Effects of Autologous Fat and ASCs on Swine Hypertrophic Burn Scars: A Multimodal Quantitative Analysis

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Background: Hypertrophic scar formation is unpredictable and poorly understood, afflicting both the pediatric and adult populations. Treatment methods with conservative and invasive approaches have low rates of compliance and high rates of morbidity. The purpose of this study was to test a reproducible scar model and investigate a new technique of scar modification through the use of adipose-derived progenitor stromal cells (ASCs).

Methods: Twenty thermal deep-partial thickness contact burns were created on the dorsum of three 8-week-old domestic swine and allowed to mature for 10 weeks. Scars were then injected with 2 cc saline, expanded autologous ASCs, or 2 cc fresh lipoaspirate and sampled at 2 week intervals up to 10 weeks postinjection. Volumetric analysis with a 3-D scanner, mechanical elasticity testing through negative pressure transduction, and standardized photography evaluation with Image J was performed. RNA sequencing was performed on scar tissue samples, cultured cells, and fresh lipoaspirate to determine relevant gene transcription regulation. Immunohistochemistry was used to verify expression level changes within the scars.

Results: Volumetric analysis demonstrates a reduction in average scar thickness at 6 weeks when injected with ASCs (-1.6 cc³) and autologous fat (-1.95 cc³) relative to controls (-0.121 cc³; P < 0.05). A decrease in overall tissue compliance is observed with fat or ASC injection when compared with unburned skin at 8 weeks (35.99/37.94 versus 49.36 mm Hg × mm; P < 0.01). RNA sequencing demonstrates altered regulation of fibroblast gene expression and a decreased inflammatory profile when scars are injected with autologous fat/ASCs over controls.

Conclusion: Early results suggest that autologous fat and/or ASCs may improve healing of hypertrophic scarring by altering the cellular and structural components during wound remodeling up to 20 weeks after injury. This may have beneficial applications in early treatment of large or cosmetically sensitive immature burn scars.

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INTRODUCTION

Survival from thermally induced injuries has significantly improved in recent history, yet advances in aesthetic and functional outcomes inflicted by the resulting hypertrophic burn scar have not kept pace. Current modalities for the treatment of hypertrophic scars are not uniformly predictable, often relying on patient compliance and may potentially increase morbidity. The traditional surgical approach aimed at prevention of hypertrophic scarring is early excision and skin grafting of deep thermal injuries.
expected to require longer than 2 weeks to heal via secondary intention. Once a hypertrophic scar has developed, conservative treatment options include the use of pressure garments, massage, and silicone-based therapy, all of which are theorized to alter collagen deposition by influencing vascularity and oxygen tension.1-3 Invasive modalities consist of surgical excision and primary closure, adjacent tissue rearrangement, radiation, fractionated laser therapy, and intralesional injections of various medications.4-8

Recognition of the regenerative capabilities of adipose-derived stem cells (ASCs) in fat has lead to significant interest in their potential clinical use to modulate healing, given their sheer abundance in the donor sites, ease in harvest, and the density of pluripotent cells in fat compared with bone marrow.9 Clinically, improvement in skin quality was noted after lipofilling and recognizing its proangiogenic potential; Rigotti et al.10 and Serra-Renom et al.11 first demonstrated its effectiveness in the setting of radiodermatitis. The indications for lipofilling have since expanded to include scar contractures, acute burns, and healed burn scars.12,13

The effects of injected fat and ASCs on scar properties have been characterized using scar scales,13 histology,14 biomechanical tools,15 immunohistochemistry,14 and molecular analysis.15 Improvements in coloration, volume, pliability, and collagen arrangement have been demonstrated with both treatment modalities.13,14 On a molecular level, upregulation of vasculogenic markers and downregulation of fibrotic markers have also been documented using both techniques.16,17 However, comparative studies for fat and ACS demonstrating quantitative short- and long-term effects have not been reported and the superiority of 1 treatment modality has not been determined. Consequently, we developed a domestic porcine hypertrophic burn scar model18 to investigate the short-term effects of saline (injection trauma), injected fat, and ASCs on scar. Specifically, we sought to quantify the changes in the biomechanical properties, erythema and scar volume of hypertrophic burn scar following treatment and assess the histologic and protein expression changes following injection.

