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IMPLICATION OF TISSUE RESPONSE TO ISCHEMIA GRADIENT IN RABBIT
EAR ON CHRONIC WOUND HEALING

By

Abhijit Prakash Mahalingashetty
B.A., Transylvania University, 2003

A Thesis
Submitted to the Faculty of the
Graduate School of the University of Louisville
In Partial Fulfillment of the Requirements
For the Degree of

Master of Science

Department of Anatomical Sciences and Neurobiology
School of Medicine, University of Louisville
Louisville, Kentucky

May 2009

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A Thesis Approved on

April 13, 2009

By the following Thesis Committee:

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ABSTRACT

IMPLICATION OF TISSUE RESPONSE TO ISCHEMIA GRADIENT IN RABBIT EAR ON CHRONIC WOUND HEALING

Abhijit P. Mahalingashetty

April 13, 2009

Ventral ear skin of the rabbit is a commonly used model for wound/ulcer studies; however the gradient effects of vascular ligation on epidermal stability has not been reported. In this study ischemic effects were studied after ligation of the central feeding vessel in one ear, while the other ear served as control. Three or six days later 9 full-thickness skin circular punches (6 mm, numbered 1-9) were removed from both ears, equidistant from each other with a proximal-distal and medial-lateral orientation. Samples were prepared for light microscopy and immunohistochemistry examinations. Quantitative analysis showed that normal and ischemic tissue differ minimally on day 3 but significantly by day 6 with respect to proximal-distal locations as well as medial and lateral locations. These findings suggest that the widely used 4-hole rabbit ear model may be well suited for studying ischemic wound healing. However, randomization of treatment placements among the four corners of the ear is necessary to reduce sample biases.

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INTRODUCTION

The rabbit ear wound model has become a staple in wound healing experiments over the last decade. The use of this model, in its various forms, is broad ranging from normal wound healing to those concerning more detailed aspects of skin wounds such as hypergranulation and hypertrophic scars. Recently, the ear model has been used to study ischemia in wounds. The problem, though, with the current rabbit ear ischemic wound model is the lack of detailed understanding of the ear tissue reaction to ischemia. The rabbit ear is highly vascular and although ischemia is induced via minimal invasive surgery, no report has established to show how revascularization and in turn the healing is affected by an ischemic gradient.

To better treat patients, greater understanding of non-healing and chronic wounds is needed. Normal healing proceeds in a stepwise fashion requiring cascade sequences and interactions at the cellular level to promote and regulate healing in a time dependent fashion (1-3). Clinically, disruption of these sequences of events, especially relating to reepithelialization, neovascularization, and oxygen perfusion, results in non-healing wounds that cause significant increases in patient morbidity and healthcare costs (4-14). Particularly, the need of oxygen for substantive healing is a well documented fact; disruption of cellular oxygen results in impaired healing as seen in diabetic wounds and other chronic wounds and ulcers. Although hypoxia is unavoidable in wounded tissue primarily because of increased cellular metabolism and damage to local blood vessels (15-19), normal healing allows for oxygen reperfusion by establishing collateral

circulation via migratory and/or proliferating vessels in the newly formed granulation tissue (20). The inability of tissue to reestablish oxygen perfusion and hence remain hypoxic results in the development of chronic wounds; thus, all chronic wounds are inherently ischemic. Yet, there are currently few animal models that mimic ischemia seen in clinical chronic wounds (17). Considering the economic impact of chronic wounds (mainly diabetic leg ulcers, ischemic ulcers, pressures sores, and venous leg ulcers) is estimated to be around \$9 billion annually (4), it is imperative that animal models be established that closely mimic such wounds.

This issue is being addressed using various animals including the rabbit ear. In such models, the goal is to prolong ischemia in the wound bed. A rabbit ischemic ear model using circumferential incision (open surgery) was developed in 1990 by Ahn and Mustoe (21) and has been used for wound studies since. The rabbit ear ischemic wound model by minimally invasive technique was developed by Chien in 2007 (22) and is the model used for this investigation. Ligation of the vessels only serves to render the ventral skin ischemic until collateral circulation from the dorsal ear and the cranium are established (around two to three weeks) (23); although, it has been suggested that re-ligation of the vessels may stimulate chronically ischemic wounds (17). Yet, ligation of the vessels is only one aspect of the rabbit ear model. The more important aspect is the circumferential tissue disruption at the ear base.

In animal wound study, rodents are still used most often due to their low cost. However, rodent wound model has several disadvantages and is extremely difficult to render ischemic. The rabbit ear offers advantages over the nude mice and other murine models. While the costs of maintaining a large population of rabbits over long periods of

time is substantially more when compared to rats or mice (17), the healing process observed in the rabbit ear model does more closely resemble that seen in humans. Due to loose skin, small animals experience rapid epithelial closure with healing occurring mainly by contraction rather than reepithelialization (24, 25). This lack of wound tension is minimal in the rabbit ear and thus healing proceeds much more slowly than most small mammal models—an advantage in similarity to human skin and for use in prolonged wound studies (17, 24, 26, 27). In addition, the rabbit ear has an avascular cartilage (as long as the perichondrium is removed) wound bed, helping to avoid vascular growth or migration from this location (17). Due to the size of the ear, multiple wounds can be placed on each ear (17). Further, the ventral surface of the rabbit ear is relatively free of hair and thus adjacent hair follicles play a lesser role in reepithelialization than in other small mammals (nude mice being exceptions) (17, 24).

Animals such as the pig have skin more similar to humans than rabbits. Evidence documents the similarities of the pig and human skin. For example, unlike small animals including the rabbit, pig and human skin have thick epidermis, dermal collagen is relatively similar, and porcine dermis is closer in elasticity to human dermis than other animals (28). While all this remains true, wounds in pigs are extremely prone to infection and the economics of maintaining pigs for long term wound healing experiments is impractical for smaller, resource starved labs (17, 24, 26-28); the rabbit model in comparison becomes more practical.

