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Histology of the thick scar on the female, red Duroc pig: Final similarities to human hypertrophic scar

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Abstract

The etiology and treatment of hypertrophic scar remain puzzles even after decades of research. A significant reason is the lack of an accepted animal model of the process. The female, red Duroc pig model was described long ago. Since the skin of the pig is similar to that of humans, we are attempting to validate this model and found it to be encouraging. In this project we quantified myofibroblasts, mast cells and collagen nodules in the thick scar of the Duroc pig and compared these to the values for human hypertrophic scar. We found the results to be quite similar and so further validated the model. In addition, we observed that soon after wounding an inflammatory cell layer forms. The thickness of the inflammatory layer approaches the thickness of the skin removed as if the remaining dermis “knows” how much dermis is gone. In deep wounds this inflammatory layer thickens and this thickness is predictive of the thickness of the ultimate scar.

Keywords

Collagen nodules; Mast cells; Hypertrophic scar; Porcine; Swine; Pig; Duroc; Myofibroblasts

1. Introduction

Hypertrophic scar is an undesirable result of deep partial thickness wounds and burns and may have devastating physical, psychosocial and vocational consequences [1-10]. The process has been studied for decades, but the pathophysiology of hypertrophic scarring is still unknown [11-20].

One major reason that the etiology of human hypertrophic scar is unknown is the absence of a useful animal model [19,21-23]. Various animal models have been described [19,22-30] but none of these have risen to being considered a “gold standard”.

Nearly 30 years ago, Silverstein, Goodwin, Raulston, and Pruitt [31] reported that deep donor sites in 12 of 12 female red Duroc pigs healed with “hypertrophic” scarring. Since there is considerable evidence that porcine skin is similar to human skin [32-44] we are attempting to validate this model. To date, we found the model quite encouraging in various respects [45-48], but mast cells, collagen nodules, and myofibroblasts in the Duroc scar have yet to be compared to human hypertrophic scar.

Various authors have suggested that mast cells may be involved in fibrosis [49-54] and that human hypertrophic scar contains more mast cells than uninjured skin and normotrophic scar [55-58]. If mast cells can be demonstrated in the Duroc wounds, the model could also be used to define timing and roles of mast cell-mediated immune responses.

Human hypertrophic scar classically is thought to be histologically defined by collagen nodules [12,59-69]. Since these nodules are not found in normotrophic scar they are thought to be characteristic of hypertrophic scars. Identification of this histological organization in deep wounds would further validate this Duroc model of scar formation.

Finally, Gabbiani et al. described modified fibroblasts in 1971 [70]. Majno et al. later termed them myofibroblasts [71]. Most investigators have determined that myofibroblast number is increased in hypertrophic scars. Nedelec et al. reported a greater number of myofibroblasts in hypertrophic scar than in normotrophic scar [72]. Santucci et al. [69] determined that early scars have large numbers of myofibroblasts and late hypertrophic scars have fewer. Ehrlich et al. [68] localized the myofibroblasts in hypertrophic scars to the collagen nodules, however, Santucci et al. and Nedelec et al. could not confirm these findings. Given these contradictory observations about localization and timing of myofibroblast infiltration into human hypertrophic scar, the Duroc model could provide a way to quantify this cellular response.

For this project, we hypothesized that the occurrence of mast cells, collagen nodules and myofibroblasts in the thick scar of the female, red Duroc pig is similar to human hypertrophic scar.

2. Materials and methods

2.1. Duroc skin and scar samples

All animal studies were performed as previously described [45-48]. In accord with Animal Care Committee permission female Duroc pigs (Toth Farm, Bellingham, WA), 6 weeks old, approximately 16–18 kg, were purchased and housed in the Harborview Medical Center Research and Training Vivarium with 12-h light/dark cycles. The animals were observed for 1 week and fed lab porcine grower diet and water ad lib. At 7 weeks, anesthesia was established with Telazol® reconstituted with 5 ml xylazine (100 mg/ml), dosage 1 ml/18 kg body weight (Phoenix Pharmaceuticals Inc., St. Joseph, MO). The hair on the back was clipped and skin cleansed with Betadine® solution and rinsed with 70% alcohol. Tangential wounds were created with a standard electric Padgett dermatome (Padgett Instruments, Kansas City, MO). Wound size was approximately 7 cm × 7 cm and eight wounds were created on the back of each pig. The wounds were allowed to granulate and re-epithelialize without application of topical agents or dressings and were photographed at the times of biopsy.

