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Real Time Analysis of the Kinetics of Angiogenesis and Vascular Permeability in an Animal Model of Wound Healing

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Abstract

The use of engineered tissue for the treatment of a variety of acute to chronic wounds has become a clinical standard, and a better understanding of the cellular mechanisms of re-vascularization and barrier integrity could enhance clinical outcomes. Here, we focus on the characterization of the re-vascularization of acellular grafts such as Integra™ in an animal model to better understand the physiological properties of blood vessels growing in the collagen-glycosaminoglycan matrix vs. wound margins. While Integra™ has been extensively studied in pre-clinical models, the re-modeling mechanisms of the capillary bed under these matrices are not well understood. Therefore, our first objective was to quantify the kinetics of revascularization. The second objective was to assess changes in vascular permeability (VP) of the wound bed compared to normal adjacent skin. The third objective was to establish a non-invasive and quantitative assay for the measurement of VP to facilitate the rapid and reproducible characterization of vascular integrity. Using an excisional wound model in mice, we characterize the appearance, growth, and maturation of blood vessels in an Integra™ graft over 28 days post-surgery. Initial appearance of blood vessels in the graft was observed at 7 days, with angiogenesis peaking between at 7–14 days. The onset of VP coincided with the increase in re-vascularization of the wound bed and there was a sustained elevation of VP that declined to baseline by 28 days. We propose that a non-invasive strategy to assess VP of the wound capillary bed to facilitate a better understanding of the cell and molecular basis of angiogenesis in wound healing.

Introduction

Treatments for both impaired wound healing resulting from systemic disease (i.e. diabetes, aging, circulation) or scar formation in acute wound healing (i.e. burns and trauma) remain a major clinical problem that yield unsatisfactory clinical outcomes. With over 3.5 million chronic ulcer patients and 1.25 million burn patients seen in U.S. annually¹, the area of wound healing as it pertains to dermal regeneration is of significant importance in the clinical setting. Yet, given the difficulties and complications that hinder normal healing, innovative strategies to evaluate the cell biology of wound healing in appropriate animal models are needed. Numerous advances in tissue engineering have yielded a range of both cellular and acellular matrices, with applications for wounds, burns, ulcers, and reconstruction^{2–6}. Here, we focus on the use of a synthetic matrix (i.e. Integra™) with a well-established clinical application in

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the treatment of full thickness burn injury, to examine the kinetics of revascularization in a full thickness excisional wound. Integra™ is an acellular synthetic scaffolding consisting of a bilayer membrane sandwiching a three-dimensional cross-linked collagen-glycosaminoglycan biodegradable matrix. This matrix is a scaffolding that is well tolerated by mice and humans and supports epithelial cell invasion and capillary growth. The clinical utility of such matrices stem from its versatility as an immediate wound dressing for coverage to a dermal regeneration template that allows for the transition from an acellular matrix to specific host tissue^{2, 6–19}.

Although others have studied the histological transitions of Integra™-mediated wound closure, the kinetics of re-vascularization and permeability have yet to be explored. Neovascularization, or growth of blood vessels, is a crucial step in the wound healing process^{13, 20–28}. It provides the vasculature to support overall repair and newly formed granulation tissue. While angiogenesis is a process that is tightly regulated, both spatially and temporally, the fact that angiogenesis is coupled during wound repair with changes in VP is less well understood. These changes in VP influence the capacity for positive and negative effectors of wound closure, including stem cells, and it is therefore an important and under-studied parameter of the vascular biology of the healing wound bed. By investigating the kinetics of revascularization as well as VP in real time, we aim to better understand mechanisms of blood vessel formation in wound healing so as to optimize therapeutic care of wounds and skin grafts.

