Published in final edited form as:

Ann Surg. 2021 January 01; 273(1): 173–180. doi:10.1097/SLA.0000000000003255.

Wounds Inhibit Tumor Growth In Vivo

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Abstract

Objective—The aim of this study was to determine the interaction of full thickness excisional wounds and tumors in vivo.

Summary of Background Data—Tumors have been described as wounds that do not heal due to similarities in stromal composition. On the basis of observations of slowed tumor growth after ulceration, we hypothesized that full thickness excisional wounds would inhibit tumor progression in vivo.

Methods—To determine the interaction of tumors and wounds, we developed a tumor xenograft/allograft (human head and neck squamous cell carcinoma SAS/mouse breast carcinoma 4T1) wound mouse model. We examined tumor growth with varying temporospatial placement of tumors and wounds or ischemic flap. In addition, we developed a tumor/wound parabiosis model to understand the ability of tumors and wounds to recruit circulating progenitor cells.

Results—Tumor growth inhibition by full thickness excisional wounds was dose-dependent, maintained by sequential wounding, and relative to distance. This effect was recapitulated by

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The authors have declared that no conflict of interest exists.

Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's Web site (www.annalsofsurgery.com).

placement of an ischemic flap directly adjacent to a xenograft tumor. Using a parabiosis model, we demonstrated that a healing wound was able to recruit significantly more circulating progenitor cells than a growing tumor. Tumor inhibition by wound was unaffected by presence of an immune response in an immunocompetent model using a mammary carcinoma. Utilizing functional proteomics, we identified 100 proteins differentially expressed in tumors and wounds.

Conclusion—Full thickness excisional wounds have the ability to inhibit tumor growth in vivo. Further research may provide an exact mechanism for this remarkable finding and new advances in wound healing and tumor biology.

Keywords

ischemic flap; parabiosis; progenitor cells; wound healing

For several decades, striking similarities between tumors and wounds have been noted.^{1,2} Tumors were first described as wounds that do not heal based on similarities between tumor stroma generation and wound healing.¹ Both processes involve hyperpermeable blood vessels, a fibrin-fibronectin provisional matrix, and mature stroma, consisting of inflammatory cells. In addition, both processes invoke vascular permeability factor (VPF), now referred to as vascular endothelial growth factor (VEGF), for angiogenesis.¹ As this comparison was first published, myriad studies have further elucidated the similarities in inflammation,^{3–5} angiogenesis,^{6,7} and stroma,⁸ as well as gene expression.⁹ However, few studies have examined potential interactions between coexisting wounds and tumors in vivo.

In this study, we examine direct in vivo tumor and wound interactions using a xenograft tumor and full thickness excisional wounds. Various wounding configurations reveal dose-dependent tumor inhibitory effects of wounds that are related to proximity of tumor and wound. Using an ischemic flap model, we recapitulate our findings using an in vivo model of angiogenesis competition. Next, we perform parabiosis studies to investigate competition of circulating progenitor cells. Using an allograft transplantation model, we demonstrate that our findings persist with an intact immune system. Finally, we utilize functional proteomics to identify differences between tumors and wounds in our allograft transplantation model.

METHODS

Mice

NSG (stock no: 005557), NSG-EGFP (stock no: 021937), and BALB/cJ (stock no: 000651) mice were purchased from The Jackson Laboratory (Bar Harbor, Maine) and housed in a 12-hour light/dark cycle and provided standard chow and water ad libitum. All animals used in these experiments were 12-week-old female mice. All animals were maintained at the Stanford University Comparative Medicine Pavilion in accordance with the Institutional Animal Care and Use Committee (IACUC) and National Institutes of Health (NIH) guidelines. All procedures in this experiment were performed in accordance with Stanford University Administrative Panel on Laboratory Animal Care (APLAC) 21308 and 28410.

