

Previous studies have attempted to analyze the effects of pressure therapy by characterizing resultant changes to the extracellular matrix (ECM), along with cell growth and signaling following treatment. However, to date, no research has evaluated pressure therapy's influence on elastin fibers present in scars following treatment.[13–19] In fact, in the same review mentioned above, the authors claim, "the literature search revealed insufficient evidence addressing the impact of pressure therapy on scar pliability."[7]

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Although elastin expression has not been analyzed during pressure therapy, the importance of elastin in scar remodeling is now widely accepted.

In large TBSA burn injuries requiring excision and grafting, dermal substitutes are often used as an adjunct to conventional grafting techniques.[20,21] Traditionally, dermal substitutes have been composed of collagen as the main component of the ECM. The use of this type of dermal substitute has been associated with improved outcomes.[20,22,23] Because of the success of collagen scaffolding substitutes, newer dermal matrices on the market are incorporating elastin within the matrix in an effort to reduce contracture and contribute to pliability in HTSs.[24–26]

In a previously described model of pressure therapy in HTS, components of the ECM, specifically collagen types I and III, were examined.[27] After 2 weeks of compression therapy, there was a significantly reduced amount of collagen type I in the dermis of pressure-treated scars when compared with untreated sham scars as demonstrated by immunofluorescent staining for collagens.[27] This decrease in collagen continued at least a week after treatment. The same conclusions were made when staining for collagen type III. These data show correlations between the use of pressure therapy and changes to the ECM. In the present study, additional ECM components are examined to determine the effect pressure may have on other fibrillar proteins, specifically elastin.

Elastic fibers are insoluble components of the ECM that are responsible for resilience and recoil in many tissues. These properties are especially important in the skin, lungs, and vasculature, which require repetitive extension and recoil functionality. Unlike collagen, which compromises a large portion of the total protein in the skin, elastin makes up only 2 to 5%.[28] While elastin's role in the ECM may appear minimal based on composition, devastating conditions may occur as a result of defects and/or abnormalities in elastin genes. For example, the condition called cutis laxa, which involves a frame shift mutation in the elastin gene, causes significant cardiovascular problems, as well as loose, sagging skin.[29,30] Other hereditary diseases such as Marfan syndrome, Buschke–Ollendorff syndrome, emphysema, Menkes syndrome, atherosclerosis, pseudoxanthoma elasticum, and Williams syndrome are also related to defects in elastin-encoding genes.[31] In normal skin, elastin proteins are important for cell signaling, and they induce many pathways including fibroblast migration and proliferation, keratinocyte migration, smooth muscle proliferation, calcium transport, ECM production and degradation, and cell survival.[28]

In early development, the elastin gene transcribes tropoelastin monomers which are secreted to the extracellular space where they self-assemble. These monomers combine with microfibrils to form elastic fibers.[28] After tissue development, the transcription of elastin stops in uninjured tissues. Because elastin has a half-life of over 70 years, normal adult skin does not undergo routine elastin turnover.[21] In adult wound healing, mature elastin is not present, and only disorganized elastic fiber networks are formed in scars.[28] In 1987, Tsuji and Sawabe[32] showed the presence of elastic fibers in postburn HTS using scanning and transmission electron microscopy. Their findings were confirmed in 1996, when another group showed the presence of elastic fibers in scars.[33] In that study, no elastin fibers were detected in scars that were less than 3 months old. However, 3 months later, there were immature elastin fiber networks observed that matured over time.[33]

Clinically, HTSs may be evaluated by a variety of scales, including but not limited to, the Seattle Scar Scale, Hamilton Scar Scale, Patient and Observer Scar Assessment Scale, Stony Brook Scar Evaluation Scale, and the Vancouver Scar Scale (VSS).[34] The VSS, the first validated burn scar assessment scale, was used in this study because the scars were all created in the same manner, and were thus fairly homogenous, providing an ideal scar to be evaluated by this particular scale.[34] The scale is composed of four metrics: vascularity, pigmentation, height, and pliability. Scars with lower scores in the pliability metric are characterized as "better," more pliable scars. Because of elastin's connection to pliability, the present study investigated whether elastin was differentially regulated in hypertrophic scar treated with pressure and whether these differences positively correlated with positive changes in the VSS.