In this study, we investigated the kinetics of revascularization of an Integra™ matrix by examining grafts using classical histology and immunohistochemistry in mice at 3–28 days after graft surgery. We quantify capillary density over the time course both in the re-vascularized graft vs. flanking normal skin and compare these findings with changes in the circulation observed by ex vivo imaging of skin grafts following perfusion with a fluorescent dextran tracer. These ex vivo analyses enable the characterization of the functional circulation in the graft over time. We observed an increase in vascular leak at early time points (i.e. 3–10 days), followed by a decrease to baseline by 14–28 days. To obtain a more dynamic and quantitative determination of VP in this model, we implemented a novel non-invasive imaging strategy to monitor and quantify vascular permeability of fluorescent permeability tracers as a function of time in anesthetized animals. The advantage of this approach was the capacity to non-invasively image the same animals at multiple timepoints, and the quantitation of VP in both intact and injured skin tissues.

Methods

Skin Grafting

Eight to ten week-old, male C57/Bl6 mice (Jackson Labs, Bar Harbor, ME) were used for all experiments. Mice were anesthetized with isoflurane, the surgical site trimmed of hair, and prepared in a routine aseptic fashion. After verifying adequate anesthesia, one full-thickness (1.5 cm diameter, marked by template) circular wound was excised from the right side of the dorsum. The excisions were deep to the panniculus carnosus, removing epidermal, dermal, subcutaneous, and fascia layers. Integra grafts (Integra LifeSciences Corporation, Plainsboro, NJ) 1.5 cm in diameter were secured with seven 6–0 silk interrupted sutures (Sherwood Davis & Geck, St. Louis, MO), equidistant from each other¹⁷. Animals were housed in separate cages with a 12 hours light/dark cycle and given access to feed and water. At various time points, 3, 7, 10, 14, and 28 days after surgery, tissue was harvested. All procedures were done according to the UCSD Institutional Animal Care and Use Committee guidelines.

Immunohistochemistry

Indirect immunofluorescence was performed on cryosections (20µm) of Integra/tissue samples using anti-CD31 (Pharmingen BD Biosciences, San Jose, Calif.) and detected with Alexa fluor-

conjugated secondary antibodies (Molecular Probes, Eugene, OR). Frozen sections were fixed in 3.7% paraformaldehyde for 10 minutes, blocked in 3% bovine serum albumin for 20 minutes, stained with CD31 at 1:100 for 45 minutes, and stained with fluorescent secondary antibody at 1:200 for 45 minutes.

Image Acquisition, Analysis and Statistics

Fluorescent images of immunostained tissue sections were acquired with an Olympus Fluoview 1000 confocal microscope using exposure-matched settings (Advanced Software V1.6, Olympus, Center Valley, PA). Quantification of fluorescence intensity of CD31 immunohistochemistry was performed using Volocity image analysis software (Improvision, MA) to measure fluorescence intensity in blood vessels by applying a constant threshold across all of the data sets to identify blood vessel regions of interests, followed by a calculation of the sum of pixel intensities in all of the blood vessels in each fluorescent image field^{29, 30}. All data were obtained from 3–6 animals from each time point and errors plotted as standard errors of the mean. Statistical significance was determined with Student t-test for each data set.

Ex Vivo Perfusion and VP Assay

Blood vessel permeability was determined by intravenous injection into the tail vein with 70 kDa FITC-dextran (Sigma, St Louis MO). 7.5 mg of FITC dextran in a volume of 150 microliters was injected and immediately after the injection, the mice were sacrificed and the tissue harvested. Micrographs were captured from fresh graft sections using a confocal microscope to detect FITC-dextran.

Non-Invasive/In-Vivo Characterization of Blood Vessel Permeability

Blood vessel permeability was determined by intravenous injection into the tail vein with 70 kDa FITC-dextran (Sigma, St Louis MO). Mice were anesthetized and five minutes after the injection, imaged for fluorescent extravasation on an IVIS Lumina CCD imaging System (Caliper Life Sciences, Hopkinton, MA). Exposure-matched images were acquired using the Living Image Version 3 software, compared with the background fluorescent images of matched graft regions in un-injected mice. Using a fixed region of interest for data analysis, the fluorescence of the graft was measured in injected mice vs. un-injected mice at each timepoint.