While it is commonly understood that there exists a vascular gradient in the rabbit ear, no study of this gradient and how it is affected by ischemia has been reported to the author's knowledge. This study looks to evaluate the different regions on the rabbit ear in

order to better understand the relationship among the vascular supply, skin histology, and ischemia on the ventral surface of the New Zealand white rabbit ear.

Typically, experiments using the rabbit ear model use 4-hole standardized excisional full-thickness wounds. This standardized model places the four-holes at the four corners of the rabbit ear (Figure 1). No study, to the author's knowledge, has been conducted on elucidating the role of vascular supply and ischemic gradient in the rabbit ear at these four locations. In view of the growing popularity of the 4-hole rabbit ear model, a new 9-hole model (Figure 2) was developed to study the ear in greater detail. Nine holes were chosen to allow for maximizing coverage of the ventral ear. This arrangement allowed for gradient analysis using both micro- and macro-analysis of the ventral ear. Micro-analysis was conducted using the individual positions as standalones in comparisons among the positions on the ear, i.e. 1 vs. 2; 1 vs. 3, 1 vs. 9, etc. Such an analysis would allow us the better locate skin wounds in the future studies while also allowing for randomization of wound placements—something that is taken for granted in current studies. However, it is also useful to see the larger picture of the gradient within the rabbit ear. For this purpose, the macro-analysis was conducted, in which each row and column was grouped to evaluate the proximal-distal and medial-lateral aspects of the gradient within the ear. To further analyze the gradient, the positions were used for comparative purposes in ischemic and non-ischemic control ears. Due to the source of the vascular supply, *the null hypothesis predicted proximally positioned wounds would have thicker ventral skin compared to distally located wounds for both control and ischemic biopsies; and overall, ischemic biopsies will have thinner skin than paired controls.*

METHODS & MATERIALS

Animals

Six young adult (8-10 week old) male New Zealand White rabbits, weighing between 1.60 kg and 1.87 kg, were obtained from Myrtle's Rabbitry (Myrtle's Rabbitry Incorporated, TN.). All procedures used in this study were in compliance with the National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee of the University of Louisville. All animals were housed in University of Louisville Research Resource Center (RRC) for this study. The rabbits were caged individually with food and water supplied and subjected to 12:12 hour light-dark cycle.

Rendering rabbit ears ischemic

The left ear on each rabbit was rendered ischemic while the right ear served as a paired normal, non-ischemic control. The ischemic ear model was created using a minimally invasive technique as reported by Chien (22). Rabbits were anesthetized using 50mg/kg, IP of Sodium Pentobarbital. The ears were then shaved, sterilized, and draped. To create the ischemic ear, 3 small vertical incisions (1-2 cm) were made on the vascular pedicles of the rostral, central, and caudal vessels at the base of the ear. The rostral and central arteries were divided and ligated. However, the caudal artery and all three veins were preserved. A circumferential subcutaneous tunnel was made through the 3 incisions (Figure 2). All the subcutaneous tissues, muscles, nerves, and small vascular branches

were discontinued to the level of the cartilage (minimally invasive). The skin incisions were closed with 4-0 or 5-0 prolene. A Duragesic patch (Sadox, Inc. Broomfield, CO) was attached to the back skin for releasing Fentanyl (25ug/hour) to reduce possible pain.

Rabbit wound model

Three rabbits were euthanized each on day 3 and day 6. After the rabbits were euthanized, nine circular full-thickness wounds were biopsied on the ventral side of each ear with a 6-mm stainless steel punch. Samples' were numbered 1-9 and were taken equidistant from each other with a proximal-distal and medial-lateral orientation. The circular tissue disks were removed and immersed in 4% formaldehyde buffer overnight. The circular disks were bisected and one half was embedded in paraffin for light microscopic and immunohistochemistry examination. The paraffin blocks were cut in 6- μ m and the slides were used for H&E staining and immuno-staining. The other half of the tissue was submitted for transmission electron microscope processing, which was thinly sliced into 3 mm³ blocks for plastic embedding after postfixation in 2% osmium tetroxide and dehydration. The polymerized blocks were cut at 1-micron thickness, toluidine stained, cut at 800 μ thickness, collected on copper grids, and stained with lead citrate and urinal acetate, before they were examined on a CM10 Philips electron microscope (North American Philips, Co., Mahwah, NJ).

Tissue preparation for microscopy use

Tissue was dehydrated using increasing concentration of ethanol (30% to 100%) and 100% propylene oxide. After dehydration, tissue was infiltrated in a mixture of propylene oxide and araldite before embedded in Araldite 502 for light microscopy use.

This plastic was created from mixing DDSA (Dodecenyl Succinic Anhydride) (Electron Microscopy Sciences, Hatfield, PA) and Araldite 502 Resin (Electron Microscopy Sciences, Hatfield, PA).

Proliferating Cell Nuclear Antigen (PCNA) Immunohistochemistry

Rabbit ear tissue was sectioned at 40 microns using a Vibratom (Electron Microscopy Sciences, Hatfield, PA). Sections were washed with PBS and blocked using 5% normal goat serum in 0.1% Triton X-100/PBS. PCNA mouse IgG2a mAb (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), the primary antibody, was administered at 4 °C overnight at a dilution of 1:100. Secondary antibody, goat anti-mouse IgG biotin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was administered at a dilution of 1:200. Samples were washed with PBS, treated with ABC reagent (Vector Laboratories, Inc., Burlingame, CA) and allowed for DAB reaction (Vector Laboratories, Inc., Burlingame, CA) for roughly 7 minutes. Following a rise with dH₂O, tissue was embedded for microscopy observations and study (See Appendix for Protocol). The cross reactivity of the mouse IgG_{2a} and the dilution of both the primary and the secondary antibodies was tested prior to the experiment. Slides were counterstained with Toluidine blue and mounted with aqueous medium.

