Surgical Stress Promotes the Development of Cancer Metastases by a Coagulation-Dependent Mechanism Involving Natural Killer Cells in a Murine Model

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Objective: To determine whether the postoperative hypercoagulable state is responsible for the increase in metastases observed after surgery.

Background: Surgery precipitates a hypercoagulable state and increases the formation of cancer metastases in animal models. Coagulation promotes metastases by facilitating the formation of microthrombi around tumor cell emboli (TCE), thereby inhibiting natural killer (NK) cell–mediated destruction.

Methods: Mice underwent surgery preceded by tumor cell inoculation to establish pulmonary metastases in the presence or absence of various perioperative anticoagulants. Pulmonary TCE were quantified and characterized using fluorescently labeled fibrinogen and platelets. The role of NK cells was evaluated by repeating these experiments after antibody depletion in a genetically deficient strain and by adoptively transferring NK cells into NK-deficient mice.

Results: Surgery resulted in a consistent and significant increase in metastases while a number of different anticoagulants and platelet depletion attenuated this effect. Impaired clearance of TCE from the lungs associated with an increase in peritumoral fibrin and platelet clot formation was observed in surgically stressed mice, but not in control mice or mice that received perioperative anticoagulation. The increase in TCE survival conferred by surgery and inhibited by perioperative anticoagulation was eliminated by the immunological or genetic depletion of NK cells. Adoptive transfer experiment confirms that surgery impairs NK cell function.

Conclusions: Surgery promotes the formation of fibrin and platelet clots around TCE, thereby impairing NK cell–mediated tumor cell clearance, whereas perioperative anticoagulation attenuates this effect. Therapeutic interventions aimed at reducing peritumoral clot formation and enhancing NK cell function in the perioperative period will have important clinical implications in attenuating metastatic disease after cancer surgery.

Keywords: surgical stress, cancer metastases, coagulation, natural killer cells, murine model

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Surgeons have long suspected that surgery, a necessary step in the treatment of solid cancers, facilitates the metastatic process. Despite the initial observation of this phenomenon in 1913,[1] it remains an area of unresolved inquiry. Animal studies using various hosts and tumor models have clearly demonstrated that surgery promotes the formation of metastatic disease,[2–9] but the mechanisms responsible for this effect are poorly understood.

Surgery induces activation of platelets[10] and of the extrinsic coagulation pathways leading to the formation of thrombin and fibrin clots,[11] resulting in a postoperative hypercoagulable state.[12] These mechanisms have also been implicated in the formation of metastatic disease. In murine studies, platelet depletion attenuates the number of experimental metastases,[13–15] and P-selectin deficiency inhibits platelet binding and results in a significant reduction in the number of tumor metastases in the lungs.[16] Antifibrinolytics (agents that prevent dissolution of fibrin clots), such as aprotinin, promote development of metastases in a murine model,[17] whereas studies using fibrinogen-deficient mice demonstrate a significant reduction in metastatic foci in both implanted and spontaneous models.[18,19] Mechanistic studies suggest that platelets, along with fibrin, form aggregates around tumor cell emboli (TCE), and this seems to be related to the metastatic potential of the tumor cells.[20,21] Platelet-fibrin coating of the tumor cells may facilitate the establishment of micrometastases by a number of mechanisms, including mediating tumor cell adherence to endothelial cells,[22,23] by release of stored proangiogenic factors and mitogenic factors or by protecting tumor cells from natural killer (NK) cell–mediated immune destruction.[10,24–28]

Clinical evidence also points to the improvement in survival in patients with solid malignancies receiving prophylactic doses of low-molecular-weight heparin in addition to standard therapy.[29] Likewise, perioperative administration of anticoagulation has also been associated with favorable cancer-specific survival in patients undergoing surgical procedures.[30,31]

The link between postoperative hypercoagulability and the significant increase in experimental metastases after surgery has not been explored. We sought to define this association using murine models of surgical stress and experimental lung metastases.

MATERIALS AND METHODS

Reagents

Cell-labeling dyes, Vybrant DiD, green fluorescent chloromethyl fluorescein diacetate, and human plasma fibrinogen Alexa-Fluor647 were purchased from Invitrogen. In vivo platelet labeling antibody, DyLight488, and platelet depletion antibody, α-mouse GPIbα, were from Emfret Analytics. Anti-asialo GM1 polyclonal antibody and nonimmune rabbit immunoglobulin G were purchased from Cedarlane Labs. The following drugs were purchased from the Ottawa Hospital Pharmacy: tinzaparin sodium (Innohep 10,000 anti-Xa IU/mL), dalteparin (5000 anti-Xa IU/0.2 mL), warfarin 5-mg tablets, and recombinant hirudin (Iprivask 15 mg of desirudin/vial equivalent to 300,000 antithrombin units). Actin FS, $CaCl_2$ 0.025 M, and Thromborel S were purchased from Siemens Healthcare Diagnostics.

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Cell Lines

Mouse colon carcinoma cell line, CT26LacZ, was purchased from American Type Cell Culture. Cells were maintained as monolayer cultures in complete Dulbecco's Modified Eagle's Medium. B16F10LacZ melanoma cell line was obtained from Dr K. Graham, London Regional Cancer Program, and maintained in complete α-MEM. Cells were resuspended in 1X sterile phosphate-buffered saline (CT26LacZ) or α-MEM without serum (B16F10LacZ) for intravenous injection through the lateral tail vein in mice. 3e5 cells at more than 95% viability were injected in a 0.1 mL volume/mouse.

Mice

Six- to 8-week-old specific-pathogen-free female Balb/c, C57Bl/6 (B6), and severe combined immunodeficiency (SCID) (B-, T-cell deficient) mice were purchased from Charles River Laboratories and IL-2Rγ-KO (NK-deficient) mice were purchased from Jackson Labs. Mice were housed in pathogen-free conditions at the Animal Care facility at the University of Ottawa. All studies were approved by the Animal Care Committee at the University of Ottawa.

Establishment of Murine Surgical Stress Model

Mice were subjected to 2.5% isofluorane (Baxter Corp) for induction and maintenance of anesthesia. Routine perioperative care for mice was conducted as per standard protocols at the University of Ottawa including pain control using bupernorphine (0.05 mg/kg) administered subcutaneously the day of surgery and every 8 hours for 2 days postoperatively. Surgical stress was induced in Balb/c mice by an abdominal laparotomy (3-cm midline incision) and partial left hepatectomy or left nephrectomy preceded by an intravenous challenge of 3e5 CT26LacZ cells to establish pulmonary metastases. Mice were euthanized 3 or 8 days after tumor cell injection and their harvested lungs were stained with X-gal (Bioshop) as described previously.[17] Total number of surface visible metastases was determined on the largest lung lobe (left lobe) using a stereomicroscope (Leica Microsytems). This correlates well with the total number of lung metastases on all 5 lobes and was therefore used for the study. The experiment was repeated in B6 mice using syngeneic B16F10LacZ cells (3e5 cells). For quantification of tumor cells on hematoxylin and eosin (H&E) slides, fixed lung tissue slices were processed and stained with hematoxylin and eosin. Ten random high power fields (at $\times$ 40) were chosen from each tissue section and quantified using a light microscope (Leica Microsystems) and analyzed with Aperio Image Scope software.