Results

Remodeling of granulation tissue in full thickness excisional wound

Several animal models have been used in grafting studies to closely approximate the repair process in human skin. Here however, with the long term goal of using the power and versatility of mouse genetics to better understand the cell biology of wound healing, we examined the vascular response to grafts in a mouse model. To characterize the kinetics of wound closure and contraction, mice were subjected to full thickness 1.5 cm diameter excisional wounds and grafted with silicone-backed Integra matrix and allowed to heal to full wound closure over a time course of 14 days (Figure 1). Analysis of hematoxylin-eosin stained tissue sections of the graft after 3 days (Figure 1, Panel A) revealed an accumulation of neutrophils in the matrix and an absence of granulation tissue and capillaries. The vascularized underlying muscle layer was apparent and clearly demarcated the graft region for up to 7 days (Figure 1). At 14 days, a newly formed epidermal layer was overlaid with a homogenous dermis fully intermeshed within the grafted matrix. Although the gross appearance of the graft is clearly distinct from the adjacent normal tissue (Figure 1, Panel F), there is a nearly homogenous integration of the matrix and granulation tissue that supports a well-formed capillary bed (Figure 1, Panels G–H, arrows).

Revascularization of the Integra™ graft in C57Bl/6 mice

To determine the kinetics of revascularization in the Integra™ graft, we performed an immunohistochemical analysis of tissue sections from the C57Bl/6 graft model. Tissue samples from the center of the graft site and normal flanking skin were prepared for cryosectioning and subjected to indirect immunofluorescent staining with an endothelial marker (anti-CD31 antibody) to localize capillaries. Exposure-matched images were acquired using a laser scanning confocal microscope on normal flanking skin, on the transition zone between the graft and the pre-existing skin and on the graft that overlays the vascularized muscle are shown (Figure 2, Panel A). Demarcation between the graft and the pre-existing skin in the transition (Figure 2, Panel A, middle column, dashed line) highlights the qualitative differences between the new vessels growing into the graft and the pre-existing vessels. A demarcation between the pre-existing vessels of the overlying graft and the adherent underlying muscle (i.e. adherent through day 7) is clearly evident (Figure 2, Panel A, right column, solid line). By 14 days however, the graft was adhesion-free (i.e. un-attached from the underlying muscle layer) and well-vascularized as determined by anti-CD31 immunohistochemistry. Quantitation of CD31-positive capillaries revealed that there was an absence of blood vessels in the graft 3 days post-surgery, a peak of staining at 10 days and a decrease in vascularization by 28 days (Figure 2, Panel B). These findings indicate that although the graft itself is devoid of blood vessels at an early timepoint (i.e. day 3), the Integra™ graft supports extensive and rapid revascularization (i.e. a peak at 7–10 days post-grafting), which eventually subsides by day 28 to capillary densities comparable to adjacent flanking skin. Furthermore, the hematoxylin/eosin staining revealed extensive neutrophil infiltration at day 3 (Figure 1), which was followed by re-organization, re-epithelialization, and re-vascularization by day 10.

Perfusion analysis reveals an initial phase of extensive vascular leak

To characterize the increase in vascular leak in the initial phase (i.e. 3–7 days post-grafting) vs. the re-vascularization phase (10–28 days), we subjected animals with grafted full thickness excisional wounds to perfusion analysis. Following an intravenous injection with 70 kDa fluorescent dextran 5 minutes prior to sacrifice, excised skin grafts were imaged with a laser scanning confocal microscope 3–28 days post-grafting to analyze the kinetics of vascular leak of blood vessels in the graft. In regions of the graft (Figure 3, dotted area), extensive VP was observed over 3–7 days, when blood vessels were largely absent, suggesting leakage from flanking damaged/angiogenic blood vessels. In contrast, the absence of VP by day 28 suggests that the blood vessels in the graft included a functional endothelial barrier. Although the integrity of blood vessels in flanking skin remained intact (Figure 3, arrows), there was an absence of dextran-filled blood vessels in the graft in the first 7 days. However, by 28 days, this dextran perfusion technique did not reveal any qualitative differences in the integrity of the blood vessels in the graft vs. surrounding normal skin. As expected, wound contracture is observed in rodent wound healing models. Because significant vascular leak precedes angiogenesis and remodeling of the wound bed, we developed a quantitative and minimally invasive technique to assess VP to further characterization of vascular integrity after wound healing *in vivo*.

Non-invasive imaging and quantitation of VP in skin grafts

To monitor changes in VP of blood vessels in the skin graft over an extended timecourse and in the same animal, we used a cooled CCD imaging system (Lumina, Caliper Life Sciences) for whole animal imaging to detect the extravasation of 70kDa fluorescent dextran (FITC-dextran) in the wound bed. Because the clearance of the FITC-dextran after each injection is sufficient to reduce any residual background to baseline after each timepoint (data not shown) this approach is amenable to multiple analyses over time in the same animal. Mice bearing the skin grafts (4–28 days) were evaluated following intravenous administration of the fluorescent

dextran (Tracer) compared to un-injected control mice with a graft (Sham) and imaging revealed both qualitative and quantitative changes in VP over the timecourse (Figure 4). While extensive VP was observed within 4 days of the graft and increased to a maximum at 10 days, a reduction in VP was observed by day 14. Quantitation of the VP in this graft model revealed that there was a statistically significant increase in VP of the graft site vs. normal skin in the same animal throughout the timecourse (Figure 4, Panel B, $P < 0.05$). In addition to the detection of the color tracer in the animals injected with fluorescent dextran (Figure 4A, right column), the ambient white light animal images reveal the contraction of the wound site over the 28 day timecourse (Figure 4A, left column, arrowhead). The combination of this non-invasive imaging technique to monitor VP with classical immunohistochemistry enables a comparison of the kinetics of re-vascularization vs. VP, two processes that are interdependent in the angiogenesis response of the wound bed. Our data support a model in which elevated VP precedes the formation of new blood vessels during wound healing in vivo.

Discussion

In this study we characterized the kinetics of re-vascularization of a synthetic graft matrix, and quantified the changes in vascular integrity in a full thickness excisional wound bed. While at 3–7 days post-grafting we observed few blood vessels, there was extensive vascular leak. By 14–28 days, however, there was a well-formed capillary bed that retained a relatively normal non-leaky VP phenotype. As expected, re-vascularization of the synthetic graft-mediated wound was accompanied by an early and extensive neutrophil infiltration concomitant with vascular leak. From an initial absence of blood vessels in the graft, and over the following 10–14 days, a well-vascularized wound bed developed which by 28 days was similar to control skin. It is particularly interesting that although these blood-filled vessels appeared intact starting from day 10, our VP measurements suggest that they remained permeable, at least to molecular tracers (70 kDa) that approximate the size of serum albumins that otherwise do not normally cross an intact endothelium. The application of these non-invasive imaging techniques to assess VP and overall vascular integrity now provides an important tool to characterize more than just blood flow in the wound bed as with laser Doppler, but provides a quantitative standard to evaluate therapeutic strategies that influence the rate of angiogenesis and patency of wound healing.

Although an extensive literature demonstrates similarity in the wound healing response of animal models, the mouse offers a unique biological opportunity to evaluate the molecular and cellular basis of wound healing and vascular remodeling³¹. While the tissue generated by the application of Integra to the wound bed is different from standard granulation tissue, this resulting tissue leads to the formation of a well-integrated and re-vascularized wound bed that is suitable for an overlay of a thin skin graft. Similar to Integra grafts in human subjects, the adhesions to muscle that are formed in the mouse grafts at their early stages up through day 10, dissipate at later time points, and lead to a pliable graft at the site of the wound. Although the leakage of vascular tracers was observed for up to 14 days post-grafting, CD31 immunostaining revealed a significantly higher density of the vascular tree at 10–14 days than later at 28 days. Immunohistochemical analyses with markers for pericytes, a cell type associated with more established and presumably ‘less-leaky’ blood vessels was not significantly different from adjacent normal skin sections. These findings support the hypothesis that the increases in re-vascularization of the wound bed at day 10–14 after injury that exceed normal skin blood vessel density must be accompanied by a mechanism to prune and remodel vessels so that the vascular density over the following 2 weeks returns to that of normal skin. The signal for this response is not known but the observation underscore the need to develop a temporal-spatial model of gene expression to explain the changing kinetics and targets of the wound healing response. While the kinetics for vascular changes in mice are rapid compared to larger vertebrate models and man, the findings here suggest that there may

be a therapeutic window to systemically deliver drugs to the wound bed that would optimally accelerate wound healing when new vessels are abundant and permeable.

The early accumulation of platelets and accompanying neutrophil infiltration that precedes by re-vascularization and integration of the synthetic graft into skin suggests that are highly organized processes that regulate the early steps of re-vascularization. For example, Dvorak and colleagues suggested that chronic vascular hyperpermeability that occurs over a period of days in full thickness wounds may initially involve the remodeling of mother blood vessels to form highly angiogenic blood vessels^{25, 32, 33}. These glomeruloid microvascular proliferations occur as part of 'mother vessels', but do not exclude other mechanisms such as sprouting angiogenesis and the recruitment of endothelial progenitor cells in the formation of new vessels. We have certainly observed here changes in vascular integrity of adjacent blood vessels during normal wound healing, and these changes may reflect a mechanism for the controlled release of growth factors/matrix proteins/cells into the wound bed. Clearly, further studies are required to assess how the kinetics of revascularization and VP are affected in wound healing defective models. To this end, the dynamic model described here and the availability of promoter driven reporter models make it possible to investigate the temporal-spatial changes in wound healing that define normal repair and regeneration.

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Figure 1. Hematoxylin/eosin analysis of Integra™ graft incorporation following surgery

A) Low power photomicrographs of hematoxylin/eosin stained Integra™ sections harvested at 3 days, B) 7 days and C) 14 days. D) High power micrographs of the graft after 3 days with prominent neutrophil infiltrate, and E) the underlying graft-muscle interface. F) High power micrographs of the Integra graft after 7 days with reduced levels of neutrophil infiltrate, and G) the re-vascularization of the integral-muscle interface (arrow). H) High power micrographs of the graft after 14 days with evidence of re-epithelialization, wound closure, and re-vascularization (arrow), as well as I) increased density of capillaries in underlying dermis. (W=Wound site, M=Margin; Bar=100µm in Panels A–I).

Figure 2. Characterization of revascularization in IntegraTM graft

A) Indirect immunofluorescent staining of skin graft cryosections was performed with an anti-CD31 antibody at 3, 7, 10, 14, and 28 days after surgery. Representative images acquired by laser scanning confocal microscopy are shown at each timepoint. Normal skin tissue (Left column) was from skin adjacent to the graft itself. Transition skin tissue (Middle column) was analyzed from the normal skin-graft interface. Dotted line demarcates the transition zone evident during days 3–14. Wound tissue (Right column) from the center of the graft. B) Quantitation of CD31-positive capillaries at each timepoint is shown using techniques described in Materials and Methods. (M=Margin, W=Wound Site, *=statistical significance $P<0.05$ is indicated between the 10 day wound site vs. 10 day margin, and 10 day wound site and 14 day wound site). Bar=100 μ m.

Figure 3. Analysis of blood vessel integrity in the wound bed following graft surgery
 Vascular leak was determined at 3, 7, 10, 14, and 28 days following graft surgery with Integra™ by intravenous administration of fluorescent 70 kDa dextran immediately before harvest as described in Materials and Methods. Extravasation of the fluorescent tracer was imaged with a laser scanning confocal microscope ex vivo on intact graft specimens (*=regions of increased vascular leak and dashed line outlines the region of the graft). Bar=100µm.

Figure 4. Non-Invasive quantitation of blood vessel permeability in wound bed

A) Representative images of changes in vascular permeability in the wound bed was determined at 4, 7, 10, 14, and 28 days following graft surgery by intravenous injection with a 70 kDa fluorescent tracer. Anesthetized mice were imaged (S=Sham and T=Tracer) in which both Sham and Tracer mice were subjected to the graft at each timepoint indicated but only the mice labeled as Tracer were subjected to an intravenous injection of fluorescent tracer as described in the Materials and Methods. Contraction of the wound over the 28 day timecourse is evident in the animals in the sham-injected column (arrowhead). B) Quantitation of changes in vascular permeability for the fluorescent tracer was performed at each time point and the relative fluorescent intensity determined for each graft (T) vs. un-injected sham (S) mice to account for background fluorescence of the subjects.