Semi-quantitative analysis of ventral ear thickness, epidermis, and keratinocyte nuclei

Initially, biopsy tissue was cut into four sections that were then sectioned and embedded onto slides. Two sections were selected randomly and were captured at 40x magnification using a Spot CCD camera (Diagnostic Instruments, Inc., Sterling Heights,

MI). Quantitative analysis of the ventral ear thickness was performed on captured images for both ischemic and control tissues using NIS Elements Basic Research imaging software (Nikon Instruments Inc. Americas, Melville, NY). The ventral skin thickness measurements were made by using a tracing tool and recorded as an area (squared microns). To ensure consistency, a width of 1.75 microns was used for all traced samples. The areas were then determined by measuring varying heights that were determined by measuring the distance from the cartilage (perichondrium) to edge of the epidermis. Each sample section provided three measurable areas, from left to right, equidistant from one another (3.50 microns apart). An average of 6 areas was measured for each biopsy. Data were collected for statistical analysis.

Epidermis thickness was measuring using a similar method. Epidermis layer was captured using the Spot CCD camera at 1000x magnification. Six area measurements were measured per section for a total of 12 area measurements for each biopsy. The width was held constant at 0.150 microns while the height was measured from the basal layer to the beginning of the stratum corneum. Measurements were taken left to right equidistant from one another (0.180 microns apart). Within each measured area, the number of keratinocyte nuclei was counted and data was collected for statistically analysis.

Statistical Analysis

All data are expressed as the mean plus or minus the standard error of the mean (mean $\pm$ SEM). Data was analyzed using either paired or independent T-tests and analysis of variance (ANOVA) followed by a post hoc multiple comparison tests using commercially available statistical software (SPSS Inc., Chicago, IL). A P value of ≤ 0.05

was considered significant for all cases. Graphs were made using GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA).

RESULTS

Skin Temperature

The skin temperature difference between the normal and ischemic ear ranged from 0.9 °C to 5.8 °C. The lowest average difference (1.32 ± 0.16 °C) occurred on the day of surgery (day 0). A large increase was seen one day postop (3.28 ± 0.35 °C) and this was statistically significant ($p < 0.005$) and this difference remained throughout the examined days (3.83 ± 0.32 °C, on average). Day 3 mean temperature was significantly different compared to day 0 mean ($p < 0.001$). Day 6 mean temperature was significantly different compared to day 0 mean ($p < 0.005$). (Figure 3)

General Histology

Ventral skin was seen to be more compact compared to dorsal skin of the ear and composed of thin skin with stratified squamous epithelium. Unlike the dorsal skin, the ventral skin showed few samples with adipose tissue in the hypodermis; instead, tissue was seen extending to the perichondrium and cartilage. In general, microscopic observations of normal and ischemic tissue were similar; observations seen in normal tissue were also seen in ischemic tissue. The epidermis contained stratified squamous epithelium and was highly cellular containing numerous keratinocytes in various maturity stages extending from the basal layer near the basement membrane to the shedding stratum corneum. Larger numbers of keratinocyte were observed at the basal layer, stratum basale, compared to apical portions of the epidermis. The number of keratinocyte

layers, those in the stratum spinosum, was observed to be varied depending on location and tissue sampled. Ventral skin was observed to have a dermal layer that was predominantly acellular; contained occasional hair follicles with accompanying arrector pili muscle; sebaceous glands located near hair follicles or extending from the dermis to the epidermis; and abundant neurovascular bundles. Nerve bundles ranged in size (observations not quantified) and contained both myelinated and unmyelinated fibers—myelinated fibers stained more intensely than unmyelinated fibers. Microvasculature contained capillaries extending through the dermis as well as one to three cell layer arterioles adjacent to numerous smaller venules. (Figure 4)

Immunohistochemistry

Proliferating cell nuclear antigen (PCNA) stains revealed greater number of stained nuclei in ischemic tissue compared to normal tissue; however, difference was not measured quantitatively and no statistical significance test was conducted. Positively stained nuclei were found in keratinocytes of the epidermis and some spindle-shaped nuclei in the dermis. Proliferating keratinocyte nuclei were positively stained in the epidermis (Figure 5) of both control and ischemic tissue. Most epidermal positive staining occurred at the basal layer. Within the dermal layer, many endothelial nuclei were found stained. The labeled nuclei lined microvessels; yet, not all nuclei surrounding the same vessels stained positive. More often, it was observed that nuclei on one side were stained while those on the opposite were not (Figure 6).

Ventral Skin Thickness Measurements

Day 3

Comparing normal skin tissue at different positions with one another revealed a pattern that was slightly different in ischemic skin. Positions 1, 2, 3, and 5 were significantly different compared to positions 4, 6, 7, 8, and 9 in normal skin. In ischemic skin, positions 1, 2, 3, 4, and 5 were significantly different compared to positions 6, 7, 8, and 9. Comparing day 3 normal and ischemic skin thickness, results differed significantly at positions 1, 3, 4, 8, and 9 ($p < 0.05$ for 1, 4, 8, and 9; $p < 0.005$ for 3). On average, the thickness deviation between distal positions, like those proximally, were minimal for both normal and ischemic tissue. It was the middle positions, as stated previously, that were different; positions 4 and 6 were thicker than 5 for both tissue types (Figure 10). There was a significant difference between proximal, middle, and distal located positions ($7.64 \pm 0.287 \mu\text{m}^2$, $6.40 \pm .266 \mu\text{m}^2$, and $4.33 \pm 0.161 \mu\text{m}^2$, respectively) for control tissue and (9.06 ± 0.390 , 6.70 ± 0.454 , and $5.02 \pm 0.249 \mu\text{m}^2$, respectively) for ischemic tissue (Figure 10). When compared, control and ischemic tissue were significantly different at distal and proximal locations ($p < 0.05$ and < 0.005 , respectively). On average, centrally located positions are thinner than medial and lateral positions for control and ischemic tissue (Figure 7).