METHODS

Animal Model

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

Two red Duroc swine were used in this study. Each animal received a 4 inch by 4 inch, full-thickness (0.09

inch in depth) excisional wound on each flank, created using a dermatome set to 0.03 inch × 3 passes. A square wound was created for ease of imaging and treatment. Many studies have shown that the position of the wound on the animal may affect the contracture rate; therefore, the midthorax was chosen for wound creation. Wounds created in cephalad positions tend to contract much quicker.[35] Prior to excision, and at weekly examinations through day 63, wounds and subsequent scars were evaluated for healing. Once scars were formed, they were evaluated each week by a clinician using the VSS. Punch biopsies (3 mm) were taken and preserved in All-Protect® reagent (Qiagen, Valencia, CA) or formalin for subsequent assays.

At 70 days postwounding, pressure therapy was initiated. In each animal, the right wound was treated with pressure, while the left, untreated wound served as a sham. Pressure treatment was applied using an automated pressure delivery system (APDS), which delivered a constant pressure of 30 mm Hg to the scar.[36,37] Of note, the APDS provides direct pressure as a perpendicular force to a discrete lesion, which is not exactly the same as the pressure that would be achieved using compression garments. This type of pressure delivery is still translatable as it is used in face masks and silicone sheet application under scar.[38] The surgical mounting and pressure recording capabilities of the APDS are described in previous work.[36,37] The sham-treated scars received device mounting, but no pressure treatment was delivered. Pressure treatment occurred from day 70 to day 84, for a duration of 2 weeks, with a midterm check at day 77. Scars were assessed using the VSS and biopsies were taken weekly through day 126.

Transcript Level Elastin Measurement

Scar biopsies were removed from All-Protect reagent and homogenized using a TissueLyser LT (Qiagen, Germantown, MD) and RNA was extracted using the RNeasy Fibrous Tissue Mini Kit (Qiagen) according to the manufacturer's protocol. RNA sample quality, as indicated by 260/280 ratio, and concentration were obtained using a Nanodrop 2000C spectrophotometer (Thermo Fisher Scientific, Amarillo, TX). RNA quality was also assessed using a Bioanalyzer (Agilent, Santa Clara, CA), and only samples with an RNA integrity number greater than 7.0 were used for analysis. RNA samples were diluted to 1 ng/µl and assayed using the iScript One-Step RT-PCR Kit with SYBR Green (BioRad, Hercules, CA) with primers (Integrated DNA Technologies, Coralville, IA) and reverse transcriptase according to the kit protocol. Reference gene, 18S rRNA (forward: 5′-CCG CGG TTC TAT TTT GTT GGT TTT-3′, reverse: 5′-CGG GCC GGG TGA GGT TTC-3′), levels were quantified in all samples in parallel with target genes. Gene-specific primers for elastin were: forward: 5′-TGC AGT GGT ACC TCA ACT CG-3′, and reverse: 5′-GTA CCA ACC CCT GGC AGC TT-3′. Reactions were run and cycled as detailed in a previous work.[39] The annealing temperature for 18S was 57.0°C and for elastin was 59.5°C. Normalization and fold change were calculated for each time point and compared with baseline, uninjured skin using the $\Delta\Delta C_t$ method.

