Wound-Induced Tumor Progression

A Probable Role in Recurrence After Tumor Resection

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Objective: To determine the effect of several wound factors on melanoma growth in a mouse model.

Design: Cohort analytic study.

Setting: Animal research facility of Roger Williams Medical Center, Providence, RI.

Study Group: Seventeen groups of 5 C57BL/6 mice each.

Interventions: A surgical wound was created in 1 hind limb, after which different concentrations of B16F10 melanoma cells were injected in adjacent subcutaneous tissue. The nonwounded hind limb in the same mouse served as a control. In this fashion, a critical tumor cell dose was determined that showed tumor growth in the wounded but not the control hind limb. Tumor growth in control hind limbs then was compared with that in the “artificially wounded” hind limbs, which were co-injected with mouse wound fluid or growth factors. Early (day 1) and late (day 10) wound fluids and tumor growth factor β (TGF-β), basic fibroblast growth factor (bFGF), both combined, and interleukin 6 (IL-6) were used.

Main Outcome Measure: Wound factors increase tumor growth, indicating potentiation of tumor recurrence at a surgical wound.

Results: The critical tumor cell dose was $10^3$ cells. All growth factors and both wound fluids showed increased tumor growth over time except IL-6. Hind limbs injected with early wound fluid showed increased tumor growth over time when compared with those injected with late wound fluid ($P<.001$), TGF-β ($P<.001$), bFGF ($P<.001$), and IL-6 ($P<.001$). Combined TGF-β and bFGF co-injection resulted in increased tumor growth compared with TGF-β ($P<.001$) and bFGF ($P<.001$), but did not differ significantly from early wound fluid ($P<.07$).

Conclusions: The healing wound and its mediators in wound fluid or purified growth factors significantly enhanced tumor growth. Combining TGF-β and bFGF increased tumor growth to a level closer to wound fluid. The inflammatory response provoked by wound healing mediators may be an important mechanism in tumor growth after ablative surgery.

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The relationship of tumor growth to wound healing is an area that has particular relevance to all surgeons with an interest in cancer management. In clinical practice, this may play a role in tumor recurrence after primary resection for cancer. Tumor growth following invasive diagnostic or therapeutic procedures for malignant disease may well be a consequence of this relationship.1,2 Significantly fewer clonogenic cells are necessary to grow a tumor in a wounded site than in nonwounded tissue.3 This finding was attributed to growth factors derived from the traumatized tissue and the inflammatory cells at the wound site. Growth factors released during wounding are not only important in the process of healing during inflammation and granulation tissue formation,4 but can also contribute to creating a suitable environment for wound-induced tumor growth.5,6 Further identification of molecular mediators should yield important clues to the mechanisms involved in tumor recurrence after tumor resection.

The aim of this study was to determine the effect of wounding on tumor growth. A critical tumor cell dose that showed tumor development in a wounded site, but not in a nonwounded site, was co-injected with wound fluids or single or combination doses of growth factors as they exist in the healing wound. A possible mechanism of tumor growth caused by wounding was evaluated.

RESULTS

The B16F10 murine melanoma cells proved to be a very aggressive, fast-growing cell line. No tumors developed later than 32 days.
MATERIALS AND METHODS

ANIMALS AND ANESTHESIA

Female C57BL/6 mice, 6 to 8 weeks old and weighing 18 to 20 g, were obtained for this study from Taconic Farms, Germantown, NY. Mice were kept at the animal research facility of Roger Williams Medical Center, Providence, RI. All surgical procedures were performed under anesthesia, which consisted of intraperitoneal infusion of sodium pentothal (60 mg/kg). Subcutaneous injections with 28-gauge needles and shaving of hind limbs the day before the experiment were performed without anesthesia. All experiments were performed with the authorization of the Animal Experimentation Committee at Brown University, Providence.

CELLS

Murine melanoma cells (B16F10) were originally obtained from I. J. Fidler, MD, PhD, M. D. Anderson Cancer Center, Houston, Tex, and have subsequently been maintained in culture in our laboratories. The B16F10 cells were cultivated in serum-free and protein-free medium (Sigma Hybri-max, Sigma Chemical Co, St Louis, Mo) supplemented with 10% fetal bovine serum, 2 mL of L-glutamine, 50-U/mL penicillin, and 50-g/mL streptomycin (Gibco, Long Island, NY).

WOUND FLUID

Rectangular polyvinyl alcohol sponges (Unipoint Industries, High Point, NC) were prepared for implantation as described previously. Six sponges per animal were implanted through a dorsal midline incision of 2 cm in 2 groups of 5 mice each. The sponges were inserted in subcutaneous pockets that had been formed by blunt dissection. Incisions were closed with a wound clip. At day 1 and 10 after implantation, the sponges were harvested after mice had been killed with carbon dioxide inhalation. In this fashion, early (day 1) and late (day 10) wound fluids could be collected. Sponges were placed in syringe cylinders contained within sterile test tubes and centrifuged for 10 minutes (400g) at 4°C. The cell-free wound fluid was passed through a 0.45-μm filter and frozen at -20°C. Early and late wound fluids were tested as mediators of tumor growth by local subcutaneous application instead of surgical wounding. All wound fluids were prepared at the Division of Surgical Research of Rhode Island Hospital, Providence.

GROWTH FACTORS

Growth factors were obtained commercially (GIBCO BRL, Life Technologies, Gaithersburg, Md). Transforming growth factor-β (TGF-β), basic fibroblast growth factor (bFGF), and interleukin 6 (IL-6) were tested as mediators of tumor growth by local subcutaneous application instead of surgical wounding.

SURGICAL WOUNDING

This series of experiments was performed to determine a critical tumor cell dose for B16F10 melanoma in this model. This dose was defined as the maximal number of tumor cells injected that would show tumor growth in the wounded but not in the nonwounded hind limb (Figure 1 and Table 1). Tumor growth in a surgi-
cally wounded hind limb was compared with the contralateral nonwounded hind limb. Hind limbs injected with $10^6$, $10^5$, or $10^4$ B16F10 cells showed tumor growth in both wounded and nonwounded hind limbs. No tumor growth was found in nonwounded hind limbs injected with $10^3$ or $10^2$ cells, whereas wounded hind limbs needed only $10^2$ cells to show tumor growth. Two groups were not suited for proper statistical analysis because of small numbers caused by animal death during anesthesia in group $10^5$ and automutilation of the wounded site in 2 animals in group $10^3$. Groups $10^2$ and $10^3$ were chosen for the second part of the study because they showed no tumor growth in nonwounded hind limbs and $10^2$ cells proved to be enough to induce tumor growth in wounded hind limbs.

**ARTIFICIAL WOUNDING**

This series of experiments was performed to determine possible mechanisms that cause tumor growth after wounding in B16F10 melanoma. Two cell doses, $10^2$ and $10^3$, were chosen from the results in series 1 owing to the fact that they showed no tumor growth in the nonwounded hind limbs. Hind limbs injected with $10^3$ cells showed only minor differences in tumor development of treated vs control hind limbs (Figure 2).

After injection with $10^3$ B16F10 cells, hind limbs co-injected with early wound fluid showed significantly more tumor growth in time when compared with controls (mean $r=0.9$; 95% confidence interval [CI], 0.69-0.97). Hind limbs co-injected with late wound fluid showed more tumor growth in time when compared with controls (mean $r=0.997$; 95% CI, 0.989-0.999). Hind limbs co-injected with growth factor gave the following results when the growth difference between hind limbs was correlated with time. Hind limbs co-
injected with TGF-β and bFGF showed more tumor growth than controls (mean r=0.93; 95% CI, 0.78-0.98), as did TGF-β (mean r=0.80; 95% CI, 0.34-0.92) and bFGF (mean r=0.998; 95% CI, 0.99-0.999). Hind limbs co-injected with IL-6 did not give more tumor growth than controls (mean r=0.08; 95% CI, -0.49-0.61) (Table 2, Table 3, and Table 4).

Early wound fluid showed increased tumor growth in time when compared with late wound fluid (P<.001), TGF-β (P<.001), bFGF (P<.001), and IL-6 (P<.001). The comparison between early wound fluid and the TGF-β and bFGF combination was not significant (P<.07). Combined administration of TGF-β and bFGF yielded a stronger response than single administration. Hind limbs co-injected with TGF-β and bFGF showed increased tumor growth in time when compared with TGF-β (P<.001) or bFGF (P<.001) co-injected hind limbs (Figure 3).
The observation of tumor growth after wounding has been described on many occasions.8-10 After surgical resection of a tumor, the microenvironment of the wound site differs from that of normal tissue in several ways. Hypoxia, fibroblast activation, and various growth factors released after wounding make the wounded site different from nonwounded tissue. It is suggested that these events are involved with increased tumor recurrence after surgical resection of a tumor. The model in this study offered the opportunity to determine the difference in tumor growth between wounded and nonwounded tissue with each animal serving as its own control. The study was designed to identify possible mechanisms of tumor recurrence after ablative surgery. For this purpose, a critical tumor cell dose was determined, which can be representative for different tumor cells shed after ablative surgery. The requirement of a lower critical tumor cell dose in wounded tissue vs control tissue suggests that the change in the wounded tissue microenvironment is responsible for an increase of tumor cell growth. Our results confirm earlier reports3 that showed that fewer tumor cells are required in wounded tissue to form solid tumors.

### Table 4. Tumor Growth in the Different Study Groups After Injecting 10^2 and 10^3 B16F10 Murine Melanoma Cells in Artificially Wounded and Nonwounded Hind Limbs

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean r</th>
<th>95% CI</th>
<th>P vs WF Day 1</th>
<th>P vs TGF-β or bFGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>WF day 1</td>
<td>0.30</td>
<td>0.69-0.97</td>
<td>. . . .</td>
<td>. . . .</td>
</tr>
<tr>
<td>WF day 10</td>
<td>0.997</td>
<td>0.989-0.999</td>
<td>&lt;.001</td>
<td>. . . .</td>
</tr>
<tr>
<td>TGF-β + bFGF</td>
<td>0.93</td>
<td>0.78-0.98</td>
<td>&lt;.07</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>TGF-β</td>
<td>0.80</td>
<td>0.54-0.92</td>
<td>&lt;.001</td>
<td>. . . .</td>
</tr>
<tr>
<td>bFGF</td>
<td>0.998</td>
<td>0.99-0.999</td>
<td>&lt;.001</td>
<td>. . . .</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.08</td>
<td>-0.49-0.61</td>
<td>&lt;.001</td>
<td>. . . .</td>
</tr>
</tbody>
</table>

* Growth difference between hind limbs is correlated with time using the Spearman rank correlation. Correlation coefficients are averaged per group after z transformation and are given with their 95% confidence interval (CI). The growth differences between hind limbs in the groups are compared using nonparametric 2-way analysis of variance (ANOVA). The levels of significance of the differences are given. WF indicates wound fluid; TGF-β, tumor growth factor β; bFGF, basic fibroblast growth factor; IL-6, interleukin 6; and ellipses, not applicable.

### Figure 3. Tumor size in millimeters (median paired difference between wounded and nonwounded hind limbs) vs time in days. Six curves comparing tumor growth stimulation of the co-injected growth factors or wound fluids in hind limbs injected with 10^3 B16F10 murine melanoma cells.

The explanation for the fact that tumor growth is increased in wounded tissue may be the facilitation of implantation of tumor cells in the wound environment. This explanation is supported by the observations of tumor growth at sites distant from the local site.14 In the same manner, however, local recurrence can occur. On the other hand, cytokinetic studies have shown that certain wound factors seem capable of shifting cells from the nonproliferative (Go or G1) phases into proliferation.11 From these observations it was concluded that increased tumor cell growth in the microenvironment of the wound was caused by growth factors. This speculation needs further evaluation.

The groups receiving 10^2 and 10^3 cells showed no tumor growth in nonwounded hind limbs. The critical tumor cell dose between nonwounded hind limbs in this study is between 10^3 and 10^4. The group with only 10^2 cells already showed substantial tumor growth in wounded hind limbs. The 10^2 and 10^3 groups were chosen for the second part of the study, regardless of the fact that there had been problems with 2 groups in the first part of the study (10^2 group, death of 2 animals during anesthesia; 10^3 group, automatical of the wounded site in 2 animals). These 2 groups were chosen because no tumors developed in the nonwounded site, whereas they did develop in the wounded site. The decision to add the 10^3 group was made because it was not known how strong the wounding effect of the externally administered wound factors would be, ie, if the dose of factors administered would be equally potent as a surgical wound. The main contribution of this study is the co-injection experiments with wound fluids or growth factors.

To come to a better understanding of the mechanism that causes increased tumor growth at a wounded site, different substances were used to mimic wounding. Early and late wound fluids contain a plethora of growth factors. These fluids differ in consistency and contain no cellular components of the wound-healing process. Both showed a strong tumor growth–promoting effect in this study. Our results show that there are substances in early wound fluid that facilitate tumor implantation and growth better than those in late wound fluids or growth factors.
fluid. No analysis of either wound fluid was performed. Future analysis should point out the factors that are contributing to the potentiation of tumor growth in this model.

To simplify the problem, 3 growth factors (TGF-β, bFGF, and IL-6) that are present in wound fluid and have been described as yielding a strong inflammatory response were applied to mimic wounding. The ability to elicit a strong inflammatory response has been associated with enhanced tumor growth. Tumor growth factor β, bFGF, and particularly the combination of both showed significantly increased tumor growth when co-injected with 10⁶ tumor cells. Co-injection of 10⁶ tumor cells with growth factors or wound fluids did not yield good tumor implantation and growth, whereas significant tumor growth was observed when the tumor cells were injected into the surgically wounded site. The observed lack of growth may be improved by co-injection with a higher growth factor dose. Tumor growth stimulation by growth factors or wound fluids is dose dependent. We adopted the present growth factor dose from a report in which increasing the growth factor dose gave a higher ratio of tumor development in Rous sarcoma virus–infected chicks. The growth factor dose in a surgical wound site is unknown.

The comparison of all wound fluids and growth factors used in this study showed that early wound fluid had the strongest effect. The tumor growth–stimulating effect over time of early wound fluid was larger than that of singly administered growth factors. The combination of TGF-β with bFGF showed higher tumor growth stimulation over time than single growth factors. The difference of tumor growth over time between early wound fluid and TGF-β and bFGF was not statistically significant. This shows that the combination of growth factors gives a stronger response, resembling early wound fluid. Further efforts must be directed toward the analysis of early and late wound fluids. Early released growth factors such as interleukin 1, IL-6, PDGF, and TGF-β may play an important role in strong tumor growth stimulation.

Additional factors play a role in wound healing. Cellular components (eg, fibroblasts, polymorphonuclear cells) and hypoxia may contribute to tumor cell growth in a surgically wounded site. The strong response elicited by the growth factors added in our study led to the conclusion that TGF-β and bFGF play an important role in the mechanism of tumor growth progression. Most likely this is due to facilitation of implantation, which is necessary for further growth. In future studies, further attention needs to be directed toward dose–response curves of different growth factors and their combinations, as well as their presence in wound fluids. After that it will be of interest to selectively block the mechanism of tumor growth progression found in the present study. Selective inhibitory factors, like anti–TGF-β or anti-bFGF may well antagonize the implantation and growth of tumor cells after wounding. Anti–TGF-β has been used successfully in the prevention of chronic inflammation. A locally applicable, prophylactic antigrowth factor combination that, when administered at the right time, decreases recurrence after ablative surgery should be the end point.

In conclusion, the healing wound and its mediators in wound fluid or purified growth factors, TGF-β and bFGF, significantly enhanced tumor growth. Combining TGF-β and bFGF increased tumor growth to a level closer to early wound fluid. The inflammatory response provoked by wound healing mediators may be a major mechanism in the tumor implantation and growth after wounding in this melanoma model and may suggest a mechanism for the common phenomenon of tumor recurrence after ablative surgery.

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We thank Wim Sluiter, PhD, Department of Endocrinology, University Hospital Groningen, Groningen, The Netherlands, for statistical analysis of the data.

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REFERENCES

Francis Moore, Jr, MD, Boston, Mass: From the point of view of the surgical oncologist, this paper seems to replicate and perhaps clarify the clinical circumstance of local recurrence after primary tumor resection, and the logic of the work would point us in the direction of preventing local recurrence in the future with an anticytokine therapy or maybe a less traumatic form of local surgery. But, from the point of view of the immunologist, this model looks a lot like a vaccination. I would like to direct your attention to what is happening in the control limb. In other words, does this model suppress growth on the control side rather than augment growth on the experimental side?

Let’s look briefly again at the data. In the initial titration of the $10^3$ or the $10^2$ cells, there was no tumor growth on the nonwounded side. But, in some of the artificial wounds, especially in those animals that received saline or IL-6, we now see that there is growth on the control side. This is particularly interesting in light of the fact that there wasn’t tumor growth on the control side in any of the animals that received saline as the control injection for the artificial wound. We are not provided any data regarding growth of tumor from a $10^2$ or a $10^3$ tumor cell inoculum in otherwise untreated mice. So, I would suggest really that the proper control in these experiments was the comparison to the mouse that only received tumor and did not have the wound on the other side.

What additional data do Dr Hofer and the authors have to support the interpretation that these results indicate wound-induced tumor progression rather than suppression of growth at a remote site?

Dr Hofer: The artificial wound model is not a model of tumor suppression on the other side, because—let me put it another way. You said you would need to inject mice without any wounds to see how many cells are needed to grow a tumor, but we chose for this model to get the same circumstances on both sides. What we’ve seen in some of the mice that grow really large tumors (and we believe that the tumors start shedding their own growth factors) that even those are mice that have no tumor on the control side for a considerable amount of time and now start growing tumors on the control side. We believe that they are stimulated by the growth factors from the growing tumor. It is not tumor suppression; tumors are stimulated by the growth factor injection.

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New Section: “Operative Techniques”

The Archives of Surgery is instituting a new section, “Operative Techniques,” wherein various simple and complex procedures will be presented. Drawings of operative photographs should clearly illustrate sequential steps in the procedure. Each drawing should be accompanied by a legend and sufficient descriptive text so that the reader is taken through the procedure in an orderly manner. Color drawings or photographs may be used if they would clearly enhance the reader’s understanding of the procedures.

We are pleased to announce that Dr Jack Pickleman, a member of the Editorial Board of the Archives of Surgery, will serve as the Director of this section.

Claude H. Organ, Jr, MD