Day 6

Comparing day 6 normal positions revealed more complexity and variation than day 3 normal positions. Normal skin thickness was consistently thinner than ischemic tissue. Positions 1, 2, 3, 5, and 6 are significantly different from positions 4, 7, 8, and 9 for normal skin. However, no significant difference was found for ischemic positions when compared to each other. Comparing day 6 normal and ischemic skin revealed greater difference in ventral skin thickness than seen in day 3 samples. Ischemic positions

1, 2, 3, 4, 5, and 9 are all significantly different ($p < 0.05$ for positions 1, 2, 5, and 9; $p < 0.005$ for positions 3 and 4) than their paired controls (Figure 11). Thickness varied proximally to distally ($7.68 \pm 0.305 \mu\text{m}^2$, $5.78 \pm .190 \mu\text{m}^2$, and $5.13 \pm 0.169 \mu\text{m}^2$, respectively) for control tissue with only proximal tissue being significantly different from distal tissue. For ischemic tissue, thickness again varied proximally to distally (8.46 ± 0.419 , 8.11 ± 0.359 , and $7.74 \pm 0.405 \mu\text{m}^2$, respectively), but without significance. Comparing ischemic tissues with their paired controls, distal and middle locations were significantly different ($p < 0.001$ for both); day 3 difference was seen in distal and proximal locations (Figure 9). By day 6, centrally located positions are statistically no different when compared to medial and lateral locations (Figure 8).

Day 3 v. day 6

To determine the difference between the two time points, normal and ischemic skin thickness data was compared for day 3 and day 6. Results indicate that only positions 3, 6, and 8 were significantly different ($p < 0.005$ for 3 and 6; $p < 0.05$ for 8) for normal skin (Figure 12) and positions 1, 2, and 5 were different ($p < 0.005$ for all) for ischemic skin (Figure 13).

Epidermis Thickness

Comparing day 3 epidermal thickness in normal and ischemic tissue showed that positions 1, 3, 4, 7, and 9 were significantly different ($p < 0.05$ for 7; $p < 0.01$ for 3; $p < 0.005$ for 3; $p < 0.001$ for 1 and 9) (Figure 14). Day 6 normal and ischemic thickness revealed positions 1 through 6 and position 9 were significantly different ($p < 0.001$ for all) (Figure 15)—these same positions were also significantly different in full thickness

comparisons (Figure 10). Comparing day 3 normal epidermal thickness to day 6 revealed that positions 1, 3, 6 and 7 were significantly different ($p < 0.05$ for 3 and $p < 0.005$ for 1, 6, and 7) (Figure 16). Days 3 and 6 ischemic epidermis comparisons revealed positions 1, 2, 3, 5, and 6 as being significantly different ($p < 0.001$ for 3; $p < 0.005$ for 1, 2, and 5; $p < 0.05$ for 6) (Figure 17).

Keratinocyte Nuclei Density

Increased nuclei density in all tissue was found to be correlated with increased keratinocyte layers in the epidermis. Positions 2, 3, 7, and 9 nuclei density were significantly different ($p < 0.05$ for 2 and 3; $p < 0.005$ for 7 and 9) for day 3 normal versus ischemic tissue (Figure 18). For day 6, positions 1, 2, 4, 5, 6, and 9 were significantly different ($p < 0.005$ for 2; $p < 0.001$ for 1, 4, 5, 6, and 9) (Figure 19). Comparing normal tissue nuclei density for days 3 and 6 revealed that positions 1, 4, 5, 6, and 7 were significantly different ($p < 0.005$ for 1, 4, and 6; $p < 0.05$ for 5 and 7) (Figure 20). Comparing ischemic tissue nuclei density between the days revealed that positions 1, 2, and 5 were significantly different ($p < 0.005$ for 1 and 2; $p < 0.04$ for 5) (Figure 21).

DISCUSSION

Studies to evaluate ischemia in the rabbit ear either have not been conducted or have not been reported. However, few articles relating to the ventral ear skin discuss regenerative capabilities of the rabbit. In their discussion of the rabbit's ability to fully regenerate ear tissue after full thickness wounds were placed on the ear, Goss and Grimes (1975) and Williams-Boyce and Daniel, Jr. (1980 and 1985) show that wounds on the ear show discriminate healing. In their evidence, these authors show that males tended to heal more successfully than females and juveniles more quickly than adults; pregnant females regenerated much more quickly than non-pregnant females or males; skin thickness was also greater in males than females; proximal wounds regenerated faster and more successfully than middle locations and these faster than distally placed wounds; and proximal tissue also tended to be thicker compared to more distal locations in both males and females (29-31). These findings suggest that there are similarities in cartilage regeneration and wound healing, however, their results should be taken cautiously because this experiment did not look at the regenerative capacity of the rabbit cartilage. No recent studies were found relating to this topic. Further, reports concerning wound healing have not reported on any recovery differences in proximally placed wounds and those placed distally. Despite the evident lack of *recent* literature, the rabbit ear model seems well suited for studying normal and ischemic wound healing.

The hypothesis had predicted that proximal placed biopsies would be thicker compared to more distally placed wounds in both normal and ischemic tissue. The results indicate that this is true for normal tissue at both days examined and ischemic tissue at day 3. However, at day 6, ischemic tissue biopsies showed a consistent thickness at proximal, middle, and distal locations (Figure 9). This study suggests that normal ear skin follows a natural gradient in which thickness varies proximal to distal (thicker to thinner, respectively). It is possible that this thickness gradient correlates to the vascular supply of the ear; the vascular pedicles serve as the major entry point for the vessels supplying the ear (17, 23). Williams-Boyce and Daniel, Jr. suggest that the differences seen in proximo-distal orientation may stem from differences in innervation and vascularity in addition to cartilage thickness differences—proximal cartilage tends to be thicker than distal cartilage (30). The second part of the hypothesis had predicted that ischemic tissue would be thinner than paired controls. The results indicate that thickness variations are dependent on position, but results, particularly, for day 6 suggests that this part of the hypothesis needs to be rejected. Ischemic tissue is consistently thicker than normal paired tissue at day 6.