Fastin Elastin Assay

All-Protect-preserved biopsies of uninjured skin and scars from days 70, 84, and 126 were analyzed using the Fastin Elastin™ assay. The assay for elastin quantification was performed according to the manufacturer's instructions (Biocolor, Carrickfergus, UK).[40–42] Briefly, biopsies were dissolved in 0.25 M oxalic acid, which was used to transform elastin to α-elastin at 100°C in 1 hr. Extracts were centrifuged at 10,000*g* for 10 minutes and the supernatant was separated from the residue for further use. Extraction was repeated three consecutive times to ensure greater than 99% extraction rate and successful α-elastin transformation. Precipitating reagents included in the kit were used to precipitate α-elastin, followed by an additional centrifugation for 10 min at 10,000g to separate the precipitants and supernatant. The precipitation was kept for staining of α-elastin by a dye included in the kit. Subsequently, the dye–precipitate solution was mixed using a mechanical shaker for 90 minutes. After staining, another 10-min centrifugation at 10,000g was performed to separate unbound dye from the dye–precipitants complex. Precipitants were kept for further use. Bound dye for all test samples were dissociated using the same amount of dissociating reagent for 10 min. Colored supernatant was kept for further analysis.

Samples were plated onto 96-well microplates (Thermo Scientific™, Amarillo, TX) and analyzed using a PerkinElmer Victor 2 Plate Reader photometer (PerkinElmer, Waltham, MA) at 510 nm. A standard curve was constructed using a solution of α-elastin standard based on the absorbance of 20 points from 5 to 80 µg in increments of 5 µg. Finally, sample absorbances were intercalated into the standard curve and the percentage of elastin in the biopsy was calculated.

Verhoeff–Van Gieson Staining

Formalin-fixed scar and uninjured skin biopsies were embedded in paraffin and sectioned at 6 µm

thickness. Slides were deparaffinized and brought to distilled water and were subsequently stained in Verhoeff's solution for 1 hr until the tissue turned completely black. Slides were rinsed in tap water with three changes and then were differentiated in 2% ferric chloride for 2 min. Differentiation was stopped with three changes of tap water and slides were then treated with 5% sodium thiosulfate for 1 min. Slides were rinsed in running tap water for 5 min and then counterstained in Van Gieson's solution for 5 min. Slides were dehydrated through an alcohol gradient and cleared in two changes of xylene, and were finally mounted in Permount (Sigma Aldrich, St. Louis, MO).

Uninjured skin and scars from days 70, 84, and 126 were imaged at 40×. Three random high-powered fields were imaged in a region of interest in the dermis directly below the epidermis. Each picture was then analyzed using Image J software to obtain the percentage of the image stained black (NIH, Bethesda, MD). Black staining was assumed to be elastin fibrils. The percentage of elastin was compared between the treatment groups and over the time course.

Elastin Immunofluorescence

Slides were deparaffinized and rehydrated through an alcohol gradient and brought to phosphate-buffered saline (PBS) where they rehydrated for 10 min. Antigen retrieval was performed using a pressure cooker that achieved a temperature of 95°C in 20 min in Tris–EDTA with 0.05% Tween buffer. Slides were cooled with running water for 5 min, and then cell membranes were permeabilized using PBS with 0.025% Triton for 10 min. Slides were blocked with Superblock Buffer in PBS (Thermo Fisher Scientific) for 20 min. The mouse monoclonal primary antibody (sc58756; Santa Cruz Biotechnology, Dallas, TX) was applied at a dilution of 1:100 and allowed to incubate overnight at 4°C. The next day, slides were rinsed with PBS with 0.025% Triton for 10 min and a goat antimouse-CY3-conjugated secondary antibody (Abcam, Cambridge, MA) was applied at a dilution of 1:100 for 1 hour at room temperature. Slides were rinsed in three changes of PBS and then counterstained with 4',6-diamidino-2-phenylindole (1 μg/ml) for 10 min. Slides were rinsed in distilled water for 3 min and mounted in aqueous mounting media.

Slides were viewed with a Zeiss Axioimager microscope with multichannel black and white camera equipped with fluorescence filters (Zeiss, Oberkochen, Germany). Uninjured skin and scars from days 35, 43, 56, 63, 70, 84, and 126 were imaged at 10× using two high-powered fields.