Tumor Xenograft/Allograft

The dorsum of NSG or BALB/cJ mice were shaved and depilated using Nair (Church & Dwight, Co, Inc., Ewing, NJ). Human head and neck squamous cell carcinoma SAS cells or mouse breast carcinoma 4T1 cells, for xenograft and allograft tumors, respectively, were injected subcutaneously in the left dorsum in 100 μ L of serum free media (DMEM; Gibco, ThermoFisher Scientific, Waltham, MA), as previously described.¹⁰ Xenograft/allograft tumors were allowed to grow for 7 days until take was confirmed with palpation. Mice were randomized into treatment and control groups and tumor measurements were initiated. Measurements for length, width, and height were taken with a digital caliper and tumor volume was calculated using the formula: $V = 0.5(LWH)$, where V is tumor volume, L is length, W is width, and H is height. Tumor sizes were measured by the same blinded observer every other day and calculated as percent growth from initial volume.

Tumor Ulceration Model

Human head and neck squamous cell carcinoma SAS cells (5×10^5) were transplanted into NSG mice ($n = 5$), as described above. Tumor xenografts were allowed to grow until ulceration occurred with tumor measurements taken every other day, as described above. Day of ulceration was noted, and mice were sacrificed 10 days after ulceration. The first derivative of the tumor growth curve 10 days before and 10 days after ulceration was calculated by determining the slope.

Wound Over Tumor Model

Human head and neck squamous cell carcinoma SAS cells (5×10^5) were transplanted into NSG mice ($n = 10$ per group), as described above. Tumor xenografts were allowed to grow for 10 days. A 6mm full thickness excisional wound was created directly over the xenograft tumor. Measurements and digital photographs of wounds were taken every other day until mice met parameters for sacrifice. Wound closure was measured as a percentage of original wound using Adobe Photoshop (Adobe Systems, San Jose, CA).

Tumor Wound Competition Model

Tumor xenografts or allografts were performed, as described above. After tumor engraftment was confirmed, splinted 6mm full thickness excisional wounds were created on the right dorsum adjacent to tumor; adjacently rostral and caudal to tumor; or distally on the right dorsum, as previously described ($n = 10$).^{11,12} Wounds were covered in clear occlusive dressing (Tegaderm; 3M, Maplewood, MN) and changed every other day during which digital photographs were taken. Wound closure and tumor growth were determined, as described above.

Tumor Ischemic Flap Model

Tumor xenografts were performed in the rostral midline dorsum using methodology described above ($n = 10$ per group). A peninsular cranial pedicled dorsal flap was created by making a full thickness U-shaped incision (1.25 cm in width and 2.5 cm in length) on the dorsum just caudal to the xenograft tumor. The flap was elevated and a 0.13mm thick silicone sheet (Invotec International Inc, Jacksonville, FL) was placed below the tissue over

the underlying muscle bed. This silicone sheet prevents revascularization from the underlying tissue bed and forces revascularization from the cranial axial blood supply. The flap was closed with simple interrupted stitches using 6-0 nylon suture (Ethilon Nylon Suture; Ethicon Inc., Somerville, NJ). Digital photographs were taken on day 0 and day 10. The degree of ischemia, calculated as the area of ischemia divided by the total flap area, was determined using Adobe Photoshop.

Tumor Wound Parabiosis

Parabiosis was performed with NSG and NSG-EGFP mice, using methodology previously described.¹³ Cross-circulation was confirmed at 2 weeks by obtaining peripheral blood from the NSG parabiont with GFP⁺ cells. Human head and neck squamous cell carcinoma SAS cells (5×10^4) were injected subcutaneously into the dorsum of the NSG parabiont and a splinted full thickness excisional wound was created just caudal to the tumor xenograft, as described above. After 10 days, the tumor and wound were harvested, digested, and analyzed via fluorescence-activated cell sorting (FACS), using techniques previously described.¹⁴ Circulating cells were defined as GFP⁺. Circulating progenitor cells or undifferentiated cells from peripheral blood were defined as GFP⁺ Lin⁻, after lineage-negative gating of erythrocytes and immune cells, using Ter119, CD4, CD8a, Gr-1, CD45R, and CD11b. Hematopoietic progenitor cells were defined as GFP⁺ Lin⁻ c-Kit⁺ and putative endothelial progenitor cells were GFP⁺ Lin⁻ VEGFR2⁺.