Normal skin, unlike ischemic skin deviates less from average thickness among the positions and contains uniformity among positions in proximal, middle, and distal orientations. Ischemic tissue showed greater variability (Figures 7 & 8). Further, skin thickness varies little in the normal skin with only minor changes documented between days 3 and 6; thickness gradient was still observed. However, ischemia seems to dislodge this gradient. By day 6, ischemic positions at the distal and middle locations gained in thickness and were comparable in thickness to proximal positions that remained largely

unchanged compared to day 3. Moreover, thickness in centrally placed positions (2, 5, 8) seem also to be enhanced after ischemia. To better understand this thickness change, the epidermis was measured. It seems that thicker ventral skin correlates to having a thicker epidermis by day 6; this is especially true for ischemic tissue. Results suggest that the time between days 3 and 6 is one of increased keratinocyte turnover in ischemic tissue compared to normal tissue. On average, nuclei density per area increased by day 6 compared to day 3 in ischemic tissue. The layers of keratinocytes in the epidermis increased but were not significant. The reasons behind the increased thickness in ischemic tissue could be accounted for—but not limited to—in multiple ways including hypoxia induced angiogenesis, neovascularization, or migration of endothelial cells and ischemia caused epithelial activity.

Collateral circulation

Rendering the rabbit ear ischemic by ligating two of the three arteries renders the ear ischemic for two to three weeks at which point collateral circulation is established (17). PCNA evidence suggests that proliferating vasculature maybe helping reestablish circulation in ischemic tissue. Collateral circulation may enhance positions 4 and 6 (closest to neighboring vessels) and this may account for their increased thickness compared to adjacent positions. Increase cellular metabolic activity can be managed for a short time in the absence of oxygen, but prolonged absence cannot supply the necessary energy needed for favorable outcomes (32). Proliferation of endothelial cells and in turn microvasculature suggests that perfusion is being established quickly in this model, but the extent of neovascularization and areas of proliferation within the tissue were undetermined. Understanding the early response to ischemia by the ear skin tissue should

reveal greater details to how wounds respond. For example, wounds by nature are hypoxic and as a result, their need for oxygen helps to initially increase the rate of proliferation and growth of vessels and other angiogenic factors in the insulted region (10, 19). However, when the tissue is ischemic to begin with, wounds should place an extra burden on tissue response. Inability to establish and maintain nutrient, oxygen, and adequate blood supply in the skin tissue is one the main reasons for the occurrence of chronic wounds (1, 10, 19). The early proliferation of vasculature in the rabbit ear might minimize the oxygen debt experienced by the wounded tissue. Nevertheless, ischemia in this model is significant and prolonged compared to rat models and does allow for repeated and continuous testing at the wound sites (21, 33). Temperature differences between normal and ischemic ears also seem to support the presence of ischemia. The initial increase in the temperature difference and the subsequent maintenance of this difference throughout the days examined indicates that blood flow has not fully recovered in any of the ischemic rabbit ears.

Growth factor synthesis and secretion seem to be reduced in chronic wounds (34) and evidence suggest that upregulation of factors such as insulin like growth factor-1 (IGF-1) (35) and keratinocyte growth factor-1 and 2 (KGF-1 and KGF-2) (34) in addition to other fibroblast growth factor family members may have positive outcomes in ischemic wounds (36). For example, IGF-1 deficiency has been shown to delay healing in ischemic wounds; increasing IGF-1 levels seem to increase collagen synthesis and cell proliferation by various cells in ischemic tissue (35). Several growth factors, however, are stimulated naturally due to loss of homeostasis in injured tissue. The result is increased levels of growth factors such as transforming growth factor beta-1(TGF- β 1),

tumor necrosis factor alpha (TNF α), epidermal growth factor, keratinocyte growth factors, basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) (20, 37, 38) associated particularly with angiogenesis.

The proliferation of endothelial cells and vessels as evidenced from PCNA positive stains suggest that there may be reestablishment of vascular supply. If so, some of the factors listed above may play a significant role during ischemia. Ischemia is believed to be a strong stimulus for neovascularization because the need for restoring oxygen delivery to wounded sites is so great (39). In fact, high level of VEGF has been reported in both uninjured ischemic tissue and injured ischemic tissue (37) and evidence suggests that it plays a more prominent role in ischemic tissue than other factors such as bFGF (37, 40). Urbich and Dimmeler have reported that VEGF was shown to induce differentiation of adult progenitor populations to endothelial cells in ex vivo culture assays and in ischemic tissue (41). VEGF seems to promote neovascularization of ischemic tissue by vasculogenesis rather than angiogenesis (39). It is also possible that ear tissue was experiencing angiogenesis in addition to vasculogenesis in certain regions of the ear. Angiogenesis occurs mainly by sprouting of new capillaries from existing vasculature (20, 38) rather than induction of progenitor cells as seen in vasculogenesis. This is possible in this experiment since the caudal artery was left intact. Migration of sprouting capillaries into the ischemic tissue could easily explain the proliferation of vessels seen in the vicinity of the artery.

