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Research Article

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Antitumor Immunity as Determining Factor for Prevention of Post-Surgery Cancer Recurrence

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Abstract

Cancer surgery is the oldest modality in cancer management and remains an effective cancer treatment that excises visible tumor and can result in long-lasting clinical cure. Yet the mechanism for the ability of surgery to cure cancer is not understood. The conventional thinking is that surgery effectively excises all tumor before it spreads and that results in cure. However, with more recent studies to detect circulating tumor cells that can still persist after tumor excision, it is now increasingly clear that early tumor spread before clinical detection is a common process of cancer development. Then the important paradoxical question is how does a local therapy such as surgical excision cure a systemic disease like cancer? We hypothesize that the interaction between tumor metastasis and antitumor immunity answers this question. The model, which we call "post-surgery tumor recurrence window model", suggests that establishment of cancer metastases has an L –shaped kinetics following removal of the primary tumor. Similarly, pre-surgery concomitant antitumor immunity will also decay following surgery due to lack of antigen stimulation. Whether a new metastasis can establish is determined by the balance between these two processes. Here we present animal study evidence to support this model. We also present examples of how this model may be applied to predict post-surgery prognosis in individual cancer patients.

Keywords: Cancer, Cancer Recurrence, Antitumor Immunity, IL-12, T cell Infiltration

Introduction

Cancer surgery is the most reliable treatment to achieve clinical cure of solid cancer. The prevailing view of this efficacy is based on the notion that successful cancer surgery is dependent on proper staging and is performed before the cancer has spread, thus the belief that "early detection leads to early treatment and cure". However, many historical observations in cancer patients suggest that cancer surgery may only achieve temporary remission and tumor recurrence can occur many years after surgical removal of primary cancer [1, 2]. Recent studies with more sensitive tumor cell detection methodology have repeatedly shown that tumor spread is an early process before clinical detection of cancer and circulating tumor cells are present long after successful cancer surgery [3-7]. Available evidence is therefore not consistent with this belief, but no replacement explanation has been provided. Tumor immunologists have long thought that the concomitant antitumor immunity is capable of controlling not only the growth of primary tumors, but also establishment of metastasis [8-10]. But despite this belief and supporting evidence in animal tumor models, this explanation has not gained clinical acceptance despite the fact that antitumor immunity has gained more popularity. For example, many studies have demonstrated the prognostic significance of T cell infiltration in surgical tumor tissues supporting the hypothesis that a strong antitumor immunity correlates with better post-surgery survival [11-17]. But even with this acceptance in concept, actual clinical application of this explanation for post-surgery prediction in individual cases is still missing. A major obstacle comes from lack of appropriate animal model evidence showing that post-surgery recurrence is prevented by antitumor immunity. This is rather technical because all metastatic tumor models are tumors without concomitant immunity and tumors with concomitant immunity do not show recurrence, especially metastasis following surgery. Although this observation is consistent with antitumor immunity being protective, but the conventional belief is that, such models lack clinical relevance due to lack of the ability of metastasis formation. In addition, the most serious challenge in the clinical setting of cancer surgery is the "delayed recurrence" in which cancer recurrence takes place years after surgery. It is not known the actual reason for this delay of recurrence, but retrospective analyses of tumor infiltration point to an immunological rather than tumor biological reason. Based on the assumption that disseminated tumor cells capable of forming metastasis will do so, the formation of metastasis by disseminated tumor cells following the removal of primary tumor should decrease in frequency by time with depletion of more and more capable tumor cell seeds. On the other hand, based on the assumption that antitumor immunity will recognize newly established metastases and destroy them but will also decay gradually in strength with

the decrease in tumor antigen stimulation, we expect the antitumor immunity decreases to a point after which time it is no longer protective against newly established metastases. The relationship between these two processes is diagramed in Figure 1A and is called "the window model of post-surgery tumor recurrence". Because the fact that the process of metastases formation is determined largely by the biological characteristics of the tumor that is individually variable and is difficult to manipulate, the most reasonable way to reduce post-surgery metastases would be to amplify and extend immune-protection phase (Figure 1B).

Three clear predictions are drawn from this model and can be tested in animal models. The first is that post-surgery tumor recurrence correlates with tumor immunogenicity in that an immunogenic tumor with strong concomitant immunity will have a very low chance of recurrence following primary tumor excision, whereas a non-immunogenic tumor without concomitant immunity will likely recur following primary tumor removal. This is testable because immunogenic and non-immunogenic mouse tumor models are well defined and available. Second, if antitumor immunity is responsible for post-surgery protection against tumor recurrence, then removing or inhibiting this immunity following surgery should result in increased tumor recurrence. This is a testable prediction as removing T cells by antibody is a well-defined method of inhibiting adaptive immunity. The function of adaptive immunity can also be interfered by extensive chemotherapy that leads to bone marrow suppression, as chemotherapy is a common post-surgery adjuvant treatment. Third, if the strength of the residual concomitant immunity after surgery is responsible for protection against tumor recurrence, increasing levels of antitumor immunity before surgery should lead to increased residual antitumor immunity and should result in decreased post-surgery tumor recurrence. This is a testable prediction because our previous studies have shown that chemotherapy in immunogenic tumor models combined with interleukin-12 (IL-12) are well-defined methods to increase the levels of antitumor immunity [18-22]. Since neo-adjuvant chemotherapy is commonly used in cancer surgery, this test is clinically relevant. In addition, this is a major method to extend post-surgery immune protection through pre-surgery manipulation (Figure 1B). If proven true, it provides a solid rationale to use stimulation of immune factors for the choice and timing of cancer surgery. In this study, we carried out experiments and analyses using animal tumor models to support these three predictions, thus proving the concept that antitumor immunity is a critical factor for post-surgery protection against tumor recurrence. With this hypothesis proven in animal models, we also present some preliminary data to translate this approach into human patients.

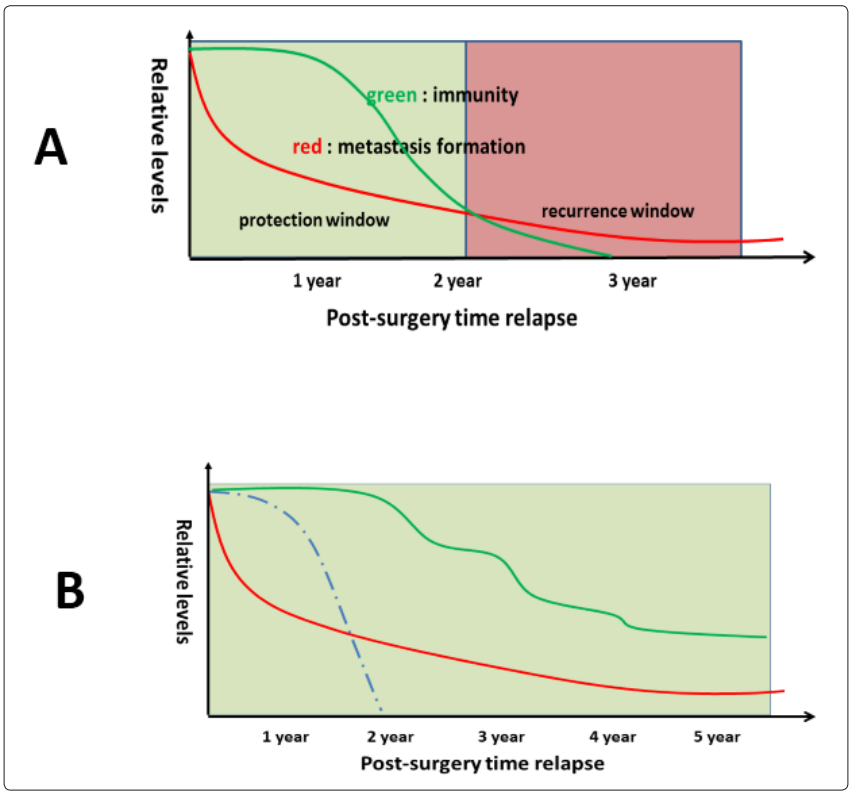

Figure 1: The proposed post-suregry tumor recurrence window model (A) and the solution to reduce post-surgery recurrence based on this model (B). The red line represents the proposed kinetics for establishment of post-surgery metastasis by disseminated tumor cells under stable conditions. The green line represents the levels of antitumor immunity decaying over time. Recurrence will take place when the protective level of immunity drops below the ability of disseminated tumor cells to form metastasis with time lapsing. In B, without changing the behavior of disseminated tumor cells to form metastasis, reduction and prevention of recurrence is achieved through delaying the decay of immunity by enhancing it (green line), thus extending the protection window to cover the entire life span of the metastasis formation. The proposed decay of the unenhanced immuntiy is marked by dotted blue lines

Materials and Methods

Tumor Models and Animals: Among the three mouse transplantable tumor Pan02 is a mouse pancreatic tumor derived from C57BL/6 mouse and is from NCI's Tumor Depository service. The C-26 is a chemically induced mouse colon tumor derived from a BALB/c mouse. It is obtained from ATCC. The MCA207 is a chemically induced mouse sarcoma derived from a C57BL/6 mouse. It is obtained from Dr. S. Young at the Surgery Branch, NCI as described before [20]. All tumor lines have been tested free of known common mouse pathogens and their use have been approved by the Animal Study Committee. All mice were obtained commercially from either Charles River Laboratory or Jackson Laboratories. Tumor lines have been maintained through in vivo passage with freshly harvested tumor cell or fragment and by freezing freshly harvested tumor fragment or cells in 10% DMSO and 90% Fetal Bovine Serum. For tumor establishment in animals, tumor monitoring and tumor size calculation, chemotherapy treatments, IL-12 administration, T cell depletion and tumor removal surgery, please refer to our previous studies [19-22].

Clinical Study and Analyses

All patients are in a group of individually consulted cases, not listed in any clinical trials. Patients came to ask for our suggestions after they have read the online posting (http://bbs.tianya.cn/post-free-3107631-1.shtml) by one of us (Tsung, K) on our views of tumor immunology and their application to explain some of well-known clinical questions/confusions. The nature of the consulting is a free exchange of our interpretation of their disease course based on our immunological view with the patients or their relatives. The pathological analyses were performed by a third-party commercial service center in Beijing, China (Lawke Laboratories, http://www.lawke.com) based on standard clinical protocols using tumor sample paraffin sections provided by the patients family. Antibodies to specific markers are common commercial ones that have been used extensively in human cancer pathology analyses. Our views after reviewing their slides were conveyed as free academic advice to patients and their family members. Patients were encouraged to discuss out suggestions and set up post-surgery treatment plans with their medical service team. All patients gave us permission to use their cases in academic exchanges such as this manuscript.

Results

Test for Prediction 1

To test for prediction 1, we have selected three commonly used and immunogenicity-known tumor models. The mouse Pan 02 is a spontaneously derived transplantable pancreatic cancer that can form subcutaneous tumors in the C57BL/6 mouse. A non-immunogenic tumor does not induce an immune response upon subcutaneous inoculation. On the other extreme, the mouse MCA207 is a chemically induced sarcoma in the C 57BL/6 mouse that induces antitumor immunity upon subcutaneous establishment that can prevent the development of the same tumor administered by a second inoculation at a distant site, thus qualifies as an immunogenic tumor [19]. The third tumor model is the mouse C-26 colon cancer, a chemically induced tumor in BALB/c mice. It is a weakly immunogenic tumor in that it can induce immune recognition upon subcutaneous establishment, but the immunity it induces is often inconsistent and not as strong as MCA207, thus it is considered intermediate between Pan02 and MCA207 [21]. If prediction one is correct, we expect an inverse correlation between tumor immunogenicity and post-surgery recurrence. Naïve 8-week old mice were first inoculated subcutaneously with 5 x 10^5 tumor cells from each of the three tumors. When the subcutaneous tumors were 7-10 mm in diameters, approximately 10-14 days following inoculation, surgery was performed to excise the tumor completely. Following surgery, the mice were observed for tumor recurrence. The results show a clear inverse correlation between tumor immunogenicity and post-surgery recurrence. For the low immunogenic tumor Pan02, post-surgery local recurrence was high 85% (Table 1). For the C-26 tumor, the post-surgery local recurrence rate was 35%. Finally, for the immunogenic tumor MCA207 tumor, the local recurrence rate was only 6%. No distant metastasis was observed in any of the experimental groups. Thus, the overall comparison of post-surgery tumor recurrence between these three tumor models fully matched the first prediction from our recurrence window model depicted in (Figure 1A).

Table 1: Observed correlation between tumor immunogenicity and post-surgery local tumor recurrence. Subcutaneous tumors were established by tumor cell inoculation. Ten days after when tumors are palpable, surgery was performed to remove tumor completely. Tumor-free mice were kept for observation of local recurrence within one-month. The rate of tumor recurrence is shown as number of mice with recurrence/total number of mice in each group (%). Data are pooled from several experiments

Tumor Model	Immunogenicity	Recurrence rate
Pan02	Low	17/20 (85%)
C-26	Medium	7/40 (35%)
MCA207	High	6/100 (6%)

Test for Prediction 2

This test is to disrupt antitumor immunity after surgery and observe for tumor recurrence. For that purpose, we used the most protective tumor model MCA207 that usually demonstrated nearly 100% post-surgery protection (Table 1). We used two methods to disrupt post-surgery antitumor immunity. First, we depleted T cells by antibodies to CD4/CD8 T cells. In the second method, we chose to apply chemotherapy after tumor surgery to suppress the anti-tumor immunity. This is a common clinical post-surgery practice that is used to affect the tumor viability but may also affect the immune function. To do this, we used the chemotherapy drug 5-FU. In pilot studies, we knew that giving this drug consecutively for 4 days would result in a significant reduction of white blood cell count to the level of bone marrow suppression. However, we did not have solid evidence for interfering with T cell function. For the test, we inoculated mice subcutaneously with MCA207 tumor cells as in experiments depicted in Table 1. Two days after surgical excision of the established subcutaneous tumors, mice were divided into three groups as listed in Table 2. One group received no treatment and served as a control. Another group received antibodies to CD4/CD8 to deplete T cells. The final group received extensive chemotherapy with 5-FU to decrease T cell number and function. After treatments, mice were left for observation for recurrence. Because we were not sure spontaneous recurrence would take place in this tumor model, we chose to inoculate 1x10^5 MCA 207 tumor cells subcutaneously on the flank opposite of the removed tumor one day after the T cell disruption treatments. In prior pilot tests, we knew that this inoculation dose would result in 100% tumor-take in naïve mice but would be rejected by most tumor-free animals following MCA

207 tumor excision surgery. Mice in the control group also received this inoculation at the same time frame. All mice were observed for spontaneous recurrence (at the surgery site) and tumor-take at the inoculation site (Table 2). As expected, all tumor-free mice in the control group that only had surgery to remove their tumors did not show any local tumor recurrence within the observational period of three months. In addition, 8 out of 10 mice in this group also rejected tumor challenge on the opposite flank. In comparison, 6 out of 10 tumor-free mice that received T cell depletion with anti-CD4/CD8 T cell antibody developed spontaneous recurrence at the surgery site and developed tumors by tumor challenge on the opposite flank. For the group that received extensive chemotherapy post-surgery, a local recurrence of tumor at the surgery site developed in 3 out of 10 mice. Each of these three mice also developed tumors at the tumor challenge site on the opposite flank. However, the spontaneous local recurrence developed in this group was small and was further suppressed for growth. This is likely due to recovery of immunity following chemotherapy. Unlike T cell depletion, which is not toxic to hosts, chemotherapy cannot be repeatedly applied, thus only a transient period of 7-10 days could be maintained for T cell suppression. The recovered T cells and their function are likely the reason for this suppressed continued development of recurrent tumor in this group. Together, these observations show that interventions that disrupt T cell number and function facilitate post-surgery tumor recurrence, possibly by weakening post-surgery immune protection. This is in direct support of the second prediction and supports the notion that antitumor immunity is a critical factor combating post-surgery tumor recurrence. It is the antitumor immunity that prevents the development of tumor nodules by disseminated tumor cells. The observation that the same mouse that developed spontaneous recurrence at the surgery site also failed to protect tumor challenge at the opposite flank indicates that this is a systemic effect, supporting a weakened immunity as a reason, and pointing out the possibility of spontaneous metastasis if such dissemination exists. The use of artificial tumor challenge is validated in this test as a way to measure the post-surgery immune protection against recurrence. This is important, as it will be the major read-out in the next test for prediction 3. The fact that extensive post-surgery chemotherapy may interfere with the protection of tumor recurrence by antitumor immunity has long been suspected and is a common belief in the clinical setting, but has not been shown previously as we have done here. This observation suggests that extensive post-surgery chemotherapy will interfere with the immune protection against cancer recurrence.

Table 2: T cell suppression following surgery leads to increased spontaneous recurrence and decreased immune protection against tumor challenge. Mice were inoculated with MCA207 tumor cells subcutaneously. Ten days after formation of palpable tumors (4-7 mm in diameter), tumor-bearing mice were subjected to surgery to remove subcutaneous tumors completely. Three days following surgery when mice were fully recovered, they were divided into three groups as listed in the table. One group received antibody to CD4/CD8 for two consecutive days followed by weekly maintenance antibody administration. The other group received 4 consecutive days of 5-FU chemotherapy. The third group did not receive any anti-T cell treatment. One day after the last chemotherapy treatment, all three groups of mice received $1x10^5$ MCA207 tumor cell challenge on the flanks opposite to the side of surgery. A group of naïve mice were also inoculated with this challenge to make sure that this dose of tumor challenge gave 100% tumor-take. Mice were kept for observation on tumor recurrence at the surgical site and tumor-take on the challenge site for one month. Recurrence was counted when a palpation (2mm) could be felt for more than a week. The rate of recurrence and tumor-take is shown as the number of mice with recurrence or tumor take/total number of mice in each group (%). Data from a single experiment are presented

Post-Surgery Treatment	Tumor-Take	
	Recurrence	Tumor inoculation
None	0/10 (0%)	2/10 (20%)
T cell depletion	6/10 (60%)	7/10 (70%)
Intensive 5-FU	3/10 (30%)	4/10 (40%)
Control naive	NA	10/10 (100%)

Test for Prediction 3

With proof of immunity as a critical protective force against tumor recurrence, the most important and practical way to improve post-surgery prognosis for cancer patients is to extend the immune protection as long as possible. Our third prediction based on the recurrence window model is about this. The test of this prediction requires an immune manipulation that increases the post-surgery protection against tumor recurrence greater than the levels seen with surgery alone. For this reason, we selected an intermediate immunogenic tumor model with room for improvement of post-surgery prognosis. The weakly immunogenic tumor C-26 is such a choice based on test in Table 1. In fact, in a previous study we have shown an experiment that demonstrated this point [21]. The diagram of that experiment and the observations are re-drawn in Figure 2. In that experiment, we used three manipulations to strengthen the pre-surgery antitumor immunity: chemotherapy, interleukin-12 and a combination of these two. Previous studies by us have already demonstrated that IL-12, chemotherapy or the two combined augment antitumor immunity [18]. The measurement of post-surgery tumor recurrence was through tumor re-challenge. The above test has indicated that this surrogate measurement can reflect the natural tumor recurrence (Table 2). The experiment is illustrated in Fig 2A. Mice were first inoculated with subcutaneous C -26 tumor cells. This model was selected because it is a weakly immunogenic tumor model with room for amplification of antitumor immunity and augmentation of post-surgery protection. The non-immunogenic tumor model Pan02 was not selected because without any evidence of pre-existing antitumor immunity, chemotherapy or IL-12 cannot augment antitumor immunity [19]. When tumors reached 7-10 mm mice were divided into 4 groups and treated with 1) nothing (tumor-bearing); 2) chemotherapy with a single dose of cyclophosphamide (Cy); 3) three injections of interleukin-12 (IL-12); and 4) Cy followed by three injection of IL-12. Ten days after treatment started, we performed surgery to remove the tumors. Ten days after the surgery, tumor-free mice were challenged with C-26 tumor cells on the opposite flanks and tumor-take as well as tumor sizes were recorded for comparison. As top bar shows, inoculation of C-26 tumors to naïve mice resulted in 0 out of 10 tumor rejection, whereas 2 out of 16 tumor-bearing mice treated by surgery alone rejected the challenge, indicating this tumor model is only weakly immunogenic. Chemotherapy had minimal effect on post-surgery protection against recurrence as only one out of 10 mice rejected the challenge. But compared to naïve mice with the same challenge, although developed tumors in most mice, mice that previously had C-26 tumor excised had a slower tumor growth rate (Fig. 2B,

bottom). A true impact on post-surgery protection by antitumor immunity was observed for the groups that were treated with either IL-12 alone or the combination of chemotherapy and IL-12. Four out of 9 (44%) mice in the IL-12 alone group and 13 out of 17 (77%) mice in the Cy+IL-12 group rejected the challenge. The few mice that had tumor-take also showed significantly slower tumor growth. These observations indicate that pre-surgery elevation of antitumor immunity may lead to elevated post-surgery immune protection against tumor recurrence, and support our third prediction shown in the recurrence window model (Fig 1). One interesting observation from this experiment is the apparent discordance between elevated post-surgery protection and the presence of elevated antitumor immunity as seen by T cell infiltration of the surgically removed tumors. (Figure 2C) shows the intratumoral immune infiltration (CD 3 T cells) from two mice from each group. It is clear that the most significant increase of T cells is consistently associated with Cy+IL-12 treatment. This elevation of T cell infiltration has been described in our previous studies [18, 20]. On the other hand, lack of elevated T cell infiltration is associated with lack of post-surgery protection in the other groups except for the IL-12 alone group. The one tumor (tumor #2) that showed elevated T cell infiltration in one of the two tumors from the chemotherapy group is from the single mouse that rejected tumor challenge in that group. Surprisingly, both tumors from the IL-12 treatment group did not show elevated T cell infiltration while both were from the mice that rejected tumor challenge. This observation, although anecdotal in nature, indicates that a visible elevation of T cell infiltration is most likely associated with better post-surgery immune protection against tumor recurrence whereas the lack of it under certain treatment condition (such as by IL-12), should not be linked to lack of post-surgery protection by modified immunity. Nevertheless, this analysis provided us with one practical way to evaluate antitumor immunity in surgical samples for an accurate assessment of post-surgery immune protection and thus the chances of tumor recurrence in cancer patients (see below).

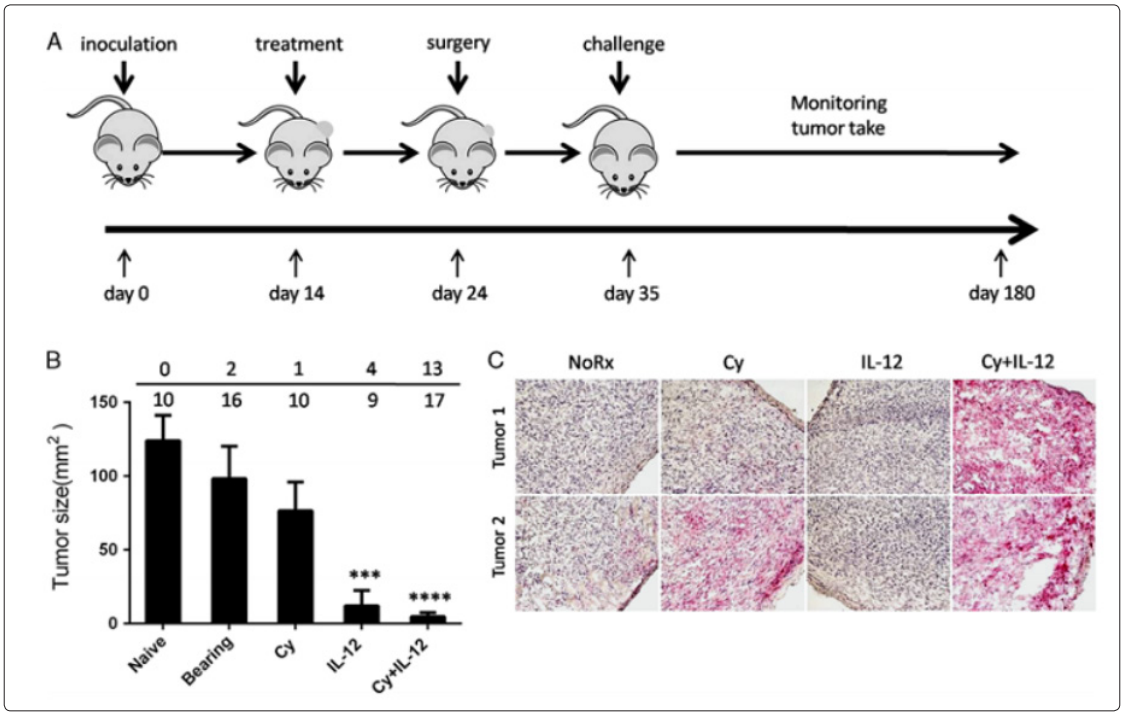

Figure 2: Elevation of antitumor immunity before surgery leads to increased post-surgery immune protection against tumor challenge. The experiment step-flow is diagrammed in panel A. Mice were inoculated with C-26 tumor cells and divided in groups when tumors became palpable in 14 days. Treatments as listed were given to each group of mice and by day 24 mice were subjected to surgery to remove all tumors completely. Ten days after surgery, tumor-free mice from each group and a group of naïve mice were challenged with $1x10^5$ C-26 cells on the flanks opposite to the side of surgery. Mice were kept for observation on tumor-take from the tumor challenge. The rate of protection against challenge is shown as the number of mice with tumor development over the number of total mice in each group. The mean sizes of the tumors developed in each group by 18 days following challenge are shown as the area of length x width in mm^2 (Panel B, column chart). *** and ****: with statistical significance of $p<0.01$, and $p<0.001$, respectively, compared to surgery alone (bearing) group. Panel C shows the examples of intratumoral T cell infiltration as determined by IHC using frozen sections from two selected tumors from each group. The identity of the selected mouse is described in the text. T cells are red in color. Micrograph amplification is 40X

Clinically Relevant Natural Tumor Recurrence Model

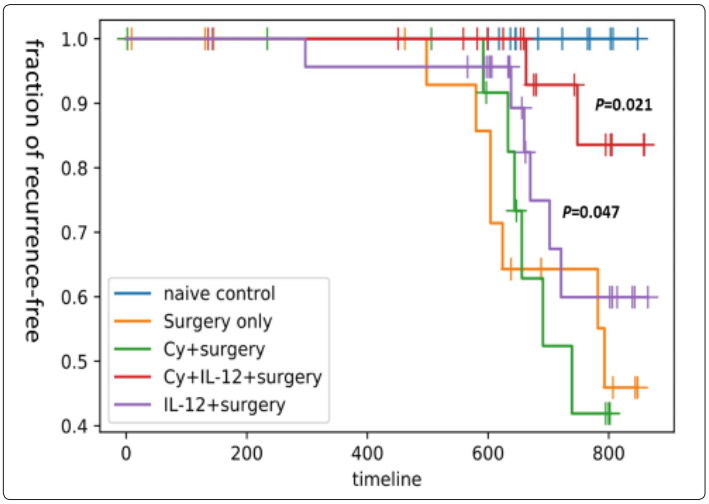

Figure 3: Elevation of pre-surgery antitumor immunity leads to delayed and reduced natural tumor recurrence in a mouse tumor model. Naïve mice were inoculated with MCA207 cells subcutaneously. Twenty days after tumor inoculation when most tumors reached 11-14 mm in diameter, mice were divided into 4 groups as listed in the figure. The number of mice in each group ranged from 20-32. Assigned treatments were initiated. 7-10 days from the start of the assigned treatment, mice with tumors were subjected to surgery to remove tumors completely. Mice were kept for observation till recurrence (drop in fraction of recurrence-free survival) or non-event death (marked by a vertical line). Data are shown as a Kaplan Meier curve of disease-free survival. One group of same aged naïve mice were kept for the entire observation period till all natural death (blue line, with 100% disease-free survival). Time line represents post-surgery observation period in days. Statistical comparison between surgery alone and other groups is calculated and significant difference was associated with IL-12+surgery and Cy+IL-12+surgery. The p values for these two groups are given in the chart.

Although all of the above experiments supported our post-surgery tumor recurrence model, these tests prove the principle but we wanted an experiment that is more clinically relevant, the biggest challenge in post-surgery tumor recurrence in cancer patients is the so-called delayed recurrence that takes places 1-3 years after cancer surgery. In these cases, surgery itself is clearly complete and there is no local recurrence so the tumor was completely removed. It is the decay of antitumor immunity that is the major problem, provided that nothing can be done to change the biological behavior of the individual disseminated tumor cells by the time of diagnosis and surgery. Is it possible to extend the post-surgery protection by augmenting immunity (Figure 1B). The above tests suggest that this can be done through pre-surgery manipulation while the tumor antigen supply is present and available. But the above tests in mouse tumor models used tumor challenge to mimic recurrence and metastasis, while in cancer patients this is a natural process taking a long time period following surgery. The similar process in a mouse tumor model has never been previously reported. For this reason, we have selected the MCA 207 tumor model for the experiment because is it an immunogenic tumor and only immunogenic tumors fail to have quick post-surgery local recurrence (Table 1) and thus may develop recurrence subsequently after host immunity weakens (Table 2). Further, whether the natural recurrence will appear as local tumor or a systemic metastatic tumor to distant sites is not known. Therefore, this experiment was carried out with the intension to elevate antitumor immunity before surgery to see whether this elevated antitumor immunity can translate into strengthened post-surgery protection against natural local or systemic recurrence. For this, we have included four comparison groups as follows: 1) surgery alone; 2) chemotherapy (Cy) followed by surgery; 3) IL-12 followed by surgery; 4) Cy+IL-12 followed by surgery. To avoid complete tumor regression by some of these treatments before surgery, we have used mice bearing large 10-13 mm tumors at the beginning of treatment. Expecting this as a long-term experiment to cover the entire mouse life span, we have also included one group of naïve mice to observe for lack of natural tumor development to indicate that any tumor developed in the experimental group would be from the MCA207 inoculation. To ensure the statistic significance of the data, we have included at least 20 mice in each group at the beginning of the experiment and 30 in the IL-12+surgery group. All mice with post-surgery short-term recurrence (within 6 month) were not considered as delayed recurrence and were excluded from the final analysis. The composed Kaplan-Meier analysis for disease-free survival of this experiment is presented in Figure 3. First, natural recurrence did take place in all groups, albeit after a long delay around two years (except one in the IL-12+surgery group). Because of the limited mouse life span, recurrences occurred two years after surgery near the end of natural life. Therefore, we did not have the time to see whether more or all of the mice in surgery alone group would develop natural recurrence. The fact that none of the same aged naïve control mice had tumor development argues that all of the tumors in the experimental groups are from MCA207 inoculation. Secondly, recurrences were observed mostly not at the surgery site, but at distant sites and often in the peritoneal cavity (not shown). Since some of the metastases were found on the opposite side from the surgery site and were in the bones and muscle (for example in the shoulder area), the metastases could not be caused by the surgical procedure, but by normal and natural dissemination of sarcoma tumor cells through the circulation from the primary tumor. Considering that the spontaneous antitumor immunity in this tumor is usually established by one week following tumor inoculation, and assuming an immune surveillance to curb tumor dissemination once immunity is in place, this presence of true metastases in this strong immunogenic tumor model underlies the ever malignant nature of cancer even under the most stringent situation. This delayed recurrence is the first ever reported for mouse immunogenic tumor models and it supports total relevance of the mouse tumor model to human cancer recurrence. This suggests that any manipulation developed using the mouse model may likely work similarly in cancer patients. Thirdly, the explanation that delayed recurrence is the result of decayed antitumor immunity is supported by the significant or nearly significant delay of recurrence in groups that had the strongest pre-surgery elevation of antitumor immunity (i.e. the IL-12 and Cy+IL-12 groups). Our previous studies have already shown these manipulations in this tumor models is highly effective in activating and augmenting antitumor immunity [18]. On the other hand, the development of two recurrences in the Cy+IL-12 group is rather shocking and unexpected because Cy+IL-12 is the strongest known manipulation to elevate antitumor immunity as it can eradicate very large tumor burdens without surgery and we have also observed life-long recurrence-free survival in the apparently cured mice [18]. The observed recurrence in mice treated with Cy+IL-12 followed by surgery suggested that the stress of surgery

may have interfered with the development of a life-long immune memory as compared to the natural clearance of tumor antigen by immunity augmented by Cy+IL-12 treatment without surgery [18]. Nevertheless, the group of mice treated with Cy+IL-12 showed the best post-surgery protection against recurrence when compared to all other groups. The association of post-surgery protection to tumor responses and reduction of tumor burden by pre-surgery treatment was not observed although the mice treated with Cy+IL-12 did have some significant tumor regression before surgery. So did mice in the group treated by Cy as we have shown before [18]. But IL-12 alone without chemotherapy did not bring tumor regression, and some of the mice had really large tumors of >15mm at the time of surgery. It was for the reason of increased chance of incomplete surgery in mice bearing large tumors that we had included more than 30 mice into this group. The actual rate of complete surgery was more than expected despite most mice in this group had much larger tumors than all other groups. Thus, the nearly significant differences in post-surgery protection between this group and the group that had surgery only without pre-surgery treatment would argue against tumor burden at surgery as a factor for better post-surgery protection. Together, results from this experiment demonstrated that natural tumor recurrence and metastasis following surgical removal of the primary tumor in an immunogenic mouse tumor model would still occur after a long time delay when antitumor immunity usually decays. The elevation of this immunity before surgery will help to further delay and reduce the recurrence. Thus at the completion of these four studies, we have provided evidence to indicate that our proposed post-surgery tumor recurrence window model depicted in Figure 1 is accurate.

Application of The Post-Surgery Tumor Recurrence Window Model in Cancer Management

If antitumor immunity is critical for post-surgery protection against tumor recurrence as our animal experiments suggest, this should have a direct application in cancer management as long as we can accurately assess the true status of antitumor immunity in a given patient following cancer surgery. For this reason, we have examined some surgical samples from cancer surgery and tried to link our animal model observations to post-surgery prognosis and outcome. We have begun doing these 3 years ago, preliminary in scale and experience initially, and gradually becoming more confident and accurate recently. These clinical analyses will be presented in another manuscript. Here we describe our rationale and practice of assessing status of antitumor immunity in cancer patients using surgical samples. Our reasoning is as follows: 1) antitumor immunity as an immune response is carried out by T cells and focuses at the site of antigen (tumor). Thus examining the tumor sample for the presence of T cells is appropriate way to evaluate antitumor immunity. 2) Not only is the number of T cells critical, T cell function is also important. Antitumor function of T cells can be reflected by two important aspects: inhibition of tumor replication and destruction of tumor structure and cells. 3) The antitumor immunity inside a tumor is also reflected by T cell distribution in relationship to tumor replication and invasion. It is like a "war-map", often not evenly distributed though out the entire tumor, but rather unevenly, thus sampling a small part of the tumor may not reflect this distribution and the critical information behind it. This point was reflected in our previous study using a mouse tumor [23]. For this reason, evaluating antitumor immunity using surgical samples is more informative than using small biopsy samples. T cell distribution information is also lost in any genetic analyses. 4) T cell subtypes such CD4 and CD8 and the activation status of T cells are critical factors to be assessed, as activated T cells are always associated with a stronger and more long-lasting antitumor activity. Based on these reasons, we choose to examine paraffin sections of the primary tumor by histologic analyses with hematoxylin and eosin (H & E), Ki-67, CD3 and CD8 staining. Tumor structure is determined by H & E staining and any disruption of defined tumor structure is readily seen by this staining (see below examples). Tumor activity (replication and metabolic activities) is determined by Ki-67 (or PCNA) staining. The positive staining reveals two pieces of critical information about tumor cells. One is the number of tumor cells in replication (proliferation index), and the other is how metabolically active the replicating cells are. This is by looking at the staining intensity of individual replicating cells. A metabolically active cell is present with intensive Ki-67 staining and enlarged nuclei. Then the status of antitumor immunity is revealed by staining T cells with CD3 (total T cells) and CD8. Since total T cells are composed mainly of CD4 and CD8 single positive cells, the ratio between CD4 and CD8 can be readily deduced from these staining. The presence, distribution and relationship to tumor activity, subtype, status of activation (preferred membrane staining with enlarged circular shape represents activated T cells), and antitumor functions are all readily revealed by looking at T cell staining in combination with other staining. This point is well illustrated by some of the examples giving below (Figures 4-7). For example, in one extreme, high grade glioblastoma is the most lethal cancer among all solid tumors with >90% post-surgery recurrence regardless of post-surgery treatment with chemotherapy and radiation. Analysis of surgical tumor samples from most glioblastoma cases showed almost total lack of antitumor immunity like the example in Figure 4. But occasionally, there are cases in which presence of antitumor immunity can be seen (Figure 5). In these cases, despite the finding that the T cell response was limited in scale (number and distribution as demonstrated in Figure 5B-F), the post-surgery recurrence was delayed significantly (for example over two years in this case without extensive post-surgery adjuvant radiation). The strong contrast in post-surgery prognosis between glioblastoma cases in which antitumor immunity was present or absent underlies the significant role of antitumor immunity in determining the fate of post-surgery tumor recurrence. On the other extreme, most accidentally detected lung cancers are stage I and are highly curable by surgery. Examination of the surgical samples reveals two typical situations. In cases in which there are malignant tumor cells (for example Ki-67 positive tumor cells) present, there is always a large number of immune cells (often CD8) co-existing inside the tumor, often suppressing tumor replication nearly completely (Figure 6). In cases where there are no clear presence of T cells (thus antitumor immunity), the tumor is not malignant (lack any Ki-67 stained cells) and non-invasive (our unpublished observation). Between these two extremes are every other cancer cases, some with strong antitumor immunity, thus faring a superior post-surgery prognosis and others with poor antitumor immunity having a poor prognosis due to tumor recurrence. An important task is to establish a reliable guideline for assessing the post-surgery prognosis for individual cases. Thus, if the presence and numbers of T cell can be evaluated, if their function assessed and their activation status identified, we believe that we can make a reliable assessment of the antitumor immune response status in a given patient. With this assessment done, combined with the tumor biology (tumor malignancy), clinical history (cancer staging and treatment responses), we often can make an individualized prediction of post-surgery prognosis that effectively correlates with subsequent

clinical course (our unpublished results). For example, Figure 7 is a non-typical case of cholangiocarcinoma. Most cholangiocarcinoma cases have poor concomitant immunity associated with poor prognosis (our unpublished observation). Even in cases in which the primary tumor is resected, post-surgery liver metastases are common. However, in this case the presence of antitumor immunity is very clear because there is a large area surrounding the only active residual tumor that is filled with immune and inflammatory cells. The typical cholangiocarcinoma structure seen in the tumor core is destroyed completely and no residual tumor cells are surviving or replicating. In this case, both the two immune functions of destruction of tumor structure and inhibition of tumor replication are demonstrated from the pathological analysis. Otherwise, simple H&E-based analysis may confuse this heavy immune infiltration with low-grade tumor differentiation (The pathology report of this case had this exact description). When combined with case history, we learned that this was a case of accidentally detected tumor during routine physical examination and check up in which an elevated tumor marker of CA19-9 was seen. In our opinion, based on the observed strong immunity, it was highly likely that if the patient had not had this tumor detected, it may have totally resolved with antitumor immunity. But with the detection and surgery, a strong antitumor immunity should be left to protect any tumor recurrence for a long time (if not reaching clinical cure). Because of this assessment, the patient was placed in observation only with no additional treatment and has been disease-free for more than three years.

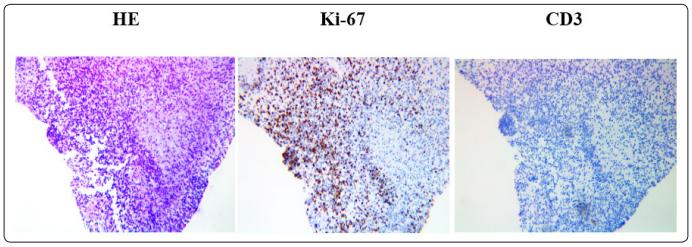

Figure 4: Microscopic views (40x) of sections from surgical sample of one case of grade IV glioblastoma stained with HE, Ki-67 and CD3 to show that there was active tumor replication while lack of T cell presence thus immunity.

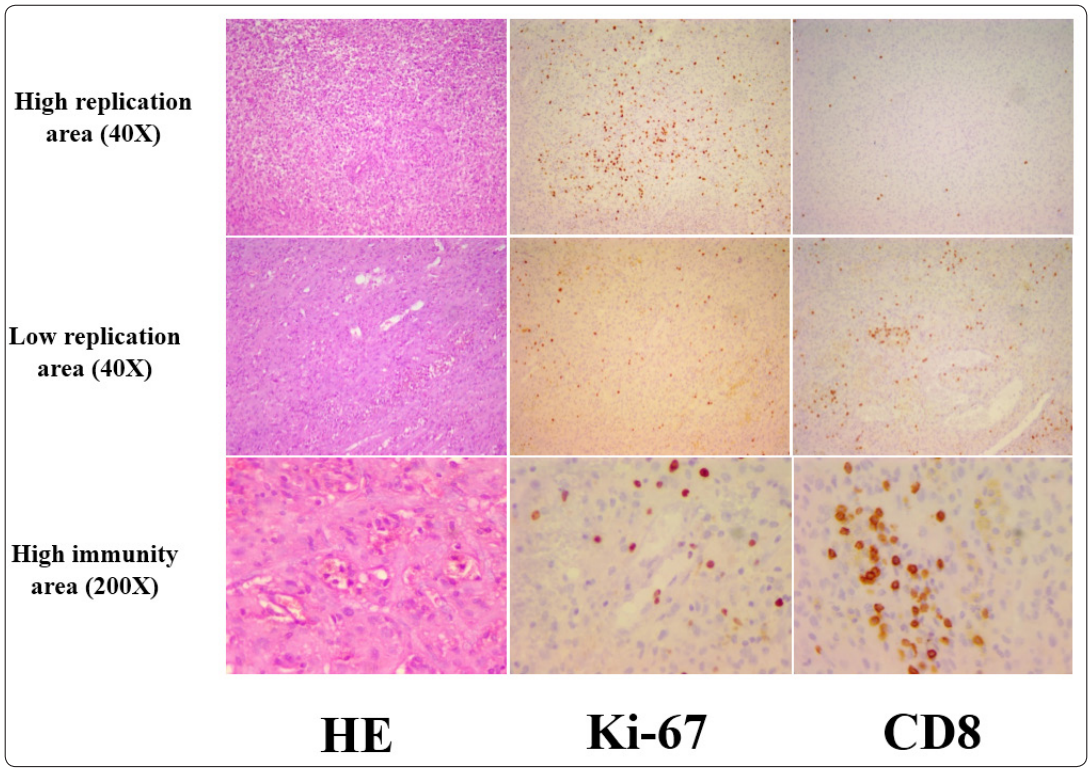

Figure 5: Microscopic views of sections from surgical sample of one grade 3-4 glioblastoma patient. The tumor was composed of areas of high tumor replication and low tumor replication/high immune presence. Examples of each area are shown. Sections were stained with HE, Ki-67 and CD8 as marked. The microscopic amplifications for each graph are also marked.

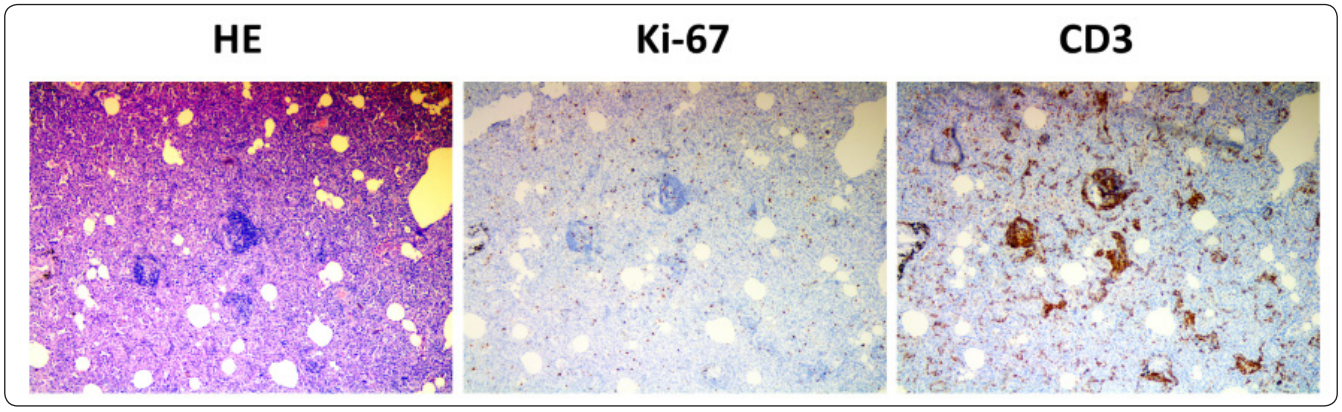

Figure 6: A typical case of early stage lung cancer discovered accidentally. The sections from surgical samples of this case were stained as marked and micrographs (40x) of the sections are shown here

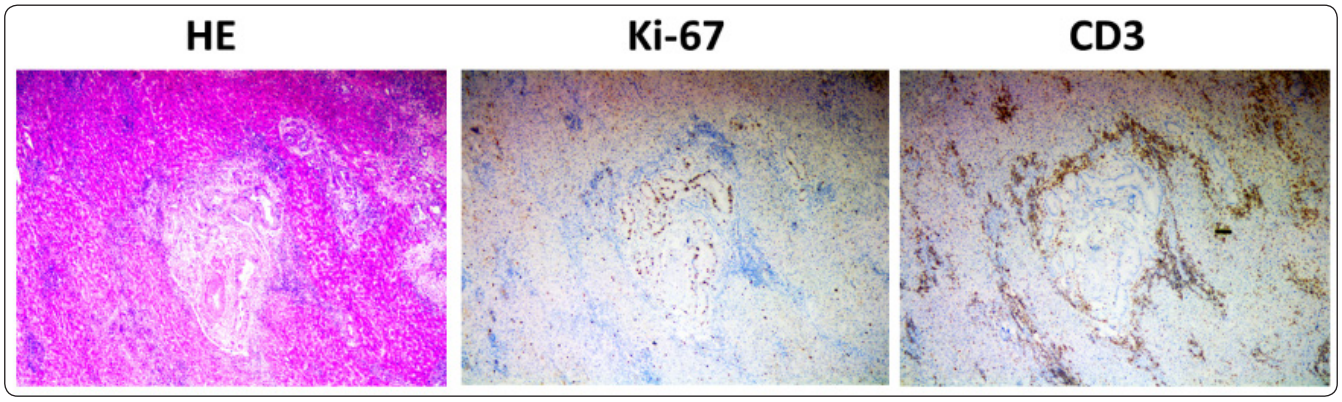

Figure 7: A rare case of cholangiocarcinoma discovered accidentally without symptoms. The sections from surgical samples of this case were stained as marked and micrographs (40x) of the sections are shown here

Discussion

In this study, we have presented supporting evidence from animal tumor models for the proposed window model of post-surgery tumor recurrence (Figure 1). Our data fully support this hypothesis and indicate that antitumor immunity is the leading determining factor for post-surgery tumor recurrence. This is based on the prerequisite that the tumor disseminates before surgery. This is clinically relevant because most cancer patients undergoing surgery meet these conditions. Although the role of concomitant antitumor immunity has long been suspected, observed, and its anti-tumor potential proposed, patients have not been treated based on its principles [2, 8, 6]. Surgeons do not perform surgery based on the status of antitumor immunity and its potential for post-surgery protection. Patients are still prescribed with post-surgery adjuvant therapies based on clinical staging that does not include the status of antitumor immunity. The explanation for the lack of clinical adaptation of its principles is disbelief of its importance. Physicians are not enthusiastic about adopting it into daily cancer management. Despite the large number of published studies showing prognostic importance of antitumor immunity documented by T cell infiltration into surgically resected tumors, post-surgery management has not considered this criterion when deciding on subsequent treatment [11-17]. In our opinion, the lack of a clinically relevant pre-clinical tumor model to demonstrate the clinical significance of antitumor immunity in post-surgery cancer recurrence is a major reason for the long ignorance by clinicians. Further, the lack of the means for accurate assessment of antitumor immunity is another. The purpose of this study in murine tumor models is to address these two problems. Findings from studies in animal tumor models and T cell infiltration of surgical samples from cancer patients appear to address and resolve these issues.

The model for post-surgery tumor recurrence in Figure 1 explains the current observations of tumor recurrence and metastases in cancer patients following surgery to remove primary tumors. It assumes that tumor dissemination has already occurred at the time of surgery and surgery only eliminates the source of new tumor dissemination. In the case of complete surgical excision of tumor, recurrence is in the form of newly established metastasis not the return of residual unremoved tumor. In that case, the establishment of metastasis follows the curve depicted in Figure 1 based on the assumption that any disseminated tumor cell that has the ability to form an individual metastasis does so at the earliest possibility when the micro-environment permits. The ability of one disseminated tumor cell to form a metastasis is determined by the biological characteristics of that cell, mostly self-driven tumor replication. But whether it can form, a metastasis is the interaction between the cancer cell and its environment, primarily elimination by antitumor immunity. For a given tumor, the biological

characteristics of all disseminated cancer cells are fixed and the post-surgery environmental conditions are constant. Thus, the rate of metastasis establishment should decay as depicted in Figure 1 until a period when all disseminated tumor cells that can form metastases are depleted. This is why after 4-5 years of disease-free survival, the case is considered a "clinical cure". So the most risky period post-surgery falls into a "window" when tumor metastases can still establish and antitumor immunity has decayed to a level that it can no longer recognize and eliminate formed metastases. Based on this model, our proposed way to improve post-surgery prognosis is to extend the period of immune protection (or any protection to that effect). In this regard, our animal study clearly indicates that it is a practical and effective approach. This should be readily applicable in the clinical setting combined with neo-adjuvant chemotherapy that has previously been demonstrated to affect post-surgery disease-free survival [23-28]. The use of IL-12, as we have demonstrated in a number of previous studies, helps to enhance the activated immunity farther by improving both quantity and quality of antitumor T cells [18-22]. IL-12 has previously been studied in clinical trials. Early work with intensive dosing has shown severe toxicity, but subsequent studies with reduced dosing and administration have demonstrated its safety [29]. What we think needs to be determined is the timing of this factor as we have shown that T cell activation is a prerequisite for its efficacy [19]. The fact that chemotherapy can activate a pre-existing antitumor immunity creates potentially useful conditions for IL-12 [22]. In a clinical setting, what needs to be determined is the immune status in individual patients at any given time (for example, before and after chemotherapy). There is no current established test to measure this, but there certainly is a need to develop one soon. It needs to be pointed out that even with the strongest elevation of antitumor immunity before surgery like the Cy+IL-12 in our experiment, there was still tumor recurrence albeit at much reduce rate and much delayed timing (Figure 3). The likely reason is in the quality of immune memory. For different antigens, for different responses, the immune memory formed after antigen clearance may vary greatly. This thought is clearly supported by clinical activities of various human disease vaccines, some lasting lifelong and others only a few years. We expect this variation in memory against individual tumor also occurs in cancer patients. For that reason, elevating pre-surgery antitumor immunity alone may not fully solve the problem of recurrence. Intermittent supply of tumor antigen under optimal immune recognition conditions should be considered as a possible strategy to improve immunity. This is the scientific basis for post-surgery tumor vaccines using tumor tissues from surgery. The timing of vaccine administration based on our window model should be before immunity drops below protective levels and not when there is adequate immune protection soon after surgery. Many previous studies of tumor vaccine did not have this timing factor in consideration. With limited material from surgery, the careful timing based on the status of immunity in an individual patient should be practiced.

The pathological analysis of human tumors presented in this study has fundamental differences from previous analyses of T cell infiltration. First, our analysis does not focus singularly on T cells, but is comprehensive it that it focuses on the relationship between T cells and tumor. Our strategy tries to answer the following questions. 1) Are there T cells present? 2) Does the presence of T cells interfere with tumor growth in terms of replication and structure? 3) Does the T cell infiltration fit the case history from the immune point of view? For example, does the presence of T cells explain an observed lack of metastases? Does it explain a patient history with lack of symptoms? This is quite different from all previous studies that only consider the number and type of tumor infiltrating T cells, but not the function or distribution of these cells. Many previous studies used a small core biopsy from paraffin blocks of tumor hoping to count the number of T cells inside a tumor. These studies may fail to identify a true picture of T cell distribution because there is often unequal distribution within a large tumor. In comparison, our analysis provides a more realistic picture of how T cells attack the tumor. For example, we often saw T cell accumulation on the opposite side from where the tumor invades. Or other times we saw T cells engaged in a "separate and destroy" formation surrounding small tumor clusters in one area at a time. The antitumor functions of immunity are only visible through the view of a large area of tumor sections with the distribution of tumor replication and T cells present simultaneously. An obvious "antagonism" between presence of T cells and decrease of tumor replication is a strong hint for possible inhibition of tumor replication by T cells. Similarly, in the area of heavy T cell presence, we often saw destroyed tumor structure and a formation of a solid area. Many pathology reports described this formation as mixed with "low differentiating tumor", but in fact, this is a consequence of immune attack, not related to tumor differentiation. A reliable way to distinguish between these two possibilities is by looking simultaneously at tumor replication activity. It is these fundamental differences between previous analyses and our comprehensive analyses that make previous analyses only significant in-group statistics but not useful in accurate prediction for post-surgery prognosis for individual patients. With the analysis described here and in subsequent manuscripts by us, we believe that a reliable guideline for assessing immune status in individual patients can be established. We aim to study this hypothesis prospectively where we identify patients with immune cell recognition of tumor, augment and amplify the concomitant antitumor immunity with IL-12 +/- chemotherapy to determine if we can generate a potent antitumor response that translate into better post-surgery prognosis. Our data in mice suggest that understanding immune cell recognition, activation and amplification of antitumor immunity will improve the outcome of patients with cancer.

Conclusions

The biggest challenge in surgical management of cancer is post-surgery tumor recurrence and metastases. Before this study, there has been no clear explanation why cancer recurrence occurs in some patient, but not others. This study has for the first time has provided a possible explanation and studied this hypothesis in animal tumor models. There are two central findings from this study. The first is that for surgical treatment of a cancer the most critical factor in long-term outcome is the level of antitumor immunity after surgery. The second is that enhancing antitumor immunity before surgery will have a direct impact on post-surgery protection against tumor recurrence. For application of these findings into cancer care, this study has also demonstrated how to elevate antitumor immunity before surgery, how to assess the status of antitumor immunity from surgical samples and make a prognostic prediction. These are significant additions to our current knowledge of cancer management and, if applied successfully into clinical practice, will change the landscape of the war on cancer all together.

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