Reverse Phase Protein Array (RPPA)

Mouse breast carcinoma 4T1 allograft tumors were generated on the dorsum of BALB/cJ mice, as described above. Splinted full thickness excisional wounds were created adjacently on the dorsum, as described above. After 10 days, both tumor and wound were excised, and tissue was snap frozen in liquid nitrogen. Specimens were sent to the MD Anderson Functional Proteomics Reverse Phase Protein Array (RPPA) Core Facility for RPPA using proprietary methodology.

Statistics

A normal distribution was used to assess whether the data met the assumptions of the statistical test utilized. An unpaired, 2-tailed

Student t test was used to compare groups. A $P < 0.05$ was considered statistically significant. Data represent mean $\pm$ standard error of measurement (SEM).

RESULTS

Ulceration or Wounding Slows Tumor Growth

Anecdotal clinical reports of cutaneous tumors and hemangiomas show that ulceration is associated with slowed tumor growth and more rapid involution, respectively.^{15,16} This was previously thought to occur when tumor growth occurs rapidly, surpassing the necessary angiogenesis for tumor survival.^{15,17} Consequently, tumor necrosis occurs centrally and tumors near the skin surface will ulcerate.¹⁸ We sought to observe this phenomenon using a human head and neck squamous cell carcinoma xenograft model. In this model, this tumor

will ulcerate relatively early and has relatively low rates of metastasis (Fig. 1A).¹⁹ A representative growth curve is depicted with point of ulceration marked (Fig. 1B). Comparison of growth rates before and after ulceration showed significant decrease after ulceration (Fig. 1C; $P < 0.05$, $n = 5$). On the basis of these data, we hypothesized that creation of a wound over a xenograft tumor would also slow its growth (Fig. 1D). When a full thickness excisional wound was created directly over a xenograft tumor before ulceration, there was significant inhibition of tumor growth by day 8 after wounding (Fig. 1E; $P < 0.05$, $n = 10$). The full thickness excisional wounds made over the tumor did not heal during the course of the experiment (Fig. S1, <http://links.lww.com/SLA/B603>; $P < 0.05$, $n = 10$), which is not surprising given these tumors will ulcerate spontaneously and prior reports with glioma spheroids demonstrate delayed cutaneous wound healing.²⁰ Taken together, these results suggest that full thickness excisional wounds can inhibit tumor growth.

Full Thickness Excisional Wounds Inhibit Tumor Growth

The above data support a tumor inhibitory effect by wounds. To explore this effect further, we placed splinted full thickness excisional wounds adjacent to xenograft tumors at a time when the tumor becomes palpable (Fig. 2A and Fig. S2A, <http://links.lww.com/SLA/B603>). Mouse wounds heal primarily by contraction due to the panniculus carnosus subcutaneous striated muscle. A splinted full thickness excisional wound allows mouse wounds to heal by granulation and reepithelialization, humanizing the temporal aspect of the wound repair process.¹¹ Placement of a single wound adjacent to the tumor resulted in tumor inhibition (Fig. 2A, B, blue vs red, and Fig. S2B, <http://links.lww.com/SLA/B603>; $P < 0.05$, $n = 10$). Placement of 2 wounds adjacent to the tumor resulted in increased tumor inhibition (Fig. 2A, B, blue vs red vs green, and Fig. S2C&D, <http://links.lww.com/SLA/B603>; $P < 0.05$, $n = 10$), suggesting a dose-dependent-like effect. Tumor inhibition was maintained when a repeat wound was created in the previously healed wound in a sequential wounding (Fig. 2A, B, blue vs red vs purple, and Fig. S2E, <http://links.lww.com/SLA/B603>; $P < 0.05$, $n = 10$). Interestingly, when the wound was placed approximately 3 times the distance, there was no significant difference in tumor size (Fig. 2A, B, blue versus red vs orange, and Fig. S2F, <http://links.lww.com/SLA/B603>; $P > 0.05$, $n = 10$). Of note, there was no difference in wound healing rate between a wound alone and a wound adjacent to a tumor (Fig. S3, <http://links.lww.com/SLA/B603>, $P > 0.05$, $n = 10$), suggesting the xenograft tumors do not influence wound healing when placed adjacently. Collectively, these data demonstrate the tumor inhibitory effect of wounds on tumors to be dose-dependent, maintained by sequential wounding, and related to distance.