Epidermis and Ischemia

The epidermis is the outermost layer of the skin varying in thickness depending on location. Our results indicated that in the ventral rabbit ear, the epidermis is quite uniform in normal tissue varying slightly in the proximal locations but not significantly. Ischemia seems to disrupt this uniformity, especially by day 6. Thickness is significantly enhanced at distal and middle locations compared to day 6 normal tissue and day 3 ischemic tissue. It is known that the epidermis is constantly turning over new cells, but how this affects epidermal thickness is unclear. By day 6, epidermis seems to be affected by ischemia. Keratinocyte nuclear density was found to be increased in day 6 ischemic tissue compared to day 6 normal and day 3 ischemic tissues. Keratinocytes, which are the dominant cell within the epidermis, are maintained by undifferentiated stem and progenitor cells in the proliferative basal layer (42). These progenitor cells constantly renew keratinocyte populations that are lost at the stratum corneum allowing the skin to maintain a strong barrier against the environment (43). While the role of epidermal cells in ischemic conditions is being currently researched, there is growing evidence that epidermal progenitor cells (EpPCs) play a large role in wound healing, especially in ischemia (41, 42).

Basal progenitor cells were once thought to be unipotent giving rise to only new keratinocytes; however, research has provided evidence of multipotency. The potential for epidermal progenitor cells to be multipotent rather than unipotent has been seen as a possible venue for chronic wound treatment (42, 44, 45). Keratinocytes and EpPCs at the chronic ulcer edges have been shown to be highly proliferative, induce granulation tissue in ischemic dermal wounds, accelerate blood flow in diabetic ischemic limbs, increase migration and proliferation of endothelial cells, increase vascularity in injured tissue, and

increase the proliferation rates of new keratinocyte in epithelial gap closure (36, 42-44, 46). The increase seen in both epidermis thickness and the nuclei density by day 6 in ischemic tissue might indicate increased activity by EpPCs and keratinocytes. EpPCs have been shown to incorporate into the endothelium of capillaries after induction of ischemia resulting in neovascularization and it has also been suggested that progenitor cells actively recruit endogenous cells to form new vessels in ischemic tissue (42). Given PCNA positive stained endothelial nuclei findings from this study, it is possible that EpPCs might have played a role in enhancing blood supply to ischemic tissue. Ischemia seems to stimulate keratinocyte growth factor-2 (KGF-2) in wounded tissue. KGF-2 has been shown to increase both epithelium thickness and migration; KGF-2 treated ischemic wounds were shown to have increased wound healing in young and aged rabbits (34).

Changes in ventral skin thickness and accompanying increases in epidermis thickness and keratinocyte population density demonstrate ischemia does alter the tissue histology. However, the exact mechanism has yet to be determined. The finding that normal ear skin adheres to a decreasing proximal to distal thickness gradient that is altered with ischemia suggests more detailed experiments should be conducted. Dermal and epidermal cell population dynamics, growth factor regulation, and progenitor cell activity should also be examined in future studies using this model. PCNA evidence suggests that proliferation of various cells is occurring in both normal and ischemic tissue; however, the cell types were not determined. Spindle shaped nuclei suggest that some of these positively stained cells could be fibroblasts. Dermal fibroblasts are actively involved in wound healing through the deposition of new collagen and ECM. Dermal-epidermal interactions are well documented and while, this experiment did not look at the

roles collagen and extracellular matrix play in ischemia, future studies should not ignore this aspect because wounds cannot heal properly without adequate collagen deposition, nor can they achieve structural integrity in ischemia as evidenced by diabetic, pressure, and venous ulcers. Future experiments should consider examining the role of angiogenesis and vasculogenesis in ischemic tissue and attention should be paid to dermal dynamics as well. While, this experiment has shown evidence of endothelial proliferation in the ischemic tissue, blood flow and oxygen perfusion should be measured to properly assess the recovery from ischemia.

Implication for wound healing research

The results of this experiment suggest that tissue responds to ischemia differently at days 3 and 6. Depending on location of the wounds, tissue also reacted differently. From this experiment, it was shown that ischemia affected the tissue at proximal and lateral positions differently than other positions. It was shown that full thickness results of ischemic tissue were significantly different at position 1, 3, 4, 8, and 9 compared to normal tissue at day 3. Additionally, positions 1, 3, 4, 7, and 9 for epidermis thickness and 2, 3, 7, and 9 for corresponding keratinocytes were also significantly different at day 3. Remembering that positions 1, 3, 7, and 9 correspond to the four-hole models, it becomes apparent that the four corners react more to ischemia at day 3 compared to the other positions. This has implications for wound healing studies that look to study early phases of wound healing. Since tissue responds differently at the four corners compared to the other locations, randomizing positions for acute and inflammatory studies is recommended. Positions 2, 4, 6, and 8 seem the logical choices. Altering the four corner model with a model that uses 2, 4, 6, and 8 may help to achieve a more detailed

understanding of the healing process in the early stages while avoiding the inherent biases that seem present at the corners. Additionally, the nine-hole model used in this study is maybe well suited for future studies as well. The information gathered from using the nine-hole model will be greater than the four-hole model and using the nine-hole model rather than the four-hole model can eliminate the need for randomizing the wounds. However, nine holes do increase the resources needed to conduct studies and additionally how skin healing occurs in such a model is unknown. For wound studies looking at longer time periods, the four corner model seems adequate.

Ischemic tissue by day 6 was found to respond to ischemia more so than by day 3. Yet, the reaction of the tissue is uniform throughout the examined ear. Ischemic tissues at positions 1, 2, 3, 4, 5, and 9 all are significantly thicker than paired control tissue. Positions 7 and 8, while not significantly different from paired control, have similar full thickness, epidermis thickness, and nuclei counts compared to all other positions. Additionally, no significant difference was observed for tissue at distal, middle, and proximal. A uniform reaction suggests that choosing any of the nine positions would be well representative of overall tissue reaction at day 6. Maintaining the four corner model would be the recommended option. How exactly the rabbit ventral ear tissue reacts to ischemia past day 6 is unknown. The granulation phase or the second phase of healing tends to last until the wounds are covered. Past studies using the rabbit ischemic model suggest that re-epithelization and wound closure usually occurs within the first two to three weeks of injury while full healing is prolonged in ischemic wounds (21, 47). Assuming that ischemic tissue is in the second phase of healing by day 6, tissue reaction

for days following 6 should be similar to that seen in day 6. If this is the case, then the four corner model maybe sufficient.