RESULTS

Vancouver Scar Scale

Pressure treatment resulted in lower VSS scores compared with sham scars both at day 84, after 2 weeks of treatment, and at day 126, 6 weeks posttreatment cessation (Figure 1A). Of the four parameters assessed using the VSS, pliability (VSS_P) was a key contributor to differences between groups. At day 70 prior to treatment, $VSS_P = 2$. Without treatment, sham-treated scars became less pliable by day 84 ($VSS_P = 3$), while pressure-treated scars became more pliable ($VSS_P = 1$). This trend continued through day 126, where $VSS_P = 1.5 \pm 0.71$ and $VSS_P = 2.5 \pm 0.71$ in pressure- and sham-treated scars, respectively (Figure 1B). Looking at the VSS total and the VSS_P, it is clear that there is a larger difference in pliability during the treatment period at day 84, and that the

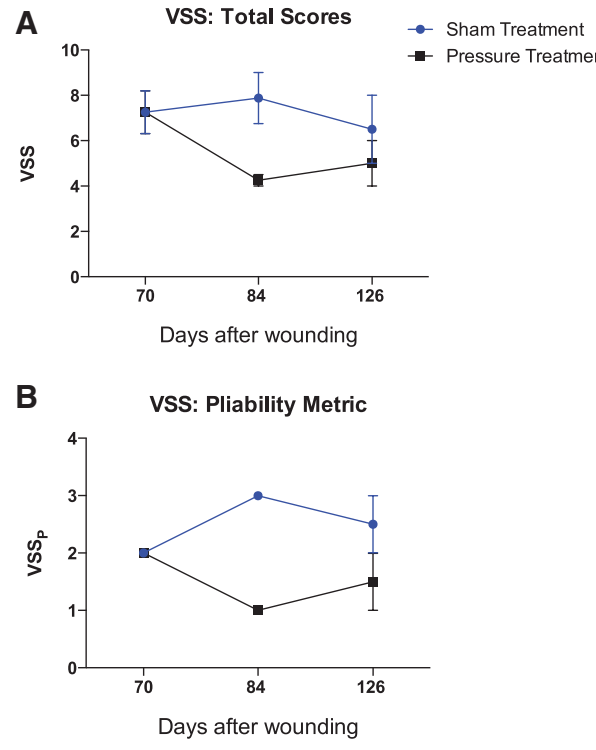

Figure 1. Pressure-treated and sham-untreated scars were evaluated using the Vancouver Scar Scale at days 70, 84, and 126. Treated scars had lower scores after 2 and 6 weeks of treatment (A). The pliability metric contributed to the differences between treated and sham scars. Treated scars became more pliable with treatment, while sham-untreated scars did not (B). Error bars represent SEM; n = 2. VSS_P, Vancouver Scar Scale pliability.

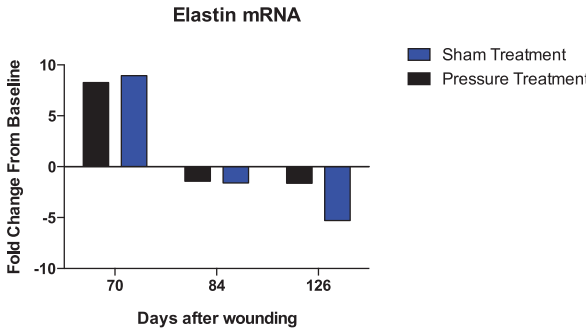

Figure 2. qRT-PCR was used to evaluate transcript levels of elastin in scars at days 70, 84, and 126. At day 70, there is an 8-fold increase in elastin transcripts compared with uninjured normal skin. This increase in transcripts was not observed after device mounting and treatment at days 84 and 126 (n = 1). *qRT*, quantitative real time.

difference continues, but becomes smaller over time. Because the VSS_p is a grade from 0 to 5, scars that were treated in the same manner sometimes showed no variability in the pliability metric (at day 70 and day 84 of pressure treatment and sham treatment, respectively).

Transcript Level Elastin Measurement

Transcription levels of elastin at day 70 postwounding were increased approximately 8-fold over the levels in baseline, uninjured skin. Whether the scar received pressure treatment (device mounting and pressure treatment) or sham treatment (device mounting and no pressure treatment), after 2 weeks there was a decrease in the transcript levels of elastin of approximately 1.6-fold. This decrease continued through day 126 (1.5-fold) (Figure 2).