Activated Factor Xa Measurement

To quantify the duration of the hypercoagulable state in mice, Factor Xa (FXa) levels were measured in the plasma from mice subjected to surgical stress (left hepatectomy) at various time points (30 minutes, 4 hours, 12 hours, or 48 hours). Nonsurgery mice were used as controls. At the endpoint, blood was collected via cardiac punctures using 3.8% sodium citrate as an anticoagulant in a 1:9 ratio (citrate:blood). Plasma was collected and analyzed using a Coatest kit (Chromogenix) according to manufacturer's instructions. The measured absorbance at 405 nm is known to be directly proportional to the amount of factor Xa.

Measurement of Plasma Soluble P-selectin Levels

Using the experimental design described for factor Xa measurement, another marker of coagulation, soluble P-selectin,[32] was used to confirm the duration of the hypercoagulable state in Balb/c mice using mouse soluble P-selectin ELISA (R&D Systems Inc) according to manufacturer's instructions.

Treatment of Mice With Anticoagulation Agents

Balb/c mice were treated with 2 low-molecular-weight heparins—tinzaparin (21.96 IU before surgery and then 7.32 IU daily[33]) and dalteparin (1 U/g before surgery and daily[34]) until endpoint-specific thrombin-inhibitor (recombinant hirudin/Iprivask—2300 antithrombin units/mouse dosed every 4 hours beginning 2 hours after surgery until endpoint[35]), vitamin-K–dependent factors II, VII, IX, and X inhibitor warfarin (0.000266 g in sterile water administered 3 days and 1 day before surgery and 1 day postsurgery[36]), and antiplatelet GPIbα (2 μg/g of body weight in phosphate-buffered saline 2 hours postsurgery and 1 hour before tumor cell injection). The antiplatelet antibody was given intravenously and all other agents were given subcutaneously (0.1–0.2 mL).

Assays for Anti-Xa, Prothrombin Time, Activated Partial Thromboplastin Time, and Platelet Depletion Measurement

Plasma anti-Xa activity level was determined after tinzaparin and dalteparin treatment using a photometric assay utilizing COATEST Heparin kit (Chromogenix). Activated partial thromboplastin time after hirudin treatment was measured using Actin FS at 30 minutes, 2 hours, and 4 hours after first injection and 30 minutes after second injection; Thromborel S was used to measure prothrombin time after warfarin treatment as per manufacturer's instructions. Untreated Balb/c mice were used as controls. Blood was collected as per protocol for factor Xa described previously. Platelet depletion was confirmed by quantifying platelets from blood smears prepared from mice with or without antibody treatment.

Assay for Interaction of TCE With Platelets and Fibrin Clots In Vivo

CT26LacZ cells were fluorescently labeled ex vivo using either DiD (5 μL/mL cell suspension—for platelet studies) or chloromethyl fluorescein diacetate (10 μM—for fibrin studies) according to manufacturer's instructions and were injected intravenously. This was immediately followed by an injection of DyLight488 (2 μg per mouse) to label platelets in vivo or fluorescently labeled fibrinogen (0.12 mg per mouse).[17] Animals underwent left hepatectomy with or without pretreatment with 21.96 IU of tinzaparin and were euthanized 4 hours after cell injection. Ten sections of each lung imaged using a fluorescent microscope (Carl Zeiss Axio Cam HR) and the percentage of TCE associated with platelets or fibrin were quantified.

Evaluation of the Role of NK, B, and T Cells in Postoperative Tumor Metastases

NK cells were immunologically depleted using α-asialo antibody (25 μL of reconstituted antibody in 50 μL volume per mouse) given intravenously 4 days and 1 day before metastatic challenge and 2 days postsurgery. For evaluation at 10 minutes and 18 hours postintervention, the third dose was omitted. This regimen was in accordance with the manufacturer's recommendations and has been confirmed in our laboratory to effectively deplete NK cells to 99% (data not shown). Control mice received equivalent amount of nonimmune immunoglobulin G. Balb/c mice received intravenous CT26LacZ cells and underwent left hepatectomy with or without perioperative tinzaparin. The lung tumor burden was quantified at 3 days postsurgery. This experiment was repeated in SCID mice and NK-deficient mice.

NK Cell–Adoptive Transfer Experiment

Donor NK cells were isolated from no surgery control or 18 hours postsurgery (lapartomy left nephrectomy) from B6 mice. Splenocytes were enriched for NK using DX5$^+$ microbeads

(Automacs; Miltenyi). 1e6 DX5$^+$ NK cells as determined by flow cytometry (data not shown) were injected intravenously via the lateral tail vein into NK-deficient mice. For all transfers, 3e5 B16F10lacZ tumor cells were injected intravenously 1 hour after immune cell transfer. Three days after immune and tumor cell injection, lungs of NK-deficient mice were isolated and quantified with X-gal.

Statistical Analysis

Data were analyzed using Graph Pad Prism 4.0 using 1-way analysis of variance followed by post hoc analysis using the Bonferroni test. Probability values less than 0.05 were considered significant.

RESULTS

Surgical Stress Increases Pulmonary Metastases and Promotes Postoperative Coagulation

Surgical stress was induced in immune-competent Balb/c mice receiving CT26LacZ cells by laparotomy and partial left hepatectomy. As seen in a representative lung photograph from a mouse 8 days following left hepatectomy (Fig. 1A), surgical stress resulted in a dramatic increase in the formation of tumor metastases compared with the nonsurgery mouse. Similar results were obtained at an earlier endpoint of 3 days when compared with control mice (Fig. 1B). To confirm these results using an independent method to enumerate lung metastases, we quantified viable LacZ$^+$ tumor cells on hematoxylin and eosin -stained lung tissue slices at high power. At 3 days after CT26LacZ and left hepatectomy treatment, there were significantly more tumor cells observed in surgically stressed mice than in no surgery control mice (see Figs. 1A, B, Supplemental Digital Content 2, available at http://links.lww.com/SLA/A308, which illustrates that surgical stress augments lung tumor metastases on × 40 hematoxylin and eosin lung tissue slides, n = 6/group, 10 high power fields selected for each mice, $^*P < 0.005$).

Using the same experimental approach, surgery was repeated in B6 receiving syngeneic B16F10LacZ cells. Surgical stress also significantly increased the number of lung metastases at 3 days, suggesting that the prometastatic effect seen after surgery is not mouse-strain or cell-type specific (Fig. 1C). The prometastatic effect of surgery did not depend on the type of surgery either, as laparotomy and left nephrectomy also significantly increased the number of micrometastases at 3 days to a similar degree as left hepatectomy (Fig. 1D). Animals receiving anesthesia had only a lung tumor burden equivalent to untouched controls (Fig. 1E). Although laparotomy resulted only in metastases equivalent to left hepatectomy, further mechanistic studies employed left hepatectomy because of the increased variability seen in the laparotomy only group (Fig. 1E).