SUMMARY AND CONCLUSIONS

The results of this experiment comply with the first prediction made by the hypothesis—ventral skin has a decreasing proximal to distal thickness gradient. Proximal skin tissue is consistently thicker compared to more distal skin tissue. However, the hypothesis also predicted that ischemic tissue would be thinner than normal control tissue. This was not the case. Day 6 ischemic tissue had increased thickness at all positions examined indicating that ischemia with time plays a role in altering the thickness gradient. In addition to changes in the whole skin thickness, the finding that the epidermis thickness and corresponding keratinocyte population density are altered as a result of ischemia is important. The epidermis plays an important role in establishing the barrier needed to protect the body from the environment. How exactly this skin thickness gradient and the epidermis are altered should be the focus of future experiments. The purpose of this experiment, however, was to outline the tissue response to ischemia and how this may affect chronic wound healing. Chronic wounds by nature are ischemic and our finding that the rabbit ear shows changes after being rendered ischemic is important. Creating new chronic ear wound models that closely resemble those seen in clinical settings is vital to gaining more knowledge about possible future treatments. The nine-hole ear model designed for this experiment can be an useful tool for elucidating information concerning the overall tissue dynamics after ischemia as well as a model for future wound healing studies that look to study the initial inflammatory stage or acute responses after injury. Although the use of nine holes increases the time and costs of

conducting research, the results obtained are more detailed than comparative four-hole studies. Yet, as discussed, researchers studying the immediate and acute responses of injury should consider either using the nine-hole model or a four-hole model that randomizes the positions of the wounds to avoid biases observed at the corners. Tissue response to ischemia by day 6 is more uniform compared to day 3 and thus researchers interested in a more long term study of ischemic wound healing can use the four-corner model without alternating wound positions. Whether deciding to use either the four-hole or the nine-hole wound model, attention should be paid to the inherent thickness gradient present in the skin.

FIGURES

Figure 1. Four-hole ear wound model. A gross image of the ventral ear skin with four 6-mm circular wounds.

Figure 2. Nine-hole rabbit wound and rabbit ear ischemic model. Current four hole models place wounds at the four corners, this model aims to look at other regions of the rabbit ear in addition to the four corners. Left ears of each rabbit were rendered ischemic by ligating two of the three arteries supplying the ear--the caudal artery and all three veins were preserved. Right ear of each rabbit served as controls.

Temperature Difference Between Ears

Figure 3. Temperature difference between ears. Difference in temperature significantly increased ($p < 0.005$) after ears were rendered ischemic ($1.32^{\circ}\text{C} \pm 0.39^{\circ}\text{C}$ on day 0 to $3.28^{\circ}\text{C} \pm 0.86^{\circ}\text{C}$ on day 1 postop). While, the rate of difference decreased as the days progressed, an average difference of $3.84^{\circ}\text{C} \pm 0.85^{\circ}\text{C}$ was observed over remaining days post surgery for all animals. Mean temperatures for days 3 and 6 were significantly different ($p < 0.001$ and $< .005$, respectively) compared to day 0.

Figure 4. General histology of the rabbit ventral skin. Ventral skin extends from the cartilage (HC) and the perichondrium (PC) to the shedding stratum corneum (SC). Epidermis (E) thickness varies according to location and is usually several cell layers thick. No observable difference was seen between normal tissue (a) and ischemic tissue (b). Dermis (D) contained neurovascular bundles (*) and were predominantly acellular. Occasional hair follicles (HF) and sebaceous glands (SG) were observed. Magnification 200x and scale bar equals 0.1 mm.

Figure 5. PCNA staining of the epidermis. Positively stained keratinocytes were found predominantly in the basal layer. Positive stains were seen in both ischemic tissue and normal tissue—no quantification was conducted. Magnification 1000x.

Figure 6. PCNA staining of the dermis. Positive staining nuclei were observed to be spindle-shaped and were present in both normal and ischemic tissue. Positive endothelial nuclei reveals proliferation of vasculature within ischemic and normal tissues. Notice in b and d that positive stained nuclei do not always surround the vessel; rather they constitute one side of the vessel. Magnification 1000x.

Figure 7. Day 3 overall skin thickness trends. For normal tissue and ischemic tissue, distal located positions tend to be thinnest compared to middle and proximal locations. Centrally located positions tend to be thinner than lateral and medial locations. The overall thickness gradient in both normal and ischemic tissue is one in which thickness decreases as you move away from the base of the ear (proximal to distal). Note Orientations: Positions 1, 4, 7 are medial positions; 2, 5, 8 are central positions; and 3, 6, 9 are lateral positions. Positions 1, 2, 3 are distal positions; 4, 5, 6 are middle positions; and 7, 8, 9 are proximal positions. Each bar on the above graph corresponds to one position, i.e. medial distal corresponds to position 1, central middle corresponds to position 5, etc. Results are shown as mean plus/minus SEM.

Figure 8. Day 6 overall skin thickness trends. By day 6, ischemic tissue is consistently thicker than normal tissue at distal and middle locations indicating thickness at these locations increased from day 3 and compared to normal tissue. Results are shown as mean plus/minus SEM.

Figure 9. Ventral skin thickness gradient. Looking just at the proximal, middle, and distal locations, the gradient becomes clear. Distally located normal and ischemic tissue for day 3 are significantly different compared to middle and proximal locations. Only proximal located positions are significantly different compared to distal located positions for day 6 normal tissue. No significant difference was found for day 6 ischemic tissue. Day 6 ischemic tissue is more uniform in thickness. Results are shown as mean plus/minus SEM.

Day 3 Normal v. Ischemic Ventral Skin Thickness

Figure 10. Day 3 Normal v. ischemic skin thickness. Results are shown as mean plus standard deviation. In day 3 tissue, positions 1, 3, 4, 8, and 9 were significantly different between normal and ischemic tissue. Notice the thickness gradient increases as you move closer towards proximal positions (7, 8, and 9). Results are shown as mean plus/minus SEM.