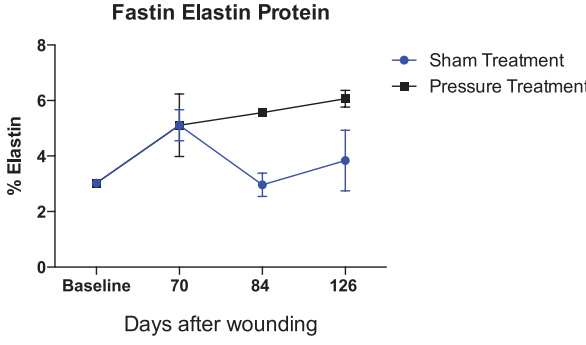

Figure 3. The fastin elastin protein levels assay was used to quantify the percentage of elastin in normal, uninjured skin and scars at days 70, 84, and 126. Elastin levels at day 70 were increased compared with baseline. The elastin percentage in pressure-treated scars increased through day 126, while the levels in sham-treated scars did not. Error bars represent SEM; n = 2.

Fastin Elastin

The percentage of elastin in scars at day 70 was increased from the amounts in baseline skin (5.11 ± 1.13% vs 3.03 ± 0.04%). At day 84, after 2 weeks of pressure treatment, the percentage of elastin increased to 5.56 ± 0.07% and continued to increase through day 126 to 6.07 ± 0.30%. Sham-treated scars did not have this increase in elastin quantity, with percentages of elastin equal to 2.96 ± 0.60% and 3.84 ± 1.55% at days 84 and 126, respectively (Figure 3).

Verhoeff–Van Gieson

Verhoeff–Van Gieson stains elastin fibers black, with a pink counterstain. The staining revealed normal, intact elastic fiber networks in baseline, uninjured skin (Figure 4, Baseline). Fibers were quantified using Image J software and the percentage of elastin in normal skin was 7.52 ± 0.51% (Figure 5). Similar to the Fastin Elastin protein data, levels of elastin fibers were higher in day 70 scars compared with baseline, uninjured skin (16.88 ± 5.32%). Stained fibers were increased in treated scars at days 84 compared with sham-untreated scars (12.33 ± 2.14% vs 8.98 ± 4.14%). The same trend was observed at day 126 (8.81 ± 1.25% vs 6.49 ± 0.13%). The VVG staining data and Image J quantification corroborated the Fastin Elastin data and followed the same trend over time.

Immunofluorescence

Baseline, uninjured skin revealed normal, intact elastic fiber networks. In the papillary dermis, thin, perpendicular elastic fibers were detected using this method. In the reticular dermis, elastic fibers were thicker than in the papillary dermis (Figure 6, baseline). Scar biopsies from days 35, 43, 56, and 63 that were immunofluorescently stained for elastin fibers revealed small, immature elastin monomers in the lower dermis right above the hypodermis. These fibers were not numerous, and there were no fibers present in the mid or upper dermis (data not shown). This is consistent with the notion that elastin fibers are formed after the first month of scar formation. At day 70, elastin fibers were detected in the upper dermis just below the epidermis, as well as throughout the dermis. Pressure-treated scars at days 84 and 126 appeared to have increased amounts of elastin immunofluorescence staining, while sham-treated scars had decreased amounts of elastin.

DISCUSSION

Scar pliability is an important component of scar quality assessments. Less pliable and contracted scars can