To define the association between the procoagulant and prometastatic effects of surgery, we measured activation of the clotting cascade (plasma factor Xa and soluble P-selectin levels) after

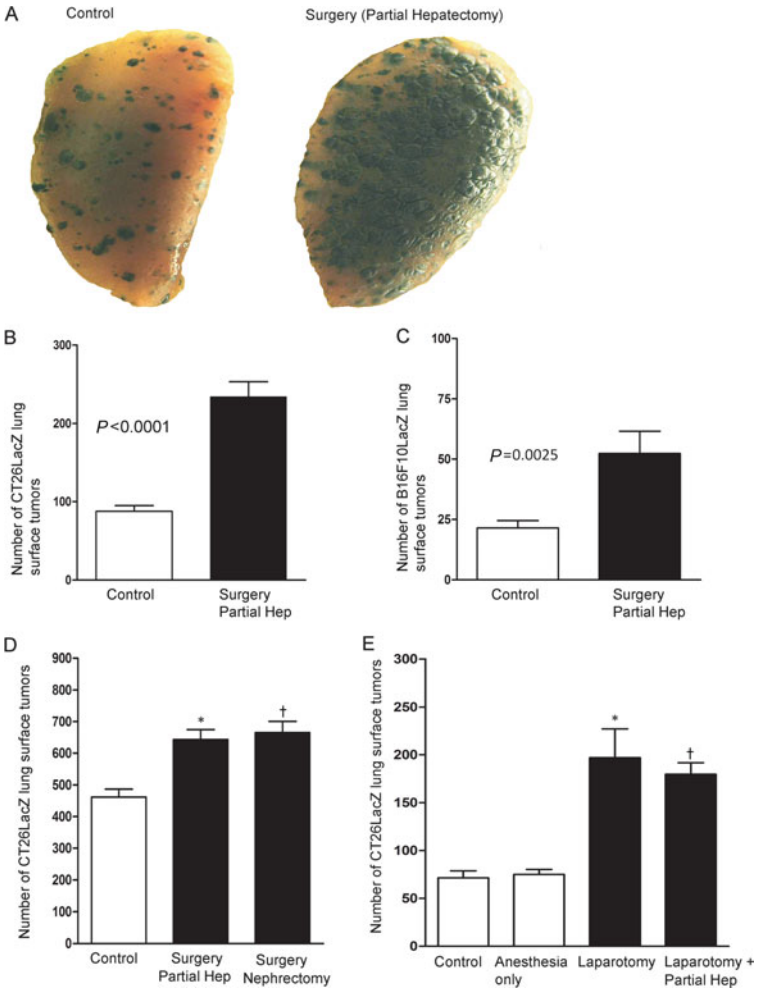

FIGURE 1. Surgical stress increases pulmonary metastases. A, Photographs of lungs from nonsurgery control (left) and animals that underwent left hepatectomy (right) at 8 days. B, Quantification of lung surface metastases from surgically stressed Balb/c mice (n = 9) and controls (n = 10) at 3 days. Compiled data from 2 independent experiments are shown. C, Quantification of lung surface metastases from surgically stressed C57Bl/6 mice (n = 10) and controls (n = 12) at 3 days. Data compiled from 2 independent experiments are shown. D, Quantification of lung surface metastases in Balb/c mice that underwent left hepatectomy (n = 9) or left nephrectomy (n = 4) at 3 days. Compiled data from 2 independent experiments are shown. $^*P < 0.001$; $\dagger P < 0.01$ compared with controls. E, Quantification of lung tumor burden from Balb/c mice that received anesthesia only (n = 3), untouched controls (n = 5), laparotomy (n = 5), or left hepatectomy (n = 4) at 3 days. $^*P = 0.0037$ compared with controls; $\dagger P < 0.0001$ compared with controls.

surgery. Factor Xa is the activated form of the coagulation factor thrombokinase, which cleaves prothrombin into thrombin, and inhibition of Factor Xa is used as a measure of anticoagulation with oral anticoagulants and low-molecular-weight heparin.[37] Soluble P-selectin is released by activated platelets and high plasma levels resulting in a procoagulant state.[32] Both markers of coagulation increased by 30 minutes, peaked at 4 hours, and stayed elevated through 12 hours, returning to baseline levels by 48 hours (Figs. 2A, B). To determine whether this timeline of postoperative coagulation mirrored the timeline of postoperative metastases, surgery was performed 30 minutes, 4 hours, 12 hours, or 48 hours before tumor cell injection, and lung tumor burden was quantified at 3 days. The timeline for the prometastatic effect of surgery was similar to that of coagulation in that tumor cell injection at 4 hours after surgery (at the peak of hypercoagulation) resulted in the greatest formation of metastases, and this effect returned to baseline by 48 hours postsurgery (when coagulation was also at baseline) (Fig. 2C).

Perioperative Anticoagulation Attenuates the Prometastatic Effect of Surgery

To further define the contribution of coagulation in the postoperative formation of metastases, 5 different anticoagulant agents, with differing mechanisms of action—tinzaparin, dalteparin, hirudin, warfarin, and platelet depletion (α-platelet GPIbα), were used. We ensured that anticoagulation was present at the time of surgery (see