Ischemic Flap Inhibits Tumor Growth

Given the tumor inhibitory effect of wounds, we sought to determine the effect of an ischemic flap placed adjacent to a xenograft tumor. Pedicled flaps are commonly used in plastic surgery to fill soft tissue defects.^{21,22} They have been shown to possess an ischemic gradient along the length of the flap.²³ The 3-sided full skin thickness peninsular flap is a common experimental mouse model of such cutaneous ischemia.²³ By creating an ischemic flap with a silicone sheet placed underneath the flap, an ischemic environment is created in the adjacent tumor (Fig. 3A and Fig. S4, <http://links.lww.com/SLA/B603>). Placement of an

ischemic flap resulted in tumor inhibition (Fig. 3B; $P < 0.05$, $n = 10$). In the ischemic flap model, the distal flap becomes necrotic. However, results from our distant wounding model suggests that the necrotic tip of the flap is unlikely to directly affect the growing tumor (Fig. 2B, orange, and Fig. S2F, <http://links.lww.com/SLA/B603>). These results suggest an inhibitory response that is related to ischemia and a competition for angiogenesis.

Wounds Outcompete Tumors for Circulating Cells

To further delineate the competition between tumors and wounds in vivo, we utilized a parabiosis model to assess recruitment of circulating cells (Fig. 4A). Parabiosis was performed with NOD-SCID-gamma (NSG) and NSG-GFP mice. Cross-circulation was confirmed at 2 weeks by obtaining peripheral blood with GFP⁺ cells from the NSG parabiont (Fig. 4B). Tumor xenograft and splinted full thickness excisional wound were created on the dorsum of the NSG parabiont (Fig. 4A, lower left). FACS analysis of the tumor and wound demonstrate significantly increased recruitment of circulating GFP⁺ cells by the wound (Fig. 4C; $P < 0.05$, $n = 5$). The wound also recruited more circulating progenitor cells (GFP⁺ Lin⁻) than the tumor (Fig. 4D; $P < 0.05$, $n = 5$). Further characterization of progenitor cells into hematopoietic (GFP⁺ Lin⁻ c-Kit⁺) and endothelial (GFP⁺ Lin⁻ VEGFR2⁺) showed preferential recruitment by wounds versus tumors (Fig. 4E; $P < 0.05$, $n = 5$, and Fig. 4F; $P < 0.05$, $n = 5$). Although this model has limitations, these data suggest the power of physiological wound healing, in terms of circulating cell recruitment, as compared to that of a xenograft tumor. The results may simply be due to the self-recognized damage patterns and recruitment factors inherent to a mouse wound versus a xenografted tumor. Moreover, the temporospatial recruitment and role of these circulating cells is yet unclear. Further research into this parabiotic tumor wound model may reveal additional insight into tumor and wound healing biology.

Tumor Inhibition Persists with Intact Immune Response

Recent research has demonstrated a role of the systemic inflammatory response in the outgrowth of tumor cells at distant anatomical sites.²⁴ Given these results, the diminished immune response in our immunocompromised mouse model may play a role in our findings. Therefore, we sought to repeat our experiments in an immune competent mouse model. We utilized an allograft transplantation model using mouse 4T1 mammary carcinoma and BALB/cJ mice. When a full thickness excisional wound was placed adjacent to the tumor allograft, there was significant inhibition of tumor growth by day 8 after wounding (Fig. S5A, <http://links.lww.com/SLA/B603>; $P < 0.05$, $n = 10$). These results suggest that tumor inhibition is independent of the immune response. Heterogeneity of tumor histology precluded accurate analysis. Therefore, we utilized functional proteomics using reverse phase protein array (RPPA) of entire tumors and healing wounds to identify 100 out of 232 differentially expressed proteins investigated in the tumor and wound (Fig. S5B, <http://links.lww.com/SLA/B603> and Table S1, <http://links.lww.com/SLA/B603>). Although detailed exploration into each of these 100 proteins has yet to be performed, further research into these proteins may reveal the exact mechanism behind our data showing adjacent tumors or ischemic flaps significantly impair tumor growth.