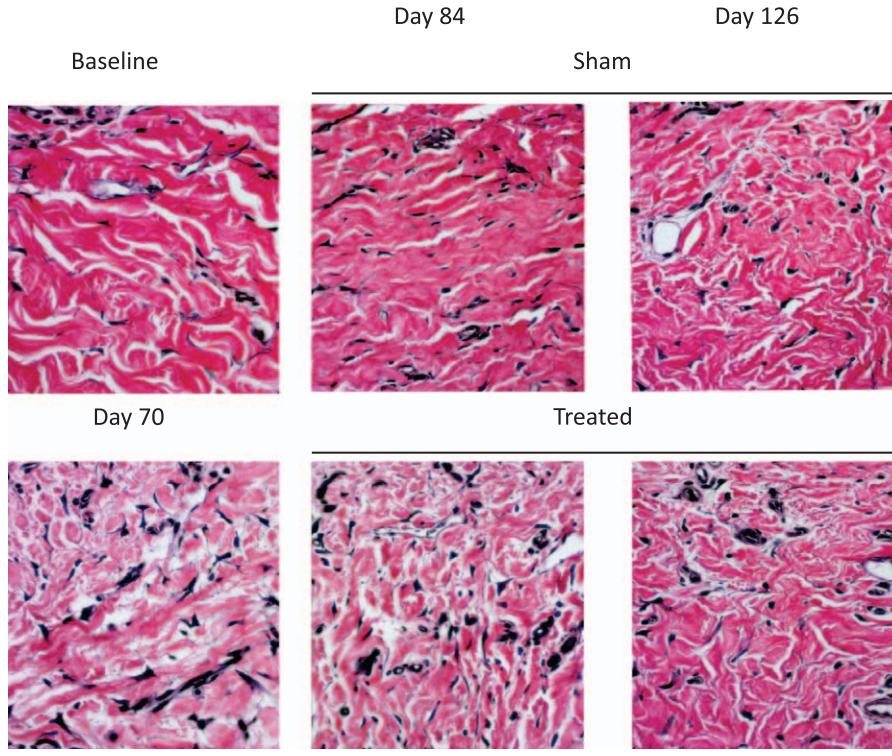

Figure 4. Verhoeff–Van Gieson staining stains elastin fibers black with a pink counterstain. Elastin was stained in baseline skin and in scars at days 70, 84, and 126.

lead to functional impairments. Significant contracture may require surgical intervention and inhibits patients from returning to their normal activities.[43,44] This is the first report in the literature characterizing elastin fibers in hypertrophic scars treated with pressure. This study has shown that pressure-treated scars became more pliable than their counterpart untreated, sham controls (Figure 1). This measurement of pliability was measured clinically by the VSS, and it was demonstrated that this change could also be seen biochemically and histologically.

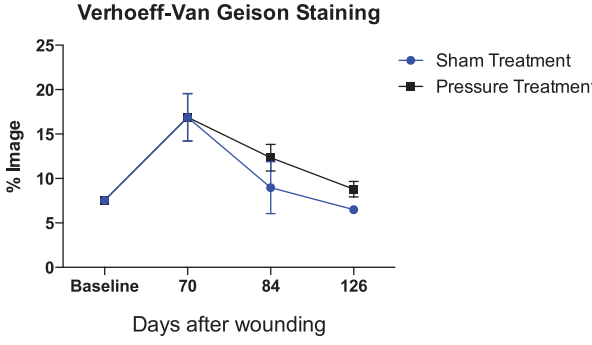

Figure 5. Verhoeff–Van Gieson staining was quantified using Image J software. There was increase elastin levels in day 70 scars compared with baseline skin. Pressure-treated scars had more elastin at days 84 and 126 compared with sham-treated scars. Error bars represent SEM; n = 2.

mRNA levels of elastin were increased 8-fold over baseline levels in normal skin (Figure 2). This increase, which was consistent among both wounds analyzed, was drastically different at day 84 and was similarly observed in both pressure-treated and sham-untreated scars. The volatility in transcript levels of elastin and other extracellular components, such as collagen, is consistent with the known high protein turnover by varied rates of degradation, synthesis, and remodeling. Instability in cell count and types in the wound may cause fluctuation in elastin transcript which is synthesized mainly by fibroblasts. Unlike other extracelluar matrix components, elastin is not found independently; rather it forms a macromolecule when its precursor, tropoelastin, is deposited on microfibril bundles and stability is further conferred by the transglutaminase and lysyl oxidase-derived crosslinks. Changes in amounts of the microfibril building block protein, fibrillin, or the rate of microfibril pericellular assembly and maturation, in addition to any variation in the amounts or activity of the several enzymes involved in the multiple posttranscriptional processing of elastin, will impart drastic consequences on quantified elastin at protein versus transcript levels. Indeed, such differences may be expected given the mounting of the pressure delivery system and the pressure applied,