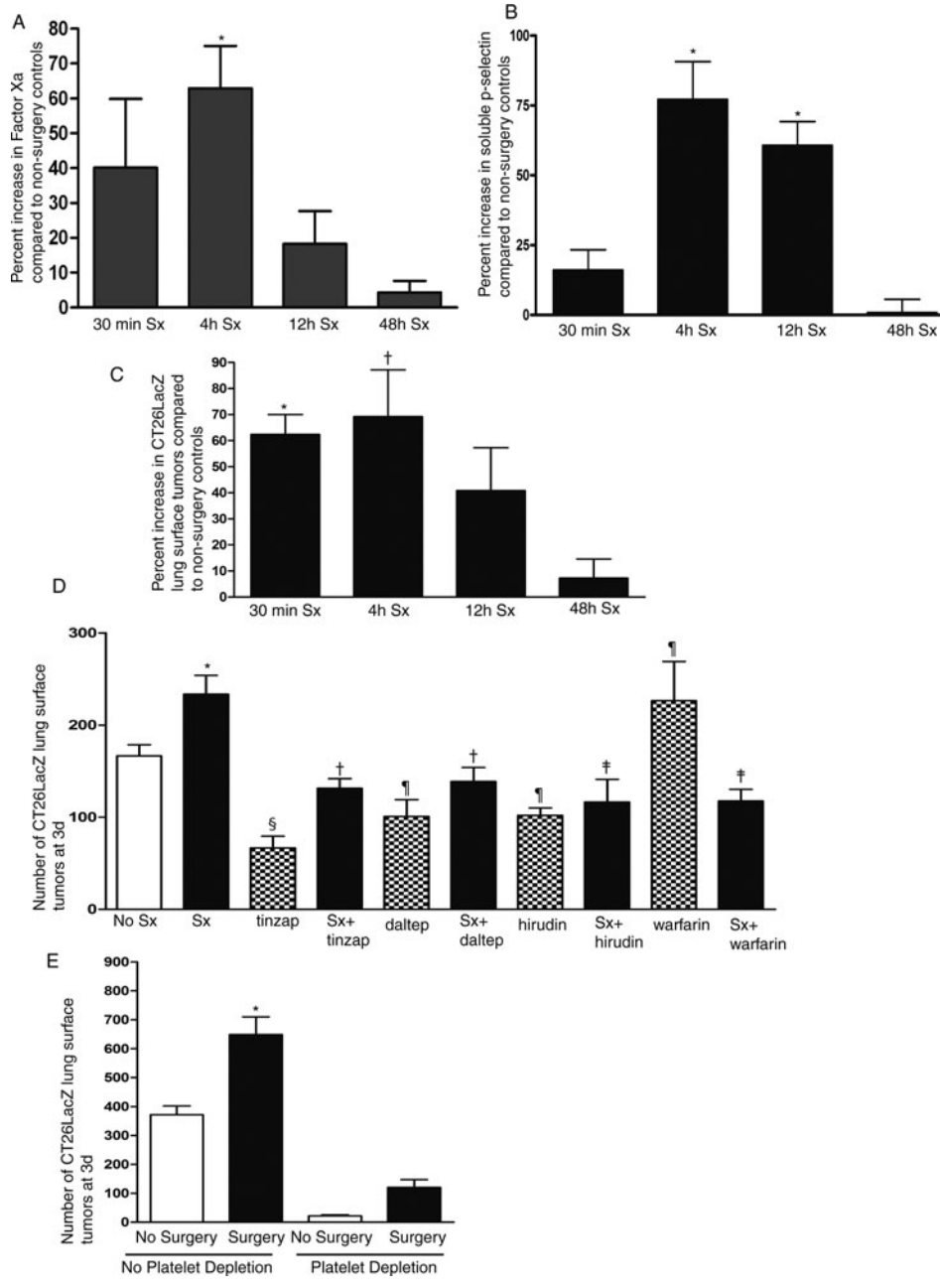

FIGURE 2. Surgical stress promotes postoperative coagulation and perioperative anticoagulation attenuates the prometastatic effect of surgery. A, Percentage increase in plasma factor Xa from Balb/c subjected to left hepatectomy for 30 minutes, 4 hours, 12 hours, or 48 hours with n = 5/group compared with nonsurgery controls. $^{*}P = 0.039$ when absorbance values compared with nonsurgery controls. B, Percentage increase in plasma soluble P-selectin based on design in A with n = 5/group. $^{*}P < 0.001$ when concentration (ng/mL) compared with nonsurgery controls. C, Percentage increase in lung surface metastases in Balb/c with left hepatectomy 30 minutes (n = 8), 4 hours (n = 5), 12 hours (n = 9), or 48 hours (n = 4) before TCI and euthanized 3 days after TCI, compared with nonsurgery controls. Pooled data from 2 independent experiments are shown. $^{*}P = 0.008$, $\dagger P = 0.013$ when counts are compared with nonsurgery controls. D, Quantification of lung tumors from left hepatectomy mice treated with perioperative anticoagulants (4–5 mice/group). $^{*}P < 0.05$ compared with nonsurgery control. $\dagger P < 0.01$; $\ddagger P < 0.001$ compared with surgery group. $\S P < 0.01$ compared with nonsurgery control. $\P P > 0.05$ compared with nonsurgery control. E, Quantification of lung tumors from left hepatectomy mice with and without platelet depletion. Pooled data from 2 independent experiments are highlighted (n = 8–11/group). $^{*}P < 0.001$ compared with nonsurgery mice with intact platelets.

Table 1, Supplemental Digital Content 1, available at http://links.lww.com/SLA/A302, which illustrates that anticoagulation was confirmed at the time of surgery using an appropriate test for each specific agent). Perioperative administration of all 5 anticoagulants resulted in a significant attenuation of the pulmonary metastases (Figs. 2D, E), demonstrating that coagulation-mediated pathways are involved in the development of tumor metastases after surgery in our murine model.

Decreased Clearance or Sustained Adherence of Tumor Cells Is Seen at Early Time Points in Surgically Stressed Mice

To investigate the early fate of tumor cells, mice were euthanized at different time points (10 minutes, 4 hours, and 12 hours) after cell inoculation with or without surgical stress (left hepatectomy) and perioperative tinzaparin and their lung tumor burden was quantified. At 10 minutes and 4 hours after cell inoculation, no significant differences were seen between the groups; however, at 12 hours, significantly more tumor metastases were seen in mice subjected to surgical stress, which was not observed when surgically stressed mice were pretreated with tinzaparin (Figs. 3A, B). This suggests that surgery impedes the clearance or promotes survival of tumor cells and that this effect is occurring between 4 hours and 12 hours after surgery. Anticoagulation with low-molecular-weight heparin is inhibiting this effect of surgery by either promoting clearance of tumor cells or impeding their survival. To further explore the early fate of tumor cells, mice were subjected to surgical stress (left hepatectomy) at different time points (0 hours, 4 hours, 12 hours, or 24 hours) after cell injection and their lung metastases were quantified at 3 days. Surgery, when performed immediately, 4 hours, and 12 hours after cell inoculation, resulted in significantly higher lung metastases whereas surgery performed at 24 hours after cell injection did not increase the formation of metastases relative to control animals (Fig. 3C). This indicates that surgery performed after tumor cell clearance or after tumor cell death has occurred does not promote the formation of metastatic disease.

Peritumoral Clot Formation Is Increased After Surgery and Inhibited by Anticoagulation With Low-Molecular-Weight Heparin

Previous studies have suggested that clot formation around TCE is associated with sustained adherence or decreased clearance of metastases.[25,27,28] To determine whether this mechanism plays a role in the postoperative period, we evaluated the association of