From our preliminary studies we determined that the settings on the Padgett dermatomes are very precise. However, this does not translate into precise wound depths for multiple reason none of which are truly controllable [73]. Moreover, the thickest setting is 0.040 in. so to achieve greater depths; multiple excisions must be done, which introduces even more error. Therefore, we do not refer to wound depth but rather to total dermatome setting. The total dermatome settings used in this study were 0.020, 0.040 and 0.060 in. creating partial thickness

wounds, shallow, intermediate and deep. The three total dermatome settings were rotated on the animals to avoid repeatedly placing one wound depth in the same anatomic location.

Under general anesthesia as described above, 5×10 mm surgical biopsies were collected on 7, 14, 21 and 28 days and 2, 3 and 5 months post-wounding. Each wound was biopsied only at one time point. Biopsies were taken from the center of each wound and uninjured skin at each time point. Biopsies were immediately fixed in neutral buffered formalin for 24 h.

2.2. Human skin and scar samples

Human skin and scar samples were acquired at the time of surgical burn reconstruction at Harborview Medical Center. All procedures were carried out in accordance of the University of Washington's Human Subjects Division. Patient demographics are included in Tables 1 and 2. Human sampling times are different from Duroc times since it is not possible to obtain human samples at specified times.

2.3. Histology

Biopsies from eight wounds on each of eight Duroc pigs were examined with H&E staining. Number of samples at each time point are listed in Table 3.

Wounds were grouped according to total dermatome settings including shallow (0.020 in.), intermediate (0.040 in.) and deep wounds (0.060 in.) at 1, 2, 3 and 4 weeks, and 2, 3 and 5 months. The thickness of the dermis left in the wound bed at the time of wounding, the inflammatory response, the new tissue at the time of epithelialization (1 week for shallow wounds and 4 weeks for deep wounds), and of the late healed skin/scar was measured with an eyepiece micrometer. We used the Wilcoxon Mann–Whitney test to determine statistical differences.

2.4. Processing for mast cell counts

Samples of uninjured human tissue (five patients) and samples of human hypertrophic scars (five patients) taken 24–30 months after injury were analyzed (Table 1). These were compared to samples of Duroc scar (five pigs) from deep wounds and samples of uninjured Duroc tissue (five pigs) taken 5 months from injury. Sections containing all the skin layers were embedded in paraffin, cut into 5 μ m sections and mounted onto slides. Histological staining was performed with Giemsa Stain Solution (Crescent Chemical Company Inc., Islandia, NY) [74–78]. Slides were baked at 60 °C for 20 min to deparaffinize and then hydrated to distilled water. Samples were incubated at room temperature for 8 min in non-filtered diluted 2% Giemsa solution, differentiated in 90% ethyl alcohol (three washes), dehydrated through absolute ethyl alcohol (three washes), cleared in xylene and cover-slipped.

An eyepiece with a standard rectangular reticule was used with a Nikon Labophot-2 standard light microscope. For mast cells quantification 20 consecutive non-overlapping rectangular fields of the upper papillary dermis were examined at 40 $\times$. Mast cells were identified by the characteristic morphology of the granules (Fig. 1); only intact mast cells with distinct blue staining were counted. Measurement was repeated five times for each field with the researcher blind to the age of the scar. The average number of mast cells was calculated for each sample and the Wilcoxon Mann–Whitney test used to determine statistical significance.

2.5. Processing for collagen nodules

Biopsies of human hypertrophic scar (25 patients, 27 scars, 9 from 3.9–12 months postinjury, 18 from 24–67 months postinjury, Table 2) were compared to deep Duroc scars (6 animals, 13 scars, 6 from less than 5 months postinjury, 7 from 5 months postinjury). Tissue was processed as for mast cell quantification but was stained with H&E. The presence or absence of nodules

was confirmed by histological evaluation with light microscopy (Fig. 2). Fisher's exact test was used to determine the statistical difference between groups.