Day 6 Normal v. Ischemic Ventral Skin Thickness

Figure 11. Day 6 normal v. ischemic skin thickness. By day 6, normal and ischemic tissues vary more compared to day 3. Statistically significant positions are 1, 2, 3, 4, 5, and 9. Unlike normal tissue, which seems to still have that inherent thickness gradient, ischemic tissue seems to have lost this gradient by day 6 as thickness has leveled out at the different locations. Results are shown as mean plus/minus SEM.

Figure 12. Day 3 v. day 6 normal skin thickness. Comparing normal skin thickness between days 3 and 6 reveals that differences are minimal. Only positions 3, 6, and 8 are significantly different. Notice the presence of the thickness gradient in both days. Results are shown as mean plus/minus SEM.

Day 3 v. Day 6 Ischemic Skin Thickness

Figure 13. Day 3 v. day 6 ischemic skin thickness. Comparing ischemic tissue for days 3 and 6 reveals that positions located distally have increased thickness while those proximally have remained relatively the same. Although there is a general lack of variation among the means, we see that positions 1, 2, and 5 are significantly different. Notice the thickness gradient is visible in day 3 tissue but this gradient is lost by day 6. Results are shown as mean plus/minus SEM.

Day 3 Normal v. Ischemic Epidermal Thickness

Figure 14. Day 3 normal v. ischemic epidermal thickness. At day 3, normal epidermis thickness does not seem to follow the overall thickness gradient; rather there is an observed uniformity for distal, middle and proximal positions. Ischemic tissue, however, has greater variability in thickness. Comparing normal and ischemic tissue reveals positions 3, 7, and 9 were significantly different. Results are shown as mean plus/minus SEM.

Day 6 Normal v. Ischemic Epidermis Thickness

Figure 15. Day 6 normal v. ischemic epidermal thickness. Day 6 epidermal comparisons reveal a greater difference between normal and ischemic skin. The same positions that are significantly different in full skin thickness are also significantly different for epidermis thickness suggesting that by day 6, difference in skin thickness may be in part due changes in the epidermis. Like full thickness measurements, positions 1, 2, 3, 4, 5, 6, and 9 are significantly different. Results are shown as mean plus/minus SEM.

Figure 16. Day 3 v. day 6 normal epidermal thickness. Comparing normal epidermal thickness for days 3 and 6 reveals that positions 1, 3, 6, and 7 are significantly different. Similar comparisons of normal full skin thickness showed that positions 3, 6, and 8 were significantly different. Results are shown as mean plus/minus SEM.

Figure 17. Day 3 v. day 6 ischemic epidermal thickness. Comparing ischemic epidermal thickness for days 3 and 6 reveals that positions 1, 2, 3, 5, and 6 are significantly different. Similar comparisons of ischemic full thickness skin showed that positions 1, 2, and 5 were significantly different. These results suggest that the epidermis is also responding to ischemia. Results are shown as mean plus/minus SEM.

Figure 18. Day 3 normal v. ischemic keratinocyte nuclei density. The finding about the epidermis prompted a closer look at the epidermis in which keratinocyte nuclei per area of measured epidermis was examined. Comparing nuclei density in day 3 normal and ischemic epidermis reveals that positions 2, 3, 7, and 9 are statistically significant. Notice the lack of a proximal-distal gradient. Results are shown as mean plus/minus SEM.

Figure 19. Day 6 normal v. ischemic keratinocyte nuclei density. Day 6 comparisons reveal a similar trend we have seen in analysis of ventral skin thickness and epidermal thickness. Positions 1, 2, 4, 5, 6, and 9 are all significantly different. Increased keratinocyte population accompanies increased thickness in ischemic epidermis. Results are shown as mean plus/minus SEM.

Figure 20. Day 3 v. day 6 normal keratinocyte nuclei density. Normal nuclei density comparisons for days 3 and 6 reveal that positions 1, 4, 5, 6, and 7 are significantly different. Results are shown as mean plus/minus SEM.

Figure 21. Day 3 v. day 6 ischemic keratinocyte nuclei density. Ischemic nuclei density comparisons for days 3 and 6 reveal positions 1, 2, and 5 are significantly different. Results are shown as mean plus/minus SEM.

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APPENDICES

PCNA monoclonal antibody

Tissue: Normal & Ischemic rabbit ear (in 1XPBS)

Section: EMS Vibratom sectioned at 40um in 1XPBS

Incubation: 96-well strip plate

Protocol:

1. 1 X PBS wash 5min x 2
2. 2% H₂O₂ 30min @RT⁰C
3. 1 X PBS wash 5min x 2
4. **Blocking** : 5% Normal Goat serum in 0.1% Triton X-100/ PBS 1.5hr@RT⁰C, shaking
5. 1X PBS wash 5min
6. 1⁰AB: (1:100, diluent by blocking solution (5%NGS in 0.1% Triton X-100/PBS))] O/N(16 hrs), 4⁰C, shaking

PCNA mouse IgG_{2a} mAb

(Santa Cruz, Cat#sc-56)

7. 1X PBS 5min x 3
8. 2⁰Ab: (1:200 diluent by blocking solution (5%NGS in 0.1% Triton X-100/PBS))]
Goat Anti-mouse IgG biotin(200ug / 0.5ml) 1hr@ RT⁰C, shaking
(Santa Cruz, Cat#sc-2039)
9. 1XPBS 5min x 3
10. "ABC" Reagent 30min@ RT⁰C
(Keep at RT⁰C for 30min before use)
11. 1XPBS 5min x 3

12. **DAB** Reaction

~7 min

(Observe under microscope for reaction time)

13. Rinse with dH₂O

2min x 2

14. Follow Embedding protocol

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