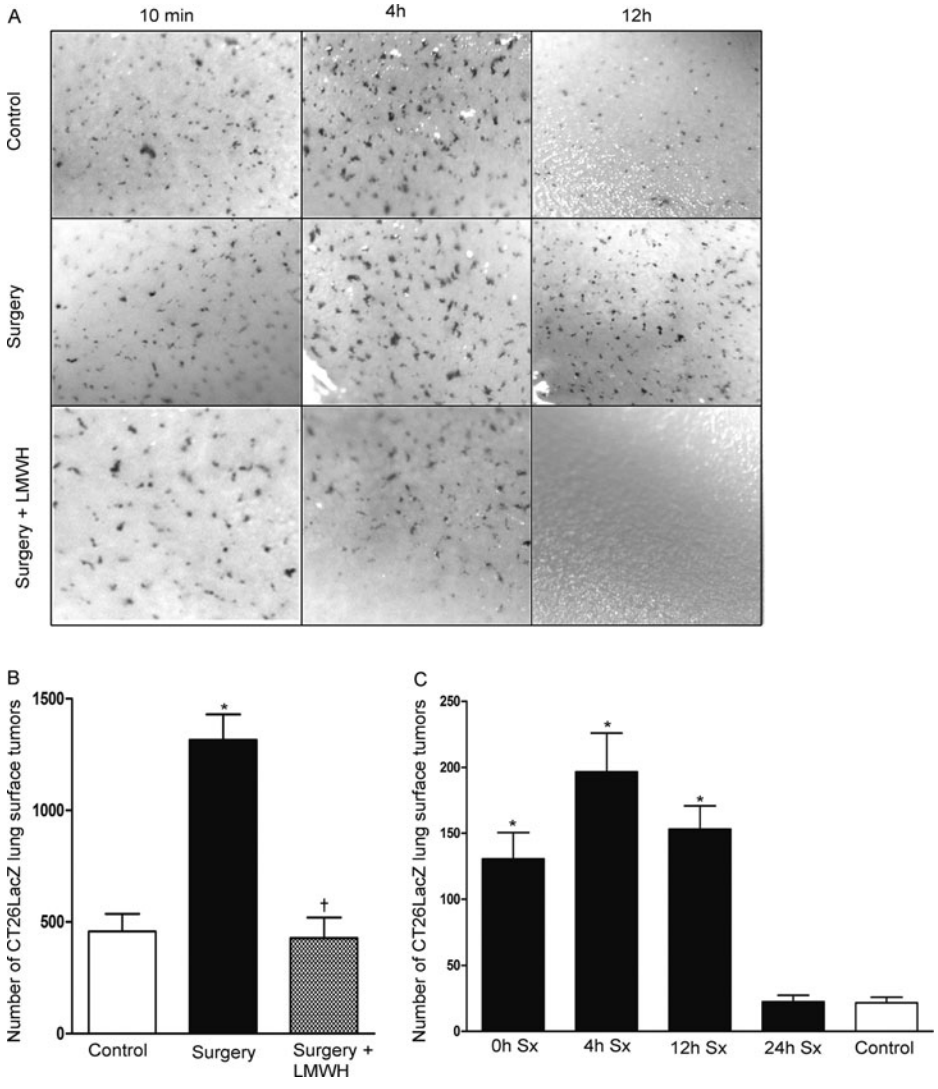

FIGURE 3. Decreased clearance or sustained adherence of tumor cells is seen at early time points in surgically stressed mice. A, Representative sections of tumor-laden lungs from Balb/c mice that underwent no surgery (*first row*), surgical stress (*middle row*), and pretreatment with tinzaparin before surgical stress (*third row*) and euthanized at 10 minutes, 4 hours, or 12 hours after TCI. Original magnification, × 12.5. B, Quantification of lung tumor burden from 12-hour group (n = 6–7/group). Pooled data from 2 independent experiments are shown. $^{*}P < 0.001$ compared with control; †$P < 0.001$ compared with surgery group. C, Quantification of lung tumor metastases from mice subjected to surgical stress immediately (n = 11), 4 hours (n = 10), 12 hours (n = 9), or 24 hours (n = 5) after TCI and euthanized 3 days after TCI. Nonsurgery mice served as controls (n = 10). Pooled data from 2 independent experiments are shown. $^{*}P < 0.001$ compared with nonsurgery controls.

fibrin and platelet clots with TCE in the lung at an early time point (4 hours) after surgery. A 2-fold increase in platelet clot formation around TCE was seen in animals that underwent surgery but not seen in surgically stressed mice pretreated with low-molecular-weight heparin (Figs. 4A, B). Likewise, surgery also increased fibrin deposition around TCE with a corresponding 3-fold increase in the percent of TCE being associated with fibrin clots compared with nonsurgery controls (Figs. 4C, D), and this was not seen in the animals pretreated with low-molecular-weight heparin.

NK Cells Are Important in the Formation of Postoperative Tumor Metastases and for the Therapeutic Effect of Perioperative Anticoagulation

Previous studies have demonstrated that NK cells play an important role in clearing tumor cells in the vasculature.[38,39] To determine whether this mechanism is operating in the postoperative period in our model, the surgical stress experiment was repeated after pharmacological depletion of NK cells. In animals with depleted NK cells, surgery did not result in an increased number of lung metastases demonstrating the importance of NK cells in the postoperative formation of metastases (Fig. 5A). Moreover, when NK cells were depleted, anticoagulation with low-molecular-weight heparin was no longer able to attenuate the formation of metastases in surgically stressed mice (Fig. 5A). This was not due to the ceiling effect reached in the model (see Figure 2, Supplemental Digital Content 3, available at http://links.lww.com/SLA/A303, NK cell depletion resulted in comparable lung tumor burden across all treatment groups when the metastatic challenge dose was reduced to 3e4 tumor cells). These findings were further confirmed by reproducing these results in NK-deficient mice (Fig. 5B). To further explore the early fate of tumor cells, mice were subjected to left hepatectomy with or without low-molecular-weight heparin after pharmacological depletion of NK cells and their lung tumor burden was quantified at 10 minutes or 18 hours postsurgery and metastatic challenge. In the absence of NK cells, no significant differences were observed between the groups at 10 minutes and at 18 hours (Figs. 5C, D), further confirming the importance of NK cells in the early clearance of postoperative tumor metastases and for the therapeutic effect of perioperative anticoagulation.

To corroborate that NK cells indeed facilitate the removal of lung tumor metastases, we set up an adoptive transfer system where we purified $DX5^+$ spleen NK cells from surgically stressed (18 hours prior) and no surgery control mice and adoptively transferred this enriched NK cell population into NK-deficient mice followed with 3e5 B16F10lacZ tumor cell inoculation. When lungs were examined 3 days after treatment, we found that the NK-deficient mice that received surgically stressed NK cells contained significantly increased lung tumor burdens compared with the group that received control NK cells transferred from animals that were not subjected to surgical stress (Fig. 5E). Taken together, these data demonstrate that surgery indeed impairs NK cells and their ability to remove pulmonary metastases. By transferring surgically stressed NK cells and recreating the effect of surgery on the formation of metastases, we have definitively established that the prometastatic effect of surgery is mediated by NK cells.

To evaluate the role of B and T cells in this model, we repeated our surgical stress experiment in B- and T-cell deficient mice (SCID) (see Fig. 3, Supplemental Digital Content 4, available at http://links.lww.com/SLA/A304, the persistence of increased lung metastases 3 days after surgery and their attenuation after anticoagulation with low-molecular-weight heparin in SCID mice suggests that B and T cells are not important in the formation of postoperative tumor metastases, n = 5/group, $^*P < 0.001$ compared with the nonsurgery control, $^{**}P < 0.05$ compared with surgery).

DISCUSSION

This study provides the first experimental evidence that surgery promotes the development of cancer metastases by a coagulation-dependent mechanism. It is well known that surgery results in hypercoagulability[1,12] and that surgery promotes the formation of tumor metastases as demonstrated by previous animal studies.[1–9] At the same time, mechanisms important in hemostasis and thrombosis have been implicated in the formation of cancer metastases.[18–20] On the basis of these observations, we hypothesized that these same mechanisms might also play a crucial role in the augmentation of tumor metastases seen after surgery. Our approach, therefore, was to study coagulation-based mechanisms in a murine model of surgery.

Using this model, we showed that surgical stress induced by 2 different means (left hepatectomy or left nephrectomy) results in significant increase in lung tumor metastases compared with nonsurgery controls (Fig. 1). Total number of lung surface metastases was quantified using X-gal-stained lungs (Fig. 1) and further confirmed with hematoxylin and eosin -stained lung tissue slides (Fig. 1, Supplemental Digital Content 2, available at http://links.lww.com/SLA/A308). These results are in line with other reports that also show that surgery indeed results in increased tumor metastases in different animal models.[1,3,4]

We have validated our model using a different cell line (B16F10 melanoma cells) and strain of mouse (B6) to show that our results are not mouse strain or cell line specific (Fig. 1). Using our surgery model, we investigated the role of coagulation-based mechanisms responsible for the development of tumor metastases observed after surgery. We, first, established that the time course of postoperative hypercoagulable state (measured by factor Xa and soluble P-selectin) mirrors the postoperative prometastatic state in our mouse model (Fig. 2), indicating that metastatic success is greatly enhanced at the peak of postoperative hypercoagulability. We next demonstrated that perioperative anticoagulation and platelet depletion, using a number of different drugs with varying mechanisms of action, attenuated the increase in tumor metastases seen after surgery (Figs. 2D, E). It is interesting that warfarin in the absence of surgical stress did not decrease metastases consistent with previous studies showing that vitamin-K antagonists are inferior to agents such as low-molecular-weight heparin.[40] However, in the setting of hypercoagulation, resulting from surgery, the efficacy of warfarin is greatly improved providing further evidence that it is the hypercoagulation that promotes the formation of metastases after surgery.

There are a number of interrelated mechanisms responsible for postoperative clotting. These include (1) initiation of the extrinsic coagulation cascade by subendothelial exposure of tissue factor and with binding and activation of FVII, leading to activation of FX and generation of thrombin (FIIa), (2) platelet activation directly by collagen or via thrombin with binding of P-selectin and degranulation, and (3) ultimately leading to the deposition of platelet and fibrin clots secondary to thrombin generation and platelet aggregation. Each of these mechanisms has been implicated in the development of metastases and different anticoagulants target different factors in the cascade. Low-molecular-weight heparin, such as tinzaparin and dalteparin, directly inhibit factor Xa and FIIa and indirectly inhibit tissue factor by releasing tissue factor pathway inhibitor from the vascular endothelium and can dampen the cascade at several steps. Warfarin inhibits vitamin-K dependent coagulation factors (FVII, IX, and X) and also acts to inhibit the generation of factor Xa and subsequently the generation of thrombin, whereas hirudin is a specific thrombin inhibitor (IIa). Each of these anticoagulants has the ability to attenuate the prometastatic effect of surgery to a similar degree and each

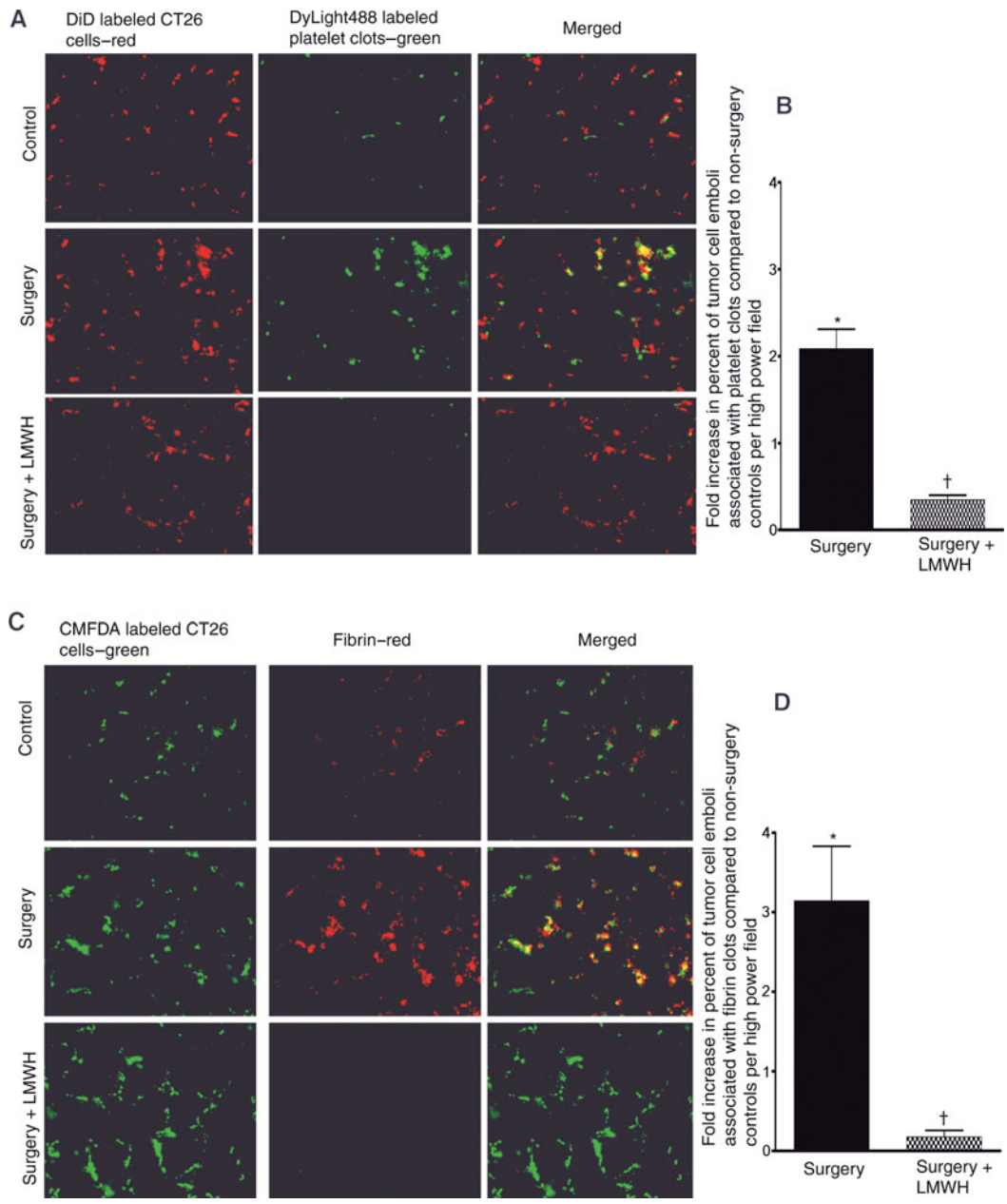

FIGURE 4. Peritumoral clot formation is increased after surgery and inhibited by anticoagulation with low-molecular-weight heparin. A, Representative fluorescent pictures from lungs of mice that underwent no surgery, surgery, or treatment with tinzaparin before surgery and euthanized 4 hours after TCI. Column 1 shows DiD (red)-labeled tumor cells whereas column 2 shows DyLight488 (green)-labeled platelets from the same lung sections. Merged pictures from the same sections are depicted in column 3. Original magnification, × 10. B, Between 5 and 10 sections of each lung from each mouse were imaged and percentage of TCE associated with platelet clots was determined per high power field from the merged pictures and converted into fold-increase compared with nonsurgery controls. n = 4–5/group. $^{*}P = 0.0004$ compared with control. $\dagger P < 0.0001$ compared with surgery group. C, Representative fluorescent pictures from lungs of mice that underwent no surgery, surgery, or treatment with tinzaparin before surgery and euthanized 4 hours after TCI. Column 1 shows CMFDA (green)-labeled tumor cells whereas column 2 shows AlexaFluro647-conjugated fibrin (red) from the same lung sections. Merged pictures from the same sections are shown in column 3. Original magnification, × 10. D, Between 5 and 11 sections of each lung from each mouse were imaged and the percentage of TCE associated with fibrin clots was determined per high power field from the merged pictures and converted into fold-increase compared with nonsurgery controls. n = 5–6/group. $^{*}P = 0.0028$ compared with control. $\dagger P = 0.0003$ compared with surgery group. CMFDA indicates chloromethyl fluorescein diacetate.

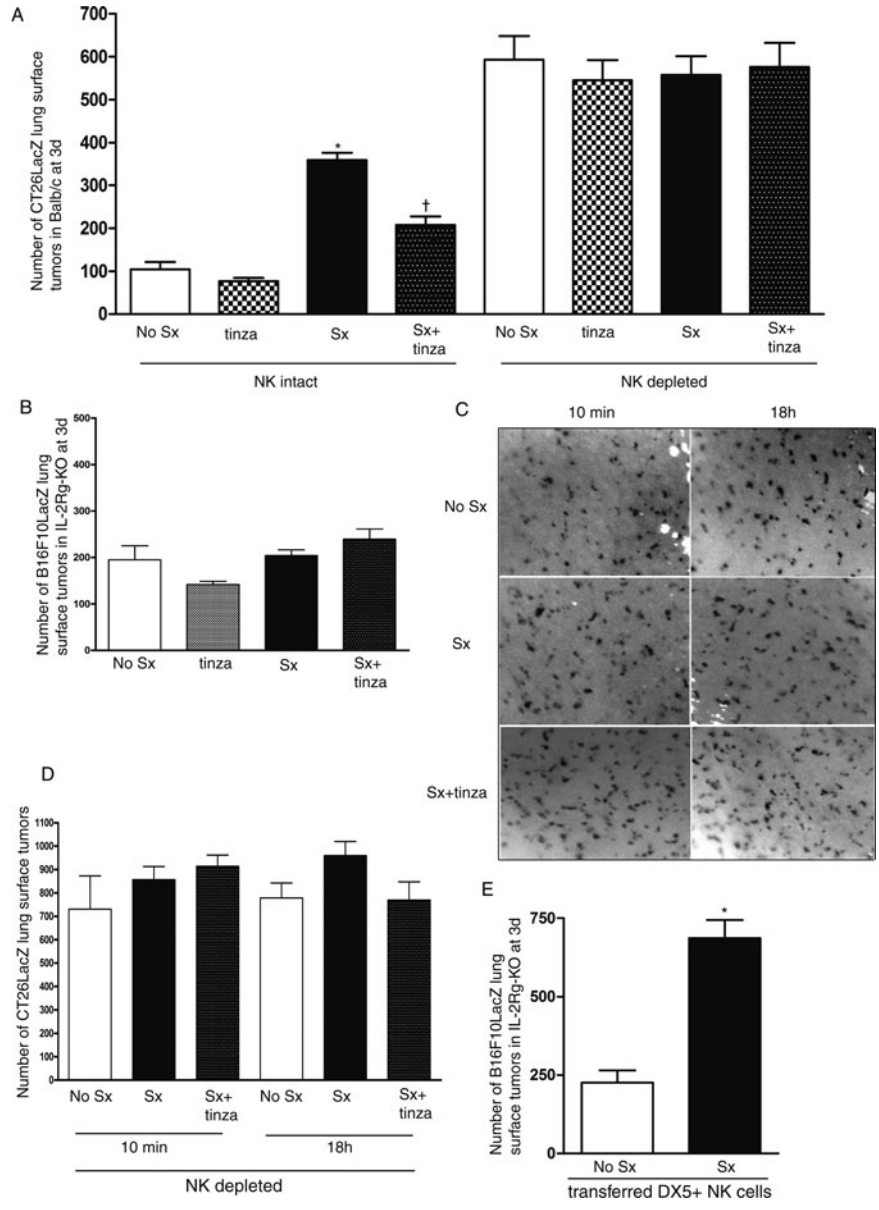

FIGURE 5. NK cells are important in the formation of postoperative tumor metastases and for the therapeutic effect of perioperative anticoagulation. A, Quantification of lung tumor metastases at 3 days from NK-intact and NK-depleted Balb/c mice subjected to surgical stress and perioperative treatment with tinzaparin. Pooled data from 3 independent experiments are shown, with n = 5–13/group. $^{*}P < 0.001$ compared with control with intact NK cells. $\dagger P < 0.001$ compared with surgery group with intact NK cells. B, Quantification of lung tumor burden at 3 days from NK-deficient mice subjected to surgery and perioperative tinzaparin, with n = 3–4/group. C, Representative lung pictures from Balb/c mice subjected to surgery and perioperative tinzaparin in the setting of NK cell depletion and euthanized at either 10 minutes or 18 hours postintervention. D, Quantification of lung tumor burden in Balb/c mice at 10 minutes or 18 hours postintervention as in C above, with n = 3–4/group. E, Quantification of lung tumor burden from NK-deficient mice receiving adoptively transferred 1e6 NK cells from surgically stressed and control mice and 3e5 B16lacZ tumor 1 hour after immune cell transfer. Mice were euthanized at 3 days postintervention. Pooled data from 2 independent experiments are shown with n = 3–5/group. $^{*}P < 0.001$.

converges on the coagulation pathway at thrombin activity, which ultimately inhibits the formation of fibrin and platelet clots around tumor cells. We have similarly demonstrated that platelet depletion inhibits the formation of metastases after surgical stress pointing to a mechanism that is common to both the extrinsic coagulation cascade and platelet activation and aggregation.

Accordingly, we aimed to identify specific mechanisms by which surgery-induced coagulation augments the development of cancer metastases. Previous studies have demonstrated that the formation of platelet and fibrin clots around TCE prevents the clearance but not the initial arrest of TCE.[27,40] We performed a time-course study evaluating the early fate of tumor cells after surgical stress and treatment with tinzaparin. Our choice of use of tinzaparin for mechanistic studies was supported by its favorable pharmacokinetic and anticoagulant properties in humans and mice. We found an increase in the number of TCE in the surgically stressed animals at 12 hours after tumor cell injection, but this increase was not seen when surgically stressed animals were anticoagulated before surgery (Fig. 3). There are 2 explanations for the changes seen in TCE numbers at 12 hours: (1) tumor cells are being cleared (by cytolysis/phagocytosis) in a manner that is inhibited by coagulation or (2) tumor cells have sustained adherence and/or viability in a manner that is dependent on coagulation. Further studies demonstrated that an increased number of TCE were associated with platelets and fibrin clots in animals subjected to surgical stress, and this was abrogated by treatment with low-molecular-weight heparin (Fig. 4). Interestingly, a 2-fold increase in the percentage of TCE associated with platelets and a 3-fold increase in the percentage of TCE associated with fibrin were found in surgically stressed animals compared with nonsurgery controls (Fig. 4), and this number correlates with the fold-increase in metastases that we have observed after surgical stress in this model. Whether this observation is merely an association or represents a cause-and-effect phenomenon requires further investigation into the potential mechanisms.

Our findings are in line with various studies of coagulation that have demonstrated the importance of coagulation in promoting experimental cancer metastases.[17,19,25,41] Platelet activation and aggregation seen after surgery could result in activation of protease-activated receptor signaling pathways leading to upregulation of adhesion molecules (P-selectin) on platelets. This can then interact with its ligands on tumor cells favoring cancer cell survival in the circulation by protecting them against mechanical stress and the immune system. Platelets may also facilitate adhesion to endothelium (via integrins) and may release growth factors such as vascular endothelial growth factor to promote angiogenesis and cell survival.[20] Furthermore, fibrin depositions have been found in and around various types of tumors providing scaffolding for angiogenesis and possibly protecting them against host defenses.[42,43] As a dimeric molecule with multiple integrin and nonintegrin binding motifs, fibrinogen might serve as an important molecular bridge between tumor cells, platelets, and endothelial cells promoting stable adhesion and helping to mechanically stabilize tumor cells at distant sites. Fibrin might provide a provisional matrix supporting migration of tumor cells out of the vasculature. Finally, fibrin(ogen)-platelet microthrombi may provide some protection to tumor cells against innate immune surveillance systems.

It is well known that NK cells are dysfunctional after surgery[44] and that anticoagulation with low-molecular-weight heparin and platelet depletion play a role in NK cell clearance of tumor metastases.[27,28] We have established that coagulation provides the link between surgery and NK cell dysfunction in the formation of postoperative tumor metastases. Specifically using NK-depleted and NK-deficient mice, we found that NK cells, but not B and T cells, play a critical role in postoperative tumor metastases (Fig. 5 and Supplemental Digital Content 3, available at http://links.lww.com/SLA/A303). Most importantly, we transferred surgically stressed NK cells into NK-deficient mice and observed enhanced lung metastases in these tumor-bearing mice compared with recipient mice who received untreated NK cells, establishing that NK cells play a crucial role in mediating tumor clearance after surgery (Fig. 5E). Furthermore, we also demonstrate that NK cells are essential to the effect of low-molecular-weight heparin on postoperative metastases in our model (Fig. 5).

Several potential mechanisms might be implicated in this context. Fibrin[19,45,46] and platelet aggregates[25] associated with micrometastatic tumor cells might present an impenetrable physical barrier to NK cells limiting their contact with target tumor cells.[26] In fact, receptors such as αm-β2 on NK cells have been shown to bind immobilized fibrinogen and platelet surface components.[47] Alternatively, the added stability conferred to tumor cell–associated platelet and fibrin aggregates may promote NK cell interactions with platelet surface proteins or platelet secretome components capable of downregulating NK cell function.[48] In fact, it has been shown that platelet-derived soluble factors, secreted after tumor cell binding or after stimulation with classic platelet agonists, impair NK cell cytotoxicity resulting in diminished granule mobilization and interferon-γ production.[49] This impaired NK cell activity has been attributed to the downregulation of activating receptor NKG2D on NK cells by platelet-derived transforming growth factor β.[49] Moreover, it has been shown that elevated transforming growth factor β1 secretion and downmodulation of NKG2D underlie impaired NK cytotoxicity in patients with cancer.[50] Finally, it could be that peritumoral clots might limit the capacity of circulating NK cells to lyse tumor cells by supporting more effective tumor cell egress out of the vasculature. This interaction can be effectively mediated by adhesion molecules found on platelets (P-selectin), leukocytes (L-selectin), and endothelium (E-selectin).[51] These potential mechanisms are not mutually exclusive, and it is conceivable that a combination of processes may ultimately determine tumor cell metastatic success. An accurate understanding of the mechanisms mediating perioperative impairment of NK cell cytotoxicity will be important in the future development of NK-specific perioperative immunomodulation strategies. Ongoing work in our laboratory is focused on characterizing the extent of the NK cell functional defect after surgery and understanding the molecular mechanism of surgery-mediated NK cell suppression in both mice and humans. Specifically, we will further characterize NK cell cytotoxicity, cytokine secretion, and cell surface marker expression defects after surgery and elucidate the mediators that inhibit these important NK cell functions.

We have established that interfering with the generation of fibrin clots or platelet clots significantly attenuates lung tumor metastases in our surgical stress model. This strongly implicates the involvement of the coagulation cascade in promoting cancer metastases in the postoperative period.

On the basis of the findings of this study, our current model of postoperative hypercoagulability and cancer metastases is as follows: surgical stress/trauma results in activation of the extrinsic coagulation cascade leading to the formation of fibrin clots and activation of platelets which form association with TCE in the circulation, ultimately leads to decreased clearance or increased survival of these tumor cells allowing them to form metastases. NK cells seem to play a crucial role in the promotion of postoperative metastases. Treatment with anticoagulants such as low-molecular-weight heparin results in decreased formation of fibrin clots and decreased activation of platelets, leading to increased clearance/decreased survival of tumor cells resulting in attenuation of tumor metastases (Fig. 6). These observations are particularly noteworthy when we consider the potential clinical implications. Surgery remains the appropriate and necessary means of treatment of most solid malignancies. Understanding the mechanisms responsible for surgery-enhanced tumor metastases will allow us to design new adjuvant therapeutic strategies that would prevent tumor recurrence after surgery. This is especially important because the perioperative therapeutic window of opportunity offers a promising means of improving patient outcome but is unfortunately underutilized. Therapeutic interventions aimed at reducing peritumoral clot formation and enhancing NK cell function in the perioperative period have important implications for the

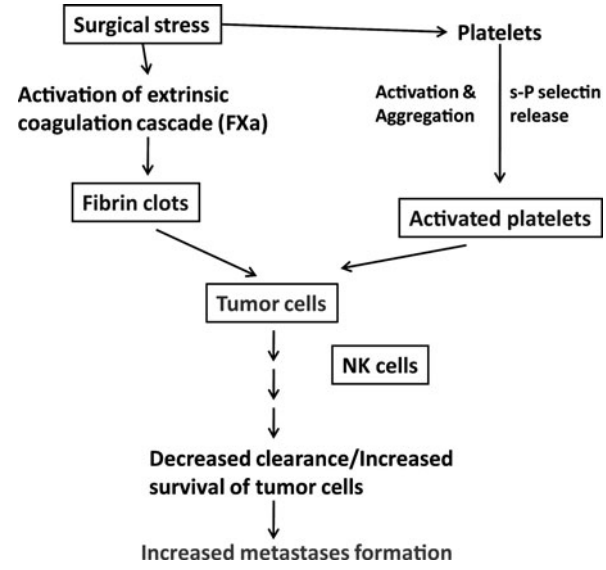

FIGURE 6. Working model of postoperative hypercoagulability and cancer metastases. See description under "Discussion."

management of surgical cancer patients and shed light on the recent finding from our group that a postoperative venous thromboembolism is associated with a worse disease-specific survival in patients who had undergone cancer surgery.[52] A multicentre randomized clinical trial of extended perioperative low-molecular-weight heparin in patients who had undergone colon cancer surgery is currently underway at multiple centers across Canada.[53]

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