2.6. Processing for myofibroblasts

Three shallow and three deep wound samples at 1–4 weeks, and 3 and 5 months were analyzed. The immunohistochemical study was performed on 4 μ m paraffin sections. The sections were microwaved in 0.1 M pH6.0 citrate buffer for 5 min, and allowed to cool to room temperature. Anti- α -smooth muscle actin monoclonal antibody (obtained from Giulio Gabbiani, Geneva, Switzerland) was diluted with PBS at 1:300 and incubated with the sections at room temperature for 60 min. Endogenous peroxidase was inactivated by incubation with 0.3% H₂O₂ in methanol for 10 min at room temperature. The secondary antibody was biotinylated goat anti-mouse antibody (Vector Laboratories, Burlingame, CA) at 1:250 dilutions for 30 min, followed by Streptavidin AH–biotin complex (SABC Kit, Zymed Laboratories, San Francisco, CA) at 1:250 dilutions for 30 min. All antibodies were diluted in the serum/TBS blocking solution and all incubations occurred at room temperature. Sections were visualized for immunoreactivity using (0.12%) 3,3'-diaminobenzidine (Sigma, St. Louis, MO) as a chromogen for 20 min and counterstained in hematoxylin (Fig. 3). An eyepiece with a standard rectangular reticule was used with a Nikon Labophot-2 light microscope. To count myofibroblasts, the designated rectangular area was started at one edge of the tissue in the upper papillary layer. Eight consecutive non-overlapping rectangular fields were examined at 20 $\times$. The Wilcoxon Mann–Whitney test was used to test for statistical differences.

3. Results

3.1. Wound healing and histology

As in previous publications [45–48], shallow wounds epithelialized at about 1 week after-wounding whereas deeper wounds required approximately 4 weeks. For shallow wounds, the healed wound consisted of normal epidermis, well-organized collagen fibers and hair follicles, and minor contraction. The healed wound was difficult to discern from uninjured skin. Deep wounds developed thick scar, disorganized collagen, no skin appendages and major contraction.

3.2. Wound inflammatory layer

By “inflammatory layer” we refer to a layer of maturing granulation tissue or cellular fibrosis (early scar) with significant inflammation. This discrete layer was identified by intense hematoxylin staining of the inflammatory cells. At 1 week after-wounding, the excised tissue was replaced by an inflammatory layer of almost equal thickness (Fig. 4). In shallow wounds the inflammatory layer was gone at 4 weeks. In deep wounds, at 4 weeks when the wounds were finally epithelialized, the inflammatory layer was still very thick (Fig. 5). Also in these deep wounds, during the subsequent weeks the inflammatory tissue layer was replaced with a thicker layer of disorganized fibrous tissue.

3.3. Wound thickness

The thickness of the wounds at the various time points is shown in Fig. 6a–c. For shallow and intermediate wounds (Fig. 6a and b) the thickness of the wounds resembles uninjured skin. For the deep wounds, the thickness of the wound was significantly greater than that of uninjured skin at 8, 12 and 21 weeks ($p < 0.05$). The thickness of the inflammatory layer increases with wound depth and the difference is significant for the deep wounds at 2, 3 and 4 weeks ($p < 0.05$).

3.4. Mast cells

We counted mast cells in four groups (Duroc scar, uninjured Duroc skin, human hypertrophic scar and uninjured human skin). For each wound type, the mean number of mast cells was calculated. The Kruskal–Wallis test was used to determine statistical differences.

Duroc scars at 5 months contained more mast cells than uninjured Duroc skin ($p < .05$) and human hypertrophic scar contained more mast cells than uninjured human skin ($p < 0.01$) (Table 4).

3.5. Collagen nodules

We grouped the sample data into two categories according to age of the scar, <5 and 5 months for Duroc scar and 0–12 and 24–67 months for human hypertrophic scar and calculated the percent of samples in each group containing collagen nodules.

Human hypertrophic scars (33%) less than 12 months old contained nodules; 81% of human hypertrophic scar greater than 24 months old ($p < .05$). Whereas no Duroc scars less than 5 months old demonstrated nodules, 50% of Duroc scars at 5 months demonstrated distinct nodular structures ($p < .05$) (Table 5).

3.6. Myofibroblasts

The counts of α -SMA positive spindle shaped cells are shown in Table 6. In the deep wounds the counts were higher than in shallow wounds at 1, 2 and 3 weeks and then declined. There was no difference at 3 and 5 months.

4. Discussion

4.1. Histology, the inflammatory layer and thickness

The thickness of the deep wounds at 5 months is greater than the thickness of uninjured skin and the thickness of shallow wounds.