DISCUSSION

The similarities between tumors and wounds have been described for several decades.^{1,2} However, direct in vivo interactions have not been examined. Dvorak¹ described that “tumors are wounds that do not heal.” This was based on the observation that, like healing wounds, tumors acquire a fibrin-based stroma that is derived from leaking plasma proteins that coalesce into a matrix. However, in wounds the fibrin matrix recedes, whereas in tumors, a VPF is persistently produced, and the fibrin matrix is maintained, which may provide a defense against the immigration of immune cells.

In recent years, our understanding of the similarities between wounds and tumors has been refined. It is clear that both tumors and healing wounds utilize many of the same growth-promoting factors, such as transforming growth factor- β , fibroblast growth factor-2, and platelet derived growth factor, to maintain their own development.²⁵ DNA microarray analysis of fibroblast cells exposed to serum, a model for wound healing, has allowed the discovery of a “core serum response,” a set of genes that are upregulated by the serum-exposed fibroblasts.⁹ The genes seen in the core serum response are involved in promoting cell replication, cell motility, and extracellular matrix remodeling. Further experiments have shown that some, but not all tumors, exhibit the same core serum response.⁹ In a series of patients with breast cancer, those whose tumors exhibited gene expression similar to the core serum response were more likely to progress to metastasis and death, implying that the core serum response confers more aggressive behavior to a tumor.²⁶ It is well known that healing wounds rely on angiogenesis that scales down appropriately when healing is complete.²⁷ Tumors benefit from abnormal angiogenesis that does not self-regulate in the same way, but tumor angiogenesis is promoted by many of the same growth factors employed in wounds, such as VEGF, fibroblast growth factor, and platelet derived growth factor.²⁸

We sought to investigate whether tumor ulceration correlated with a slowing of tumor growth. It has been presumed that tumor ulceration occurs as a result of rapid tumor progression whereby the tumor outgrows its blood supply leading to ulceration.¹⁷ By comparing tumor growth before and after ulceration, we show a slowing of tumor growth after ulceration. Although the change in tumor growth slope is significant, the physiological significance is unclear. Anecdotally, wounds have been observed to stimulate regression of benign tumors, particularly hemangiomas.¹⁵ By placing a wound directly over the xenograft tumor, we show tumor growth was inhibited, although the extent may not be physiologically significant. The wound placed over the tumor does not heal, which is consistent with prior studies of skin incisions placed adjacent to glioma spheroids.²⁰ Studies have shown the propensity for chronic wounds to develop tumors (eg, Marjolin ulcer) and mechanisms have been investigated.²⁹

We next created an experimental mouse model in which xenograft or allograft tumors were placed adjacent to splinted full thickness excisional wounds in different configurations or an ischemic flap. Our studies show that full thickness excisional wounds inhibit tumor growth in vivo. This effect was shown to be dose-dependent, where placement of 2 wounds augmented tumor inhibition. This inhibition could also be maintained by creating another wound after the wound had healed. We showed that the inhibition of tumor growth is

abrogated when the wound is placed farther away from the tumor, implying that local factors are involved, and that the inhibition of tumor growth is not due to systemic factors or a global inhibition of cell growth. Similarly, when an ischemic flap was placed adjacent to a tumor, there was inhibition of tumor growth. This suggests an angiogenic mechanism, as the ischemic flap preferentially competes for blood supply.

Wound healing is a highly complex process that is thought to have evolved to rapidly protect following injury, allowing survival and procreation.³⁰ Therefore, it is not surprising that the wound repair process is able to outcompete tumor progression. Our results demonstrate that a wound will preferentially recruit more circulating cells than a tumor. Further research is necessary to understand the exact mechanism behind our findings, and how it relates to clinical observations, such as involution of hemangiomas upon ulceration. A recent study has shown that the wound healing response to surgery triggers the outgrowth of otherwise dormant metastases using subcutaneously implanted sterile polyvinyl acetate sponges.²⁴ This suggests further complexity by a systemic response that has been suggested in other studies.³¹ Continued exploration between tumor wound interactions promises to provide new insight into both wound healing and cancer biology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

The authors would like to thank Jennifer G. Gonzalez, Christina N. Thomas, Alexander T.M. Cheung, and Samir Malhotra for their technical assistance.

