

Surgical stress promotes the development of cancer metastases by a coagulation-dependent mechanism in a murine model

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ABSTRACT

Surgery precipitates a hypercoagulable state and has been shown to increase the development of cancer metastases in animal models, however mechanism(s) responsible for this are largely unknown. We hypothesize that the prometastatic effect of surgery may be secondary to postoperative hypercoagulable state. Surgical stress was induced in mice by partial hepatectomy or nephrectomy, preceded by intravenous injection of CT26-LacZ or B16F10-LacZ cells to establish pulmonary metastases with or without perioperative anticoagulation and their lung tumor cell emboli (TCE) were quantified. Fibrinogen and platelets were fluorescently labeled prior to surgical stress to evaluate TCE-associated fibrin and platelet clots. Surgery significantly increased metastases while anticoagulation with five different agents attenuated this effect. Fibrin and platelet clots were associated with TCE significantly more frequently in surgically stressed mice. Surgery promotes the formation of fibrin and platelet clots around TCE and this appears to be the mechanism for the increase in metastases seen following surgery.

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TABLE OF CONTENTS

| | |
|--|------|
| Abstract | ii |
| Acknowledgements | iii |
| List of Abbreviations | vi |
| List of Figures | viii |
| List of Tables | ix |
| 1. Introduction | 1 |
| 1.1 Cancer metastases | 1 |
| 1.2 Risk factors for progression of metastases during the perioperative period | 1 |
| 1.3 Surgical stress promotes cancer metastases | 2 |
| 1.4 Surgery activates coagulation pathways resulting in a hypercoagulable state | 3 |
| 1.5 Hemostatic disturbances associated with malignancy | 4 |
| 1.6 Activation of coagulation pathways have been implicated in cancer metastases | 5 |
| a) Role of tissue factor in cancer metastases | 5 |
| b) Role of fibrin in cancer metastases | 8 |
| c) Role of platelets in cancer metastases | 8 |
| 1.7 Role of NK cells in tumor surveillance | 10 |
| 1.8 Linking activation of coagulation pathways and NK cells in tumor metastases | 12 |
| 1.9 Role of anticoagulants in attenuating metastatic disease | 13 |
| 1.10 Rationale and Study Objectives | 14 |
| 2. Materials and Methods | 17 |
| 2.1 Reagents | 17 |
| 2.2 Cell lines | 17 |
| 2.3 Mice | 18 |
| 2.4 Establishment of murine surgical stress model | 18 |

| | |
|---|-----------|
| 2.5 Plasma activated Factor X measurement | 19 |
| 2.6 Measurement of plasma soluble p-selectin levels | 20 |
| 2.7 Treatment of mice with anticoