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Original Article

Characterization of surgical models of postoperative tumor recurrence for preclinical adjuvant therapy assessment

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Abstract: Purpose: Nearly 30% of cancer patients undergoing curative surgery succumb to distant recurrent disease. Despite large implications and known differences between primary and recurrent tumors, preclinical adjuvant therapy evaluation frequently occurs only in primary tumors and not recurrent tumors. We hypothesized that well characterized and reproducible models of postoperative systemic recurrences should be used for preclinical evaluation of adjuvant approaches. Experimental Design: We examined traditional animal models of cancer surgery that generate systemic cancer recurrences. We also investigated models of systemic cancer recurrences that incorporate spontaneously metastatic cell lines and surgical resection. For each model, we critiqued feasibility, reproducibility and similarity to human recurrence biology. Using our novel model, we then tested the adjuvant use of a novel systemic inhibitor of TGF-β, 1D11. Results: Traditional surgical models are confounded by immunologic factors including concomitant immunity and perioperative immunosuppression. A superior preclinical model of postoperative systemic recurrences incorporates spontaneously metastatic cell lines and primary tumor excision. This approach is biologically relevant and readily feasible. Using this model, we discovered that “perioperative” TGF-β blockade has strong anti-tumor effects in the setting of advanced disease that would not be appreciated in primary tumor cell lines or other surgical models. Conclusions: There are multiple immunologic effects that rendered previous models of postoperative cancer recurrences inadequate. Use of spontaneously metastatic cell lines followed by surgical resection eliminates these confounders, and best resembles the clinical scenario. This preclinical model provides more reliable preclinical information when evaluating new adjuvant therapies.

Keywords: Surgery, recurrence, models, surgical oncology, concomitant immunity, perioperative immunosuppression, TGF-β

Introduction

Systemic cancer recurrences occurring after “curative” surgical resection account for more than 300,000 deaths each year [1]. These recurrences often result from the proliferation of micrometastatic disease that was undetected at time of surgery [2]. These metastatic foci are established during primary tumor growth and are the result of hematogenous and/or lymphatic spread to distant locations [3].

In attempts to prevent morbidity and mortality from systemic cancer recurrences, surgical candidates often receive adjuvant protocols incorporating chemotherapy, targeted biologics, or immunotherapy. Despite common use in an adjuvant setting, few of these modalities have been critically analyzed using preclinical models of recurrent diseases. This is at least partially driven by the paucity of such recurrence models readily available to the translational researcher. Instead, preclinical analysis has been carried out in models of primary cancer then assumed to be applicable to recurrent disease scenarios. Such an assumption is concerning given well-documented immunologic changes that occur
after surgery and differences between primary and recurrent disease [4]. With this in mind, our group has become interested in investigating adjuvant therapies in preclinical models incorporating surgery and systemic recurrence development. More specifically, we sought to critically evaluate and develop murine models of systemic cancer recurrences that occur following surgery. Using a biologically relevant and reliable model of systemic recurrence, we examined the impact of immunotherapy (systemic TGF-β) on postoperative recurrences. This novel model revealed an impressive synergy between surgery and immunotherapy that would have been overlooked in traditional preclinical tumor models that fail to incorporate surgical resection.

**Materials and methods**

**Animals**

Female C57Bl/6, BALB/c, and B6x129/J1 hybrid (6-8 weeks old) mice were purchased from Charles River Laboratories, Inc. (Wilmington, MA). All mice were maintained in a pathogen-free animal facility for one week prior to experimentation. The Animal Care and Use Committees of the Wistar Institute and University of Pennsylvania approved all animal study protocols described in this publication, and experiments were conducted in compliance with the Guide for the Care and Use of Laboratory Animals.

**Cell lines**

The murine malignant mesothelioma cell line, AB12, was derived from an asbestos-induced tumor and has been previously described in detail [5]. The murine esophageal carcinoma cell line, AKR, was derived from mouse esophageal squamous epithelia with cyclin D1 over expression via Epstein-Barr virus ED-L2 promoter in p53 deficient genetic backgrounds [6]. The murine lung cancer cell line, TC1, was derived from mouse lung epithelial cells immortalized with HPV-16 E6 and E7 and transformed with the c-Ha-ras oncogene [7]. The spontaneously metastatic murine lung cancer line, LKR, was derived from an explanted pulmonary tumor from an activated KrasG12D mutant mouse that had been induced in an F1 hybrid of 129SvJ and C57Bl/6 [8]. The metastatic NSCLC cell line, murine Lewis lung carcinoma (LLC), was obtained from American Type Culture Collection (Manassas, VA).

AB12, LKR, AKR, and LLC cell lines were cultured and maintained in high-glucose DMEM (Mediatech, Washington, DC) supplemented with 10% fetal bovine serum (FBS; Georgia Bio-technology, Atlanta, GA), 5 Ag/mL penicillin/streptomycin, and 2 mmol/L glutamine. The TC1 cell line was grown in vitro in RPMI, 10% FBS, 5 Ag/mL penicillin/streptomycin, and 2 mmol/L glutamine. Cell lines were regularly tested and maintained negative for *Mycoplasma* spp.

**Animal models**

Mice were injected subcutaneously on the flank with 5x10⁵ AKR tumor cells (C57Bl/6 mice), 1x10⁶ AB12 cells (BALB/c mice), 1.2x10⁶ TC1 cells (C57Bl/6 mice), 2x10⁶ LLC cells (C57Bl/6 mice), or 2x10⁶ LKR cells (Bl/6x129/J1) unless otherwise noted. Tumor cells for subcutaneous injections were suspended in 100μL PBS. Tumor volume was calculated using the formula \((3.14 \times \text{long-axis} \times \text{short-axis}^2)/6\). Lungs were harvested at various time points and fixed in formalin to assess for pulmonary tumor burden in LKR and LLC models.

Intraperitoneal (i.p.) and intravenous (i.v.) tumor cell inoculations utilized the same quantity of tumor cells as in flank inoculations unless otherwise noted. Intraperitoneal cell inoculants were suspended in 500μL PBS, and injected into the peritoneal cavity. Cells for i.v. injections were suspended in 300μL PBS. Pulmonary involvement was determined by the percentage of total lung cross-sectional area with tumor involvement.

**Surgery**

Mice with flank tumors were anesthetized using ketamine (80mg/kg) and xylazine (10mg/kg) then shaved. A 1 cm incision was made adjacent to the tumor. Full resection was achieved by excising 100% of the tumor (including vascular supply) using standard blunt dissection. Mice undergoing sham surgery were anesthetized using ketamine (80mg/kg) and xylazine (10mg/kg), the flank contralateral flank was shaved, a 1cm incision was made. Sterile silk 4-0 sutures were used to close wounds. Buprenorphine (0.1mg/kg) was administered immediately following surgery and 4 hours after as
Systemic recurrence models

Table 1. Summary of tested models of cancer recurrence (* Best approaches)

<table>
<thead>
<tr>
<th>Model</th>
<th>Advantages</th>
<th>Disadvantages</th>
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<tbody>
<tr>
<td>Systemic Recurrence</td>
<td></td>
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<tr>
<td>I. Two Nodules</td>
<td></td>
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<tr>
<td>IA. Concurrent Injection</td>
<td>• Technically simple</td>
<td>• Various growth patterns among different cell lines</td>
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<tr>
<td></td>
<td>• Easy to monitor primary and metastatic nodules</td>
<td>• Timing does not resemble sequence of events in patients with cancer</td>
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<tr>
<td></td>
<td>• Consistent growth within each cell line</td>
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<tr>
<td>IB. Interval Injection</td>
<td>• Technically simple</td>
<td>• Growth of the metastatic tumor is inconsistent and varies between cell lines</td>
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<tr>
<td></td>
<td>• Easy to monitor both nodules</td>
<td>• Primary and metastatic tumor do not differ in phenotype</td>
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<tr>
<td></td>
<td>• Good representation of sequence of events that occur in patients with cancer</td>
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<tr>
<td>IC. Rechallenge and Post-Op Rechallenge</td>
<td>• Easy to monitor both nodules</td>
<td>• Preoperative immunosuppression is an induced immune response that allows for rechallenge</td>
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<tr>
<td></td>
<td>• Consistent growth of metastatic tumor in selected cell lines</td>
<td>• Rechallenge is only feasible in selected cell lines</td>
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<tr>
<td></td>
<td></td>
<td>• Metastases are not established at time of surgery</td>
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<tr>
<td>IIA. Spontaneously Metastatic Cell Lines*</td>
<td>• Technically simple</td>
<td>• Monitoring recurrent disease is difficult</td>
</tr>
<tr>
<td></td>
<td>• Consistent, characterizable patterns of systemic recurrence</td>
<td>• Requires larger sample sizes (n=15-20) to allow for periodic monitoring &amp; biological variation</td>
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<td></td>
<td>• Resembles events occurring in human cancer biology</td>
<td>• Processing of samples analyzed is time consuming</td>
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postoperative analgesia.

*Transforming growth factor-β (TGF-β) inhibitor, 1D11*

The anti-murine TGF-β mAb, 1D11, neutralizes the three isoforms of TGF-β [9], while the murine IgG1 mAb against Shigella toxin, 13C4, serves as an isotype control. Antibodies were provided by the Genzyme Corporation. Antibodies (1D11 and 13C4) were suspended in 0.2 ml of distilled water and administered intraperitoneally at a dose of 25 mg/kg. Once begun, antibodies were given three times per week for three weeks [10, 11].

Statistical analysis

Differences among two groups were compared by Student t-test or one-way analysis of variance (ANOVA) for multiple comparisons with appropriate post hoc testing. Fisher exact test was used for categorical data. Results are expressed as the mean and the standard error of the mean, unless otherwise noted. Time to disease progression was defined as the time from surgery until the recurring tumor reached a volume of 500 mm³. Best-fit linear regressions for models were generated using time from surgery as the independent variable with mean tumor volume or surface area as dependent variables. A p<0.05 was considered a statistically significant result.

Results

*Manipulation of non-metastatic cell lines to model systemic recurrences*

We performed a literature search in the U.S. National Library of Medicine (PubMed) and identified small animal models of cancer surgery that model postoperative systemic recurrences. A frequent approach involves simultaneously establishing multiple tumor sites in a mouse [12-17]. One tumor is dubbed the “primary tumor” while other site(s) is designated “metastatic”. Surgery is then performed on the primary site, and the other lesions are monitored as systemic recurrences. There are multiple variations of this model documented in the literature: the second nodule is established at the time of surgery, after the primary tumor, at the time of surgery, or following surgery (Table 1) [12-16]. The “metastatic foci” may be located in the flank (subcutaneous injection), peritoneum
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Simultaneous tumor nodules

The simplest approach incorporates concurrent injection of tumor cells at two locations (“primary” tumor and “metastatic” tumor) followed by resection of the primary tumor at a later date. To evaluate this approach, we injected an equal number of tumor cells simultaneously into subcutaneous locations on both the left and right flanks of mice. We tested this model using three cell lines in separate experiments: TC1-lung cancer, AKR-esophageal cancer, and AB12-mesothelioma. We observed bilateral tumor growth in more than 90% of the mice, and cell lines developed two distinct growth patterns. The first trend, exemplified by the AB12 cell line, demonstrated similar growth patterns in both nodules (similar rates with less than 15% variability in the dimensions between the two nodules) (Figure 1A). The second pattern, exemplified by TC1 and AKR cell lines, was characterized by a “dominant” and a “non-

Figure 1. Concurrent injection of two flank tumors as a model of systemic recurrence after surgery. Growth was consistent and predictable for AB12, AKR and TC1 cell lines. Larger tumors were resected at a volume of 500 mm$^3$. A. Initial growth of AB12 flank tumors is similar between both nodules. Following removal of one nodule, the second nodule rapidly enlarges. B. Initial growth of AKR is different between the tumor nodules. After resection of the dominate nodule, postoperative growth of the remaining, smaller nodule rapidly increases. C. TC1 demonstrates a similar finding to AKR. *denotes statistical difference at time point ($P \leq 0.05$).
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dominant nodule". One nodule often became the dominant nodule and displayed continued growth; the other non-dominant nodule would plateau in size following an initial burst of growth (Figure 1B and 1C). Regardless of the initial growth pattern, we performed surgery on the larger nodule (our primary lesion). In all animals, the remaining nodule displayed continued growth after the primary lesion was excised (Figure 1A, 1B and 1C). All mice were ultimately euthanized due to tumor burden. Although this approach was technically feasible and provided predictable growth within given cancer models, it poorly mimics the sequence of events occurring in patients with cancer. Instead, this approach better mimics the scenario of synchronous primary lesions.

Injection of a second tumor inoculum before surgery

A second frequent approach in the literature includes injecting tumor cells at a distant site after the first flank nodule is established, followed by resection of the first (primary) nodule. This has been argued to be biologically relevant as it better reflects clinical sequences that occur in patients with metastatic disease. To test this method, tumor cells (either TC1, AKR, and AB12) were injected into the flanks of mice. After tumors were fully established and measured 500mm³ (approximately 2 weeks later), tumor cells were injected into the contralateral flank.

Growth of the second tumor was observed in less than 30% of mice in each cell line (except for non-immunogenic Lewis Lung Cancer, LLC [12]. Tumor cells used in the second injection were confirmed to be viable by simultaneously injecting them into a tumor naïve mouse (data not shown). We repeated these experiments and injected two- to four-fold more tumor cells in the second inoculum. Despite increasing the number of tumor cells by several orders, growth of the second nodule was unsuccessful (data not shown).

To determine if the second flank position was in a suboptimal location due to inadequate blood supply, we experimented with alternate locations. For example, the second inoculum was injected into the peritoneal space rather than a subcutaneous location. Abdomens were then followed for signs of tumor burden (ascites and tenderness) for 3 weeks. After four weeks of observation, no mice displayed signs of abdominal disease. On necropsy, no other evidence of abdominal or systemic disease was found. Again, after increasing cell inoculums by two- and three-fold, no growth was observed (data not shown). We also injected tumor cells intravenously via the tail vein as a second inoculum. Again, systemic tumor involvement was not observed. Even after doubling and tripling the inoculum, additional growth was not observed. Although theoretical benefits exist for the interval injection of flank tumors, the growth of a second nodule is inconsistent once a cell line has established a tumor in a syngeneic immunocompetent mouse.

Evolution of concomitant immunity

Given our above observations, we performed additional experiments to sequence the timing of tumor immunity that develops when using syngeneic models of murine cancer. We injected fifty mice with various tumor cell lines (AB12, AKR, TC1, LLC) into a subcutaneous flank location. At variable time periods following the initial injection (Day 3, Day 6, Day 9, Day 14), ten mice in each group were injected with an equal number of the same initial tumor cell line into the contralateral flank.

In the AB12 and TC1 cell lines, we observed consistent growth (greater than 95% of mice) of the second inoculum when injected within three to six days of the first. Following this period, fewer than 30% of the second tumors would implant (Figure 2A). Interestingly, in AKR and LLC, cell lines which have lower levels of immunogenicity, growth of the second inoculum was more common than in the AB12 and TC1 cell lines at all time points (Figure 2A). We repeated these experiments and injected two- to four-fold more tumor cells in the second inoculum. Despite increasing the number of tumor cells by several orders, growth of the second nodule remained unsuccessful (data not shown). This propensity of the host immune system to recognize and eradicate the second inoculum likely results from development of concomitant immunity.

Second tumor inoculum (rechallenge) at the time of surgery-perioperative immunosuppression

Previous reports on surgical models have described growth of a second tumor cell inoculum...
when injected immediately after excision of the first nodule [16]. We thus examined whether surgical resection of the primary tumor allowed growth of a second inoculum of tumor cells. Mice were injected with syngeneic tumor cells (either AB12, AKR, LLC, and TC1) into a subcutaneous location in the right flank. At Day 14, a time when we found establishment of a secondary to be unreliable, we fully excised the primary tumor using sterile surgical technique. While under anesthesia, fresh tumor cells were injected into the contralateral flank.

For the AB12, AKR, and LLC cell lines, over 80% of the tumor re-challenges grew consistently if injected at the time of primary flank tumor surgery (Figure 2B). However, if the second inoculum was injected more than 3 days following surgery, less than 20% of the tumor rechallenges implanted (data not shown). All secondary tumors that grew were detected within 7 days of surgery. Growth rate of the secondary tumor was similar to that of the primary tumor (data not shown). For the TC1 cell line, injection of the second inoculum of cells immediately after surgery resulted in no tumor growth. Of note, previous studies have identified TC1 as a very immunogenic cell line which likely explains this finding [12]. These findings likely the phenomena of perioperative immunosuppression.
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which describes a short window of immune system dysfunction that follows a surgical intervention [18, Markovic, 1993 #546, Akiyoshi, 1985 #550, Ogawa, 2000 #373].

These results suggest that previous models that incorporating “metastatic focus” establishment at the time of “primary focus” excision are dependent on complicated immunologic principles, which confound preclinical drug evaluation. Further, second inoculum establishment is only feasible in certain tumor lines and poorly mimics the order of events observed in human cancer biology (when metastatic foci are present prior to surgical excision).

Spontaneously metastatic tumor cell lines create rigorous systemic recurrence models

Given the described immunologic confounders associated with previous models of postoperative systemic cancer recurrences, we evaluated recurrence models utilizing cell lines that spontaneously metastasize [19, 20]. We characterized systemic recurrences after surgery in LLC cells and a new lung cancer cell line developed in our lab, LKR [8].

To characterize systemic recurrences (pulmonary metastases) following surgery, we began by injecting LLC or LKR tumor cells into the right flanks of C57Bl/6 and B6x129/J1 fifty mice, respectively. At day 9, 16, 23, and 30, ten mice underwent complete resection of the flank tumor. A control group underwent sham surgery at Day 9. Forty days after the injection, mice were sacrificed, lungs were harvested, and pulmonary tumor burden was quantified. After day 9, despite flank tumor resection, all mice undergoing complete resection of LLC flank tumors were had evidence of pulmonary disease (Figure 3A). Similarly, all mice undergoing LKR flank tumor resection after day 16 were found to have evidence of pulmonary involvement (Figure 3B and 3C). In both models, it was also noted that delayed excision of flank tumors cor-

Figure 3. Spontaneously metastatic murine NSCLC cell lines (LLC and LKR) as models of systemic recurrence following surgery. A. Even when fully resecting LLC flank tumors as early as 9 days, lung metastases are seen. Later flank tumor resection was associated with increased metastatic involvement at Day 35. This trend was observed until flank resection at Day 23, after which point lung burden at Day 35 remained consistent and similar to controls. B. After 45 days of LKR flank tumor growth, a significant amount of metastatic burden is found in the lungs. C. Even after resecting LKR flank tumors as early as 16 days, lung metastases are seen. Later flank tumor resection was associated with increased metastatic involvement. There was no difference in lung involvement at Day 45 when compared to mice undergoing flank tumor resection at Day 37. ns signifies lack of statistical difference in percentage of lung surface area with tumor involvement ($P > 0.05$).
related with increased pulmonary tumor burden (Figure 3A and 3C).

In attempts to monitor for pulmonary metastasis with non-invasive imaging, we transduced LKR cells with several bioluminescent gene products include luciferase, Katushka, and Tomato Red prior to injecting them into mice [21, 22]. The flank tumors initially grew as large as 150mm³ but eventually fully regressed (data not shown). On necropsy, no evidence of pulmonary involvement was found. When analyzing residual tumors, bioluminescent genes were no longer present, presumably due to an immune response against tumors cells containing foreign gene products.

In conclusion, flank tumors that develop spontaneous metastases despite complete surgical resection are practical and much more closely mimic the systemic recurrence chronology observed in cancer patients. Unfortunately, optimal quantification of systemic disease requires animal sacrifice and imaging probes have the potential to alter the natural history of this disease.

**Surgical models of systemic recurrence allow for accurate evaluation of adjuvant immunotherapy (TGF-β inhibitor, 1D11)**

Multiple preclinical studies have demonstrated that systemic TGF-β blockade impedes tumor progression in murine models of mesothelioma [23, 24], gliomas [11], NSCLC [25] and renal cell carcinomas [26]. Additional studies have suggested that TGF-β blockade, when combined with tumor vaccination, generates augmented anti-tumor responses [10, 11]. Given these potent effects, a role for TGF-β inhibition as a surgical adjuvant has been proposed despite only being evaluated in non-surgical models. Using spontaneously metastatic models along with surgical resection, we sought to determine the efficacy of adjuvant TGF-β inhibition with the soluble anti-murine TGF-β mAb, 1D11.

We began by determining the role of 1D11 as monotherapy without a surgical intervention in treating the spontaneously metastatic lung cancer model, LKR. To do this we injected 2x10⁶ LKR tumor cells into the flank of Bl/6x129/J1 mice. Once tumors were established (approximately 300 mm³), mice (n=20) were randomized to 1D11 or control antibody protocols which consisted of intraperitoneal injections at 2 times per week for 3 weeks. After 40 days of tumor growth, mice were sacrificed and lungs were analyzed for tumor invasion. Mice randomized to 1D11 therapy were found to have a modest reduction in metastatic lung involvement compared to mice randomized to control antibodies, 15.8% of lung surface area was found burdened by cancer in mice receiving control antibody versus 13.7% in mice receiving 1D11 (p=0.83). B. Experiments were repeated and flank tumors were completely excised on Day 16. Three days after excision of the primary subcutaneous focus, mice were randomized to 1D11 (n=6) or its control antibody, 13C4, (n=6). At 40 days following flank tumor inoculation, mice were sacrificed and lungs were assessed for tumor burden. The use of postoperative 1D11 provided more than a 50% reduction in lung surface area involved with tumor; 14.4% in mice randomized to surgery with control antibody versus 4.89% in mice receiving adjuvant 1D11 with surgery (p=0.03).

We next focused attention to evaluate 1D11 as monotherapy without a surgical intervention in treating the spontaneously metastatic lung cancer model, LKR. To do this we injected 2x10⁶ LKR tumor cells into the flank of Bl/6x129/J1 mice. Once tumors were established (approximately 300 mm³), mice (n=20) were randomized to 1D11 or control antibody protocols which consisted of intraperitoneal injections at twice weekly for 3 weeks. After 40 days of tumor growth, mice were sacrificed and lungs were analyzed for tumor invasion. Mice randomized to 1D11 therapy were found to have a modest reduction in metastatic lung involvement as compared to mice randomized to control antibodies: 15.8% of lung surface area was found burdened by cancer in mice receiving control antibody versus 13.7% in mice receiving 1D11; p=0.83 (Figure 4A).
surgical adjuvant using the systemic recurrence cancer model. Mice were inoculated with LKR flank tumors, and flank tumors were completely excised once the tumors reached a size of 300 mm³. Three days after excision of the primary subcutaneous focus, mice were randomized to 1D11 (n=6) or its control antibody, 13C4, (n=6). At 40 days following flank tumor inoculation, mice were sacrificed and lungs were assessed for tumor burden. The use of postoperative 1D11 provided more than a 50% reduction in lung surface area involved with tumor: 14.4% in mice randomized to surgery with control antibody versus 4.89% in mice receiving adjuvant 1D11 with surgery; \( p=0.03 \) (Figure 4B). Of note, no mice randomized experienced wound breakdown.

Using systemic recurrence cancer models (spontaneously metastatic cell lines with complete flank tumor resection), we determined a large decrease in metastatic lung lesions in mice randomized to surgery with adjuvant 1D11 versus surgery alone. It is important to appreciate that this significant advantage would have been underestimated if traditional non-surgical models of systemic disease had been utilized as described in previous reports. These results demonstrate the necessity for accurate surgical models to evaluate adjuvant treatments in preclinical studies.

Discussion

In this study, we examined a series of animal models with the goal of simulating a common, important, but understudied clinical scenario: systemic cancer recurrences after surgery. Using our optimal models of local and systemic recurrences, we evaluated the efficacy of an adjuvant systemic TGF-β inhibitor (1D11) in eliminating postoperative recurrences.

Each model displayed distinct advantages and disadvantages (summarized in Table 1). Although previous approaches are reproducible and feasible, they fail to account for several important themes which render them of limited utility.

First, as previously described, metastatic foci are established during primary tumor growth [3]. Unfortunately, multiple flank tumor models previous models poorly recapitulate these important steps in disease progression. For example, simultaneous injection of two flank nodules, a technique used widely in the literature, poorly mimics the time course of systemic metastasis developing, rather, it more closely resembles the circumstances of synchronous primary tumors (given that lesions are injected simultaneously). Similarly, the “rechallenge” approach fails to consider the biology of metastatic foci which exist prior to surgical resection.

A second consideration that is overlooked in traditional models is concomitant immunity. The concept of “concomitant immunity” was first reported by North and colleagues in 1984, and describes the acquired ability of a host with a progressive tumor to reject a second inoculum of the same tumor at a distant site [27]. The mechanism of this immunity against tumors is primarily due to a protective T-cell memory that develops during the initial tumor exposure [28, 29]. Although this immune response is not powerful enough to eliminate the established tumor, it is able to eliminate a second inoculum. This immunity variably disappears (depending on the tumor and mouse strain), due to induction of suppressor cells, most notably T-regulatory cells [29]. Our experiments support the early work described by both North and Bursuker and the more recent work conducted by Turk and her colleagues [27, Bursuker, 1986 #33, Turk, 2004 #45]. Due to concomitant immunity, we found it was difficult to inject multiple flank sites with cancer cells and have a reproducible model of metastasis. Further such findings likely confound data acquired using such approaches.

A third consideration involving previous models of postoperative cancer recurrences is perioperative immunosuppression. In our experiments, resecting the primary tumor indeed did provide a temporary window in which tumor growth occurred. Many studies suggest that surgery generates a transient immunosuppression that allows increased tumor growth [4, 30-32]. This window is thought to result from inflammatory, neural, and hormonal factors. One arm stems from general anesthesia, which has a role in decreasing natural killer cell activity [33]. The other arm, surgery itself, impairs production of IL-2 [34] and generates immune suppressor cells [35]. These immunologic forces confound the already complicated task of developing clinically relevant animal models to study post-surgery recurrences.
To summarize, the balance between these complex factors results in "windows of opportunity" for the injected tumor cells to grow (Figure 5), which are somewhat cell type dependent.

Based on our experiences and the considerations previously described, we still believe that an optimal model for post-operative systemic recurrences includes spontaneously metastatic flank tumors and surgical resection of the primary (flank) tumor focus. Accurate results can be obtained with diligently planned experiments and data collection techniques. This model is technically simple and parallels the sequence of events observed in human metastatic cancers. In several cell lines (LLC and LRK), we have characterized the metastatic (pulmonary) involvement which is observed following flank tumor resection. The most challenging aspect to this model pertains to monitoring systemic disease because metastatic lung lesions do not produce symptoms until the tumor burden is far advanced. This makes accurate monitoring of recurrent lung disease difficult, requiring periodic imaging (i.e. micro-CT scans) or timed sampling of experimental groups with necropsy to examine tumor infiltrating into the lung. In addition, we found that experiments which require sacrificing animals to measure the quantity of disease in the lung may necessitate 15 to 20 animals per treatment group to obtain statistically significant results. These issues add to the time needed and expense of the model. Despite hurdles, spontaneously metastatic models are predictable and resemble human cancer biology, making them potentially highly useful for studying systemic post-operative recurrence biology (Table 1).

Although encouraging, it is essential to understand our findings in the context of recent advances in cancer biology. Spontaneously arising orthotopic tumors in transgenic models have proven effective for studying tumor microenvironment, growth kinetics, and immune system changes. However, with the exception of breast and skin cancers, surgical resection of orthotopic lesions cannot be reliably or safely performed. Furthermore, these models often require prolonged periods for tumor development, which results in low throughput systems.

In order to validate our approach of studying systemic cancer recurrences, we investigated that the role of systemic TGF-β inhibition in preventing systemic recurrences. TGF-β is a cytokine with multiple immunological effects including powerful immunosuppressive features [36]. It directly suppresses activation and maturation of innate and adaptive immune cells including CD3+ T cells, natural killer cells and antigen presenting cells [37]. Tumors and suppressor myeloid cells have been implicated in the production of TGF-β [38], and TGF-β inhibition has been proposed in patients as an adjuvant therapy to other chemotherapeutic and immunological approaches [39]. This cytokine therapy has been thought to be safe, non-toxic and can be administered for long periods of time without side effects [40].

We have over a decade long experience with TGF-β therapy and recently started a clinical trial in TGF-β inhibition of malignant mesothelioma. Therefore, we chose this agent because we were familiar with this approach, and it would inform a clinical trial combining cytore-
ductive surgery for malignant pleural mesothelioma with immunotherapy. Interestingly, in non-surgical models we found no observable decreases in systemic disease associated with TGF-β inhibition. However, when coupling TGF-β blockade with surgical resection we appreciated dramatic decreases in systemic tumor burden. Potential benefits of this approach may have been overlooked if not evaluated in a proper surgical model aimed at studying postoperative recurrences.

Incorporation of accurate systemic recurrence models following cancer surgery has been a challenge primarily due to the lack of systematically evaluated models. After assessing both current and newly proposed techniques, we conclude that the optimal model of systemic recurrence incorporates spontaneously metastatic cell lines followed by complete flank (primary) tumor excision. Once learned, these techniques offer realistic pre-clinical vehicles to study adjuvant therapies.

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References


an oncolytic herpes simplex virus expressing IL-12 effectively treats the parent tumor and protects against recurrence after resection. Cancer Gene Ther 2003; 10: 215-223.