Experiments from this study were performed at the MD Anderson Functional Proteomics Reverse Phase Protein Array (RPPA) Core Facility, a facility funded by the National Cancer Institute (NCI, #CA16672). M.S.H. was supported by the California Institute for Regenerative Medicine (CIRM) Clinical Fellow training grant TG2-01159 and the Stanford University School of Medicine Transplant and Tissue Engineering Fellowship Award. M.S.H., H.P.L., and M.T.L. were supported by the American Society of Maxillofacial Surgeons (ASMS)/Maxillofacial Surgeons Foundation (MSF) Research Grant Award. G.C.G., H.P.L., and M.T.L. were supported by the Hagey Laboratory for Pediatric Regenerative Medicine and The Oak Foundation. H.P.L. was supported by National Institutes of Health (NIH) grant R01 GM087609 and a gift from Ingrid Lai and Bill Shu in honor of Anthony Shu. H.P.L. and M.T.L. were supported by NIH grant R01 GM116892. I.L.W. and M.T.L. were supported by the Gunn/Olivier fund. M.T.L. was supported by NIH grant U01 HL099776.

This work was supported by grants from the National Cancer Institute (NCI), California Institute for Regenerative Medicine (CIRM), Stanford University School of Medicine, American Society of Maxillofacial Surgeons (ASMS)/Maxillofacial Surgeons Foundation (MSF), Hagey Laboratory for Pediatric Regenerative Medicine, The Oak Foundation, National Institutes of Health (NIH), a gift from Ingrid Lai and Bill Shu, and the Gunn/Olivier fund.

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FIGURE 1.

Tumor ulceration or wounding slows tumor growth. (A) Schematic showing natural progression of tumor (brown) with ulceration (red) in our in vivo xenograft tumor model. (B) Representative graph of tumor growth curve with point of ulceration noted by arrow. Blue line indicates slope of tumor growth before ulceration. Red line indicates slope of tumor growth after ulceration. (C) Bar graph representing tumor growth curve slopes before and after ulceration (* $P < 0.05$, $n = 5$). (D) Schematic showing experimental model

(brown=tumor; red=wound). (E) Tumor growth curve of tumors without or without full thickness excisional wounds directly above tumor ($*P < 0.05$, $n = 10$).

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FIGURE 2. Adjacent wounds inhibit tumor growth. (A) Schematic of tumor and wound competition model showing 1. Tumor only (blue), 2. Tumor wound (red), 3. Tumor 2 wounds (green), 4. Tumor sequential wounds (purple), and 5. Tumor distant wound (orange). (B) Tumor growth curve of 1. Tumor only (blue), 2. Tumor wound (red), 3. Tumor 2 wounds (green), 4. Tumor sequential wounds (purple), and 5. Tumor distant wound (orange) (n = 10).

FIGURE 3.

Ischemic flap inhibits tumor growth. (A) Schematic showing experimental model in which a cranial pedicled ischemic flap is placed caudal to a xenograft tumor. (B) Tumor growth curve of tumors with or without ischemic flaps (* $P < 0.05$, $n = 10$).

FIGURE 4.

Wounds outcompete tumors for circulating cells. (A) Schematic of parabiosis model. Following establishment of crosscirculation, wounds and tumors are created on nongreen mouse (lower left panel). (B) Fluorescence image of peripheral blood taken from NSG parabiont at 2 weeks confirming cross circulation. (C) Fluorescence-activated cell sorting (FACS) analysis plots of tumor and wound showing GFP⁺ cells (left) and bar graph (right; **P* < 0.05, *n* = 5). (D) FACS analysis plots of tumor and wound showing GFP⁺ Lin⁻ cells (left) and bar graph (right; **P* < 0.05, *n* = 5). (E) FACS analysis of daughter plots from (D)

of tumor and wound showing GFP⁺ Lin⁻ c-Kit⁺ cells (left) and bar graph (right; * $P < 0.05$, n = 5). (F) FACS analysis of daughter plots from (D) of tumor and wound showing GFP⁺ Lin⁻ VEGFR2⁺ cells (left) and bar graph (right; * $P < 0.05$, n = 5).