**MATERIALS AND METHODS**

**Animal Study**

The research was approved by the Institutional Animal Care and Use Committee at Cincinnati Children’s Hospital Medical Center (Protocol #1d12106). Three 8-week-old domestic swine (Sus scrofa domesticus) were purchased at around 35 kg weight and individually housed in the Animal Research Core at CCHMC. Cincinnati Children’s maintains a current Animal Welfare Assurance with the U.S. Department of Health and Human Services, Office of Laboratory Animal Welfare.

**Porcine Surgical Technique**

Using our established model,18 thermally induced, rectangular, deep-partial thickness contact burns were then created bilaterally approximately 5 cm from the dorsal midline. Each animal received 20 thermal wounds, 10 per side (Fig. 1). All eschars were allowed to auto-debride, and no intervention was employed to address chronic granulation tissue.

**Lipoaspirate Technique and Expansion of ASCs**

Ten weeks following the initial thermal injury, 60 (n = 60) epithelialized burn scars were divided into

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**Fig. 1.** Deep-partial thickness contact burns created an eschar that persisted for 6 weeks with no intervention before resulting in raised, hyperemic scar tissue as seen by week 10.
4 treatment groups. Eighteen scars (n = 18), 6 on each animal, were intradermally injected with: (1) 2 mL of normal saline; (2) 2 mL of resuspended ASCs or (3) 2 mL of autologous fat. Six (n = 6, 2 on each animal) scars were assigned as positive controls with no injection to maximize the number of treatment sites (Fig. 2). Scars were treated in a randomized controlled fashion along the dorsum of each animal to account for varying hypertrophic scar formation as evident in our pilot study.18

To obtain autologous fat and ASCs, liposuction was performed on each animal's ventral neck, yielding approximately 50–100 mL of lipoaspirate per animal. A superwet liposuction technique was utilized for fat harvest. The lipoaspirate fluid was separated from the adipose component. The adipose component of the aspirate was washed repeatedly with phosphate-buffered saline until the infranatant was free from blood. It was subsequently digested with collagenase I [0.1% in 1% bovine serum albumin in phosphate-buffered saline] for 30 minutes at 37°C with gentle shaking. Processed lipoaspirate (PLA) cells were pelleted by centrifugation and were resuspended in media consisting of Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) with 10% fetal bovine serum and 100X antibiotic/antimycotic, and then filtered through a sterile 100 μm nylon filter to remove debris before plating.

PLA cells were plated at a concentration of approximately 100,000 nucleated cells/cm². All expanded cells were third passage cells. Lipoaspirate used in the immediate autologous fat injection treatment group was harvested using a closed system technique with the supernatant allowed to separate by gravity facilitating removal. Syringes will filled to 2 cc of volume without the supernatant. All treatment groups had injections of material by a Coleman micro-droplet technique. PLA cells were resuspended in 2 cc of normal saline before injection so that all treatment groups had 2 cc of total volume added to each scar.

RNA Sequencing

RNA sequencing was performed on all treatment groups and compared with normal skin. Additionally, 2 × 10⁶ ASCs and 2 mL of fresh autologous adipose tissue harvested from 1 animal were sequenced. Four millimeter full-thickness punch biopsies were performed on all scars at weeks 1, 2, 6, and 10 postinjection. Samples frozen in RNase at -80° F had the RNA isolated and evaluated with the use of an RNeasy Mini-kit (Qiagen, Valencia, Calif.). RNA transcriptome gene analysis was performed and creation of a cDNA library to be compared with known genes and proteins involved in the inflammatory and wound healing cascade through the National Center for Biotechnology Information’s Entrez Gene Database (http://www.ncbi.nlm.nih.gov/gene).