It is interesting in our Duroc model that the thickness of the inflammatory layer at 1 week after-wounding is essentially the same as the thickness of the tissue removed. It suggests a delicate monitoring system that communicates to the local cells how much tissue is missing and how much tissue is needed to mend the defect. At 1 week in shallow and deep wounds, the amount of skin removed was replaced with almost equal amount of inflammatory tissue (Fig. 4).

The events in shallow and deep wounds are very different. In shallow wounds, the inflammatory tissue layer rapidly disappears and the skin is essentially the same as uninjured skin. In deep wounds, however, the inflammatory tissue layer continues to increase in thickness and, in time, becomes a thick layer of disorganized scar. This suggests that if the inflammatory tissue layer could be affected perhaps the thick scar could also be minimized.

The concept that cellular communication between different segments of the skin regulates response to injury is intriguing. It has been hypothesized that epidermal–dermal interactions are involved in skin morphogenetic responses including repair [79–84]. Disruption of epidermal–mesenchymal communication due to a delay in epithelialization increases the frequency of developing fibrotic conditions in skin [85]. By using a tissue-engineered model of reconstructed human skin [86], Bellemare demonstrated that hypertrophic scar keratinocytes plays a role in the development of pathological fibrosis by influencing the proliferation of dermal cells and matrix accumulation, reflected in the balance of synthesis and degradation of collagen.

4.2. Mast cell counts

Mast cell proliferation and infiltration is common in various pathologic skin conditions including wounds, however, their interactions with other cell types such as fibroblasts, endothelial cells and neurons is still unclear. Their presence may prove to be a valuable indicator of fibroblast cell activation and extracellular fibrosis [49-54]. This along with their hematopoietic derivation and strategic location between vessels and neurons suggests a key role in the formation of scar. Human hypertrophic scar has been reported to exhibit as many as four times more mast cells than normal skin [55-58].

We found that Duroc scars at 5 months contained 2.4 times more mast cells than uninjured Duroc tissue ($p < 0.05$) and that human hypertrophic scar contained 4.2 times more mast cells than uninjured human tissue ($p < 0.01$). These results are similar to that reported in the literature for human hypertrophic scar. This further validates this model of scarring and may also validate the Duroc model for the study of mast cell role in responses to injury.

4.3. Collagen nodules

Formation of nodules with whorl-like patterns, hyalinized collagen, and spheroid delineation has been described as characteristic of human hypertrophic scar [12,59-69]. We noted that in the early papers nodules were thought to be present in “all” human hypertrophic scar whereas the recent paper by Santucci et al. [69] clarified that early hypertrophic scars do not always contain nodules.

We found that in both Duroc scar and human hypertrophic scar demonstrate increasing numbers of nodules with age confirming the literature findings and further validating the model. Since we have demonstrated nodule formation in these thick Duroc wounds, these histological findings may provide one way to measure effects of scar modulation by mechanical perturbations of scarring such as pressure garments.

4.4. Myofibroblasts

Santucci et al. [69] demonstrated that myofibroblasts are common in human hypertrophic scars less than 12 months old and then decline. Kamath et al. [87] also reported on the presence of myofibroblasts over time and found counts to be highest at approximately 2 months and declining thereafter. The time course in the thick Duroc scar was similar although greatly compressed compared to the Santucci et al. report but similar to the Kamath et al. report. These findings further validate the model and suggest that the model may be useful in studying the role of myofibroblasts in scarring.

5. Conclusions

The purpose of this study was to quantify the number of mast cells, collagen nodules and myofibroblasts in Duroc scar and human hypertrophic scar to further validate the female, red Duroc model of hypertrophic scarring. We found that increased numbers of myofibroblasts, mast cells and collagen nodules are present in Duroc scar similar to human hypertrophic scar.

We also found that the thickness of the inflammatory tissue layer correlates with the thickness of the ultimate scar.

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Fig. 1.
Intact mast cells stained with Giemsa in human (a) and Duroc (b) skin, 40×.

Fig. 2. Collagen nodules in human (a) and Duroc (b) scar, 4 $\times$. Human scar is 10 months since injury and Duroc scar 5 months.

Fig. 3.
Myofibroblasts in deep wound at 2 weeks, 20 $\times$.

Fig. 4.