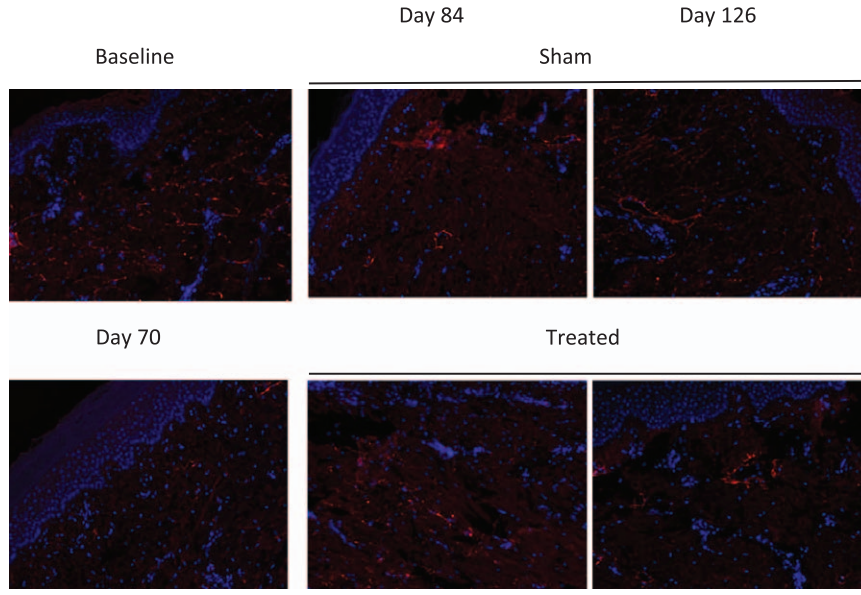

Figure 6. Immunofluorescence staining for elastin was performed in baseline skin and scars at days 70, 84, and 126. Normal elastic fibers are visualized perpendicular to the epidermis in the papillary dermis. Elastic fibers were present in scars at days 70, 84, and 126.

which have been shown to modify essential biological processes during scar formation, including ischemic conditions induced by modulated circulation, collagen turnover and subtypes, cellularity, and global gene expression (manuscript in preparation). Hence, it can be safely hypothesized that the apparent discrepancy between transcript and protein levels of elastin at days 84 and 126 may be due to either posttranscriptional or posttranslational modification of tropoelastin monomers in the pressure-treated scars compared with the shams. This requires further investigation in future work.

Differences in the VSS were more pronounced at day 84 (during treatment) as compared with day 126 (6 weeks posttreatment). The treatment period of 2 weeks was chosen for the study as a preliminary time course to see if improvements in scars could be seen in this short interval. In clinical practice, it is recommended that pressure treatment garments be worn 23 hr/day for 12 months at a pressure of 20–30 mm Hg.[7] The results of the present study, suggestive of the effect a short period of pressure treatment may have on elastin quantity, may indicate that if pressure therapy is continued for an increased period of time, there might be even larger changes to elastin content. The change to elastin content could lead to more pliable scars and, as such, better healing outcomes.

An inherent weakness of the present study is a small biological sample size. Because of the complexities of working with this model and resource limitations, only two animals were used each with two wounds on either flank. Therefore, no statistical analysis could be run to compare pressure-treated vs sham-untreated scars because each treatment arm only contained two wounds after pressure treatment initiation. Although this is the case, through the use of multiple biochemical and histological assays, we have shown reproducible and consistent changes to elastin quantity and morphology. Future work will be aimed at validating findings with additional wounds in additional animals.

CONCLUSIONS

Pressure treatment results in higher protein level expression of elastin compared with sham-treated scars. The increase in elastin amounts correlates with increased pliability in treated scars, evaluated by the VSS. These findings further characterize the ECM's response to the application of pressure as a treatment. These findings may contribute to the advancement and refinement of rehabilitation practices.

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