Immunohistochemistry

A portion of the tissue biopsies was embedded in paraffin with immunohistochemistry performed on samples by the Cincinnati Children’s Hospital Medical Center Pathology Research Core. Myofibroblasts were stained using the antibody alpha smooth muscle actin (α-SMA), the Chromogen Substrate DAB, and the counterstain Mayer’s Hematoxylin. No antigen retrieval was required. Under
light microscope 20× magnification, myofibroblast cell nuclei were manually counted on 5 separate fields within each slide and means recorded.

**Statistical Analysis**

The treatments were evaluated at weeks 4, 6, and 10 using linear mixed models procedures with time (week) as the repeated parameter with significance of \( P < 0.05 \) (SPSS, SPSS, Inc., Chicago, Ill.) with site, side, and pig number as covariates. Pairwise comparisons were made using least significant differences, and results were not corrected for multiple comparisons. Week 2 data were analyzed using general linear mixed models to determine initial differences since measurements were not made at baseline (week 0, the time at injection).

All data were expressed as averages and SD within each group. Student \( t \) tests were used for all other factor comparison with statistical significance accepted with a \( P \) value of < 0.05. Analysis was performed using Systat 13 (Systat Software, Inc., Chicago, Ill.).

**RESULTS**

There were significant differences for elasticity, elastic deformation, laxity, and ultimate deformation based on the anatomic site of the untreated hypertrophic scar (Fig. 3; Table 2). Caudal hypertrophic scars demonstrated less elasticity and elastic deformation compared with scars located more cranially. Table 2 reports the statistical results for each biomechanical characteristic for each week and treatments are coded as follows: 1 = burned untreated, 2 = saline, 3 = ACS, 4 = Fat, and 5 = normal skin (unburned).

**Elasticity and Elastic Deformation**

At week 10, unburned skin (control) and treated burn scar demonstrated different elasticity from each other and all other treatments as indicated by * \( (F = 99.0; \)
The saline-treated scars were more elastic than those treated with fat, as noted by the Elastic deformation of weeks 2, 4, 6, and 10 (F = 40.3; P < 0.001). The elasticity of each treatment group was significantly decreased. The saline and ASC treatments demonstrated the greatest reduction in scar volume compared with the control (F = 2.8; P = 0.04; Fig. 5). Similar trends were found at weeks 4 and 6 but did not reach statistical significance.

**Effect of Treatment on Erythema**

The surgery itself had an effect on burn scar erythema (excess erythema) as determined by comparing redness in high resolution images of scars before surgery and 2 weeks later. Excess erythema of untreated burn scars did not differ over course of observation. However, excess erythema was significantly higher for the saline and ASC injected groups (P < 0.05) and was higher for the fat treatment group (P = 0.07) 2 weeks after surgery.

At week 10, excess erythema was significantly higher for the ASC and fat injected groups versus untreated burn scar (F = 2.8; P = 0.04; Fig. 5). Similar trends were found at weeks 4 and 6 but did not reach statistical significance.

**Effects of Treatment on Topographical Volume**

The greatest reduction in scar volume occurred from weeks 2 through 6 in scars that were injected with autologous fat and ASCs when compared with the control (-1.95 cm³ and -1.60 cm³ versus -0.12 cm³, respectively).
Due to the rapid growth of the pigs, the scars could only be measured up to 6 weeks postinjection, prohibiting image overlay and software analysis.

**RNA Sequencing**

One hundred forty-eight genes within our bioinformatics database, known to be involved with the wound healing cascade, fibroblast differentiation, and cellular signaling were analyzed. There were significant differences in gene expression levels in 99 of these known genes across treatment groups compared with untreated scar ($P < 0.05$; Fig. 6). The ASCs and autologous fat samples were also compared with the treatment groups. It is evident that the scars treated with autologous fat or ASC injection developed a heat signature similar to the isolated adipose or ASCs when comparing these 148 genes (Fig. 8).

One particular protein-encoding gene with significant differences in gene expression levels is the BARX2 gene. Known to be involved with myofibroblast migration and differentiation, the gene was upregulated in the untreated scar and saline injected scars up to 10 weeks postinjection (Fig. 9). There was more than a 2-fold decrease in expression levels of this gene when scars were injected with ASCs ($0.69 \pm 0.22$) or autologous fat ($0.79 \pm 0.06$) compared with untreated scar ($1.76 \pm 0.48$) over 10 weeks ($P < 0.03$; Fig. 9). A second notable gene with upregulation in the autologous fat group and downregulation in the untreated group was the SPRY2 gene, involved in fibroblast growth ($P < 0.05$; Fig. 7).
factor receptor ligand binding and activation. At 6 weeks, there was a 1.25 ± 0.45 normalized increase in expression at 6 weeks when fat injected and 1.05 ± 0.18 normalized decrease in expression in the nontreated scars at 6 weeks (P < 0.05).

**Immunohistochemistry**

To determine if downregulation of BARX2 after fat or ASC injection results in reduced myofibroblast presence, samples were stained with alpha-smooth muscle actin antibody (α-SMA antibody). Unburned skin showed a focus of SMA-positive endothelial cells activity at the dermal/epidermal junction (Fig. 10A). Vertical vessels were present, increased vascularity deep into the dermis, and a flattening of the papillary junction is evident at 6 weeks and 10 weeks in an untreated scar (Fig. 10B, C). Scars treated with autologous fat appear to have diminished vessel presence (Fig. 10D); however, myofibroblast cell counts did not differ significantly in the small sample size.

**DISCUSSION**

Hypertrophic scars are described as overly projected, thickened, erythematous, indurated, fibrotic skin confined within the perimeter of the original tissue insult, the defining pathognomonic feature. Coupled with the appearance, common clinical symptoms may include pruritus, the sensation of tightness, decreased range of motion across joints and pain, ultimately causing significant morbidity.5,22 Both conservative and surgical treatment strategies have demonstrated variable success in improving these symptoms; however, the regenerative capacity of fat and its associated stem cells have shown promise as an alternative therapy. Multiple studies have documented the biomechanical improvements, histologic changes, and genetic expression of hypertrophic scar treated with fat and ASCs.23–26 Domestic swine were chosen due to the amount of tissue for analysis, similar skin characteristics to humans...
with regard to epidermal/dermal thickness, wound healing rates, and the propensity to develop hypertrophic scars following deep thermal injuries. The goal of this study was to quantify the changes in the biomechanical properties, color and volume following saline (injection trauma), fat and ASCs injection of hypertrophic burn scar using an established hypertrophic burn scar model in domestic swine. We hypothesized that the hypertrophic scars injected with fat and ASCs would improve over a 12-week period compared with untreated and saline-injected scar.

Unexpectedly, the biomechanical properties of hypertrophic scars injected with fat and ASCs did not improve over the 10 weeks following treatment. In addition, erythema also increased across all treatment groups but was statistically significant for the ASC and fat-injected scars compared with untreated hypertrophic scar. The scars injected with autologous fat or ASC, however, did show a statistically significant reduction in volume compared with the saline and control groups. Unfortunately, the rapid growth of the swine doubling of their weight over 20 weeks led to a significant elongation of the torso. Thus, our analysis of the scar volumes using image overlay after 6 weeks was not technically feasible.

The senior authors have observed clinically different results after human scars are injected with fat, whereby the scars become softer and more pliable after fat injection. We surmise that differences observed in this study are a result of increased tissue pressure resulting from injecting cells and fluid into a confined space. Scars are still in the collagen remodeling phase at 10 weeks postinjection and may have softened with further observation. To address the rapid growth of this animal model over time, we will use the Red Duroc or mini pig for further studies, which was previously cost-prohibitive in this pilot study. This will allow for longer observation times with less affect to scars by animal growth. The Red Duroc pig is also a more reliable animal model for consistent hypertrophic scar formation.

Adipose-derived stem cells have regenerative capacity, are abundant, and are easy to harvest and isolate. Our results with RNA sequencing demonstrate that the addition of ASC or fresh lipoaspirate does have an effect on the wound healing of scars in this pig model. Interestingly, the heat signatures from samples injected with autologous fat or resuspended ASCs becomes more similar to autologous fat or ASCs alone when looking at genes involved with fibroblastic regulation. The results also suggest that even up to 10 weeks postinjection, there is a signature of gene regulation that implies the transplanted cells or downstream effects persist. This is evident again by the similar heat maps generated by the harvested fat and isolated ASCs to the autologously injected scars.

RNA sequencing measures gene expression levels or transcriptomes in a particular sample with variance leading to possible altered phenotypic expression. BARX2 is a homeobox transcription factor that is involved in myofibroblasts, cell adhesion, remodeling of actin myoskeleton, differentiation of smooth and skeletal muscle and is involved in the Wnt signaling pathway. Within hypertrophic scars, there is evidence of decreased myofibroblast apoptosis, which may contribute to the functional pathology as a result of excess contractile element production in the wound. Myofibroblasts have also been shown to produce increased deposition of extracellular matrices, and collagen I and III (Col1, Col3). BARX2 expression levels in scars decreased 2-fold 10 weeks following intradermal injection of ASCs or lipoaspirate. Confirmation with polymerase chain reaction of the 2-fold increase in expression levels of BARX2 is part of our ongoing studies. Although α-SMA staining appeared to demonstrate less myofibroblast penetration and localization around diminished vessel presence on light microscopy, significance could not be achieved likely due to low sample size. If the down-regulation of BARX2 leads to a decrease in myofibroblast differentiation and migration, this would be expected to possibly alter overall tissue compliance and wound contracture with increased apoptosis of the proliferated fibroblastic cells in the hypertrophic scar.

Moreover, a significant study by Plikus et al. demonstrates evidence that myofibroblasts show plasticity and
can transform to adipocytes through bone morphogenetic protein and ZFP423 pathways or when hair follicles and ASCs are present. Additionally, they showed that human keloid fibroblasts can be reprogrammed in to adipocytes when cultured with BMP2 or BMP4. Substantiating the effects of ASC treatment or influence on these embryonic pathways and mechanisms may translate to improved clinical outcomes and help determine relative time to treat in the wound healing process.

Further studies may yield relevant data on multiple other genes with significantly altered expression levels following ASC or autologous fat injection. Furthermore, SPRY-2 is known to be a down-regulator of fibroblast growth factor receptors, notably FGF-2, involved in myogenesis pathways and also cellular apoptosis.\(^ {43,44}\) PLD1 encodes for phospholipases with cellular functions relating to inflammation, cell growth, signaling, and cellular death.\(^ {45}\) A recent study The heatmap reveals a down regulatory effect when fat or ASCs are injected and increased expression on transcriptome levels in untreated or saline-treated scars. LSG1 is involved in cell viability. This study revealed 99 genes with significantly altered expression levels from lipoaspirate/ASC injection that may lead to changes in regulatory pathway involved in wound healing dynamics after thermal injury. Other areas of limitations to this study are many. The scars were still remodeling through the end of this study, and a longer sampling period would likely have a difference on overall tissue characteristics. As a pilot study, ongoing projects are aimed at longer observation times. It was technically challenging to place fat injections precisely into the dermis without some injected material traveling deep to dermis or out a puncture wound, so some test scars received varying amounts of treatment. Although 3 different animals were used, systemic or paracrine effects could not be accounted for as all treatment groups were performed on each animal. Further evaluation of fibrotic scar transformation will additionally focus on targeted pathways involving TGF-β/SMAD, fibroblasts, and keratinocytes. Lastly, the small amount of tissue gathered in the cone for negative pressure transduction measurements could have only represented the very surface of the scar and not overall compliance throughout. These concerns are all being addressed in current ongoing studies with the red Duroc pig model.

**CONCLUSIONS**

We created a reproducible, testable swine scar model in an effort to accelerate translational treatment approaches to achieve a softer, less erythematous, and flattened scar with less invasive techniques. ASC and fat injections appear to increase the rate at which erythema resolves and reduce the overall thickness of the scar in early wound healing. However, overall compliance of the tissue is decreased during wound remodeling. RNA sequencing shows persistent influence of injected fat cells, an increase in regulation of fibroblast gene expression, influence on inflammation, and demonstrates a gene expression profile in treatment groups similar to the cell of origin’s genes (ASCs).

**REFERENCES**


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