Thickness of residual dermis, removed tissue, inflammatory layer, healed skin/scar, and uninjured skin in shallow, intermediate and deep Duroc wounds.

Fig. 5. Wound (0.060 in.) at 1 month, 4×. Note the thick, blue inflammatory layer, i.e. the layer of maturing granulation tissue or cellular fibrosis (early scar) with inflammation.

Fig. 6.

(a) Thickness of shallow wound compared to uninjured skin. There is very little difference. (b) Thickness of intermediate wound compared to uninjured skin. The difference is not significant.

Table 1

Patient demographics for mast cells comparison

Group	Age	Race	Sex	Body part
Hypertrophic scar	33	Asian	F	Face
Hypertrophic scar	9	Caucasian	F	Chest
Hypertrophic scar	15	Hispanic	F	Face
Hypertrophic scar	17	Multi-racial	M	Arm
Hypertrophic scar	15	Hispanic	M	Right leg
Uninjured skin	48	African American	Unknown	Sternum
Uninjured skin	59	Caucasian	F	Back
Uninjured skin	45	Caucasian	F	Abdomen
Uninjured skin	57	Unknown	M	Back
Uninjured skin	18	Caucasian	M	Arm

Table 2

Patient demographics for collagen nodule comparison

Group (months)	Age	Race	Sex	Body part	Age of scar (months)
0–12	27	Caucasian	M	Abdomen	3.9
0–12	11	Caucasian	F	Chest	4.9
0–12	60	African American	M	Dorsum hand	5.6
0–12	51	Caucasian	M	Chest	6.2
0–12	8	Caucasian	M	Face	6.9
0–12	19	Caucasian	M	Arm	8
0–12	5	Caucasian	M	Cheek	9.6
0–12	48	Caucasian	F	Face	10.1
0–12	6	African American	F	Neck	10.3
24–67	17	Multi-racial	M	Arm	24.1
24–67	15	Hispanic	F	Face	25.3
24–67	16	Hispanic	M	Leg	25.7
24–67	4	Caucasian	M	Sternum	26
24–67	4	Caucasian	M	Face	27.3
24–67	38	African American	M	Arm	27.5
24–67	7	Asian	F	Arm	27.8
24–67	9	Caucasian	F	Chest	27.9
24–67	Unknown	Unknown	F	Leg	28.8
24–67	33	Asian	F	Face	29.6
24–67	3	Caucasian	M	Cheek	29.8
24–67	9	Caucasian	M	Back	36.5
24–67	46	Caucasian	M	Shoulder	37.2
24–67	45	Caucasian	M	Leg	62.2
24–67	12	Hispanic	M	Chest	64.1
24–67	16	Caucasian	F	Arm	67

Table 3

Number of samples at each time point and each type of wound

	1 week	2 weeks	3 weeks	4 weeks	2 months	3 months	5 months
Uninjured	4	4	3	2	3	5	5
Shallow	4	4	4	3	3	5	7
Intermediate	2	2	2	2	1	3	4
Deep	4	2	2	4	3	2	6

Table 4

Mast cell counts

Tissue type	Age of scar (months)	N	Mean $\pm$ S.D.	<i>p</i> -Value
Duroc scar from deep wounds	5	5	55 $\pm$ 34	<0.05
Uninjured Duroc skin	5	5	20 $\pm$ 9	
Human hypertrophic scar	24–30	5	272 $\pm$ 109	<0.01
Uninjured human skin	24–30	5	53 $\pm$ 13	

Table 5

Presence of collagen nodules

Type of scar	Group (months)	Nodules		<i>p</i> -Value
		Absent	Present	
Duroc scar from deep wounds	<5	6	0	<0.05
	5	3	4	
Human hypertrophic scar	5–12	6	3	<0.05
	24–67	4	14	

Table 6Counts of α -SMA positive cells

Time	Shallow wounds (mean $\pm$ S.D.)	Deep wounds (mean $\pm$ S.D.)	<i>p</i> -Value
1 week	59 $\pm$ 92	259 $\pm$ 85	<0.05
2 weeks	6 $\pm$ 5	276 $\pm$ 92	<0.05
3 weeks	8 $\pm$ 3	179 $\pm$ 162	<0.05
3 months	2 $\pm$ 1	2 $\pm$ 2	
5 months	1 $\pm$ 1	1 $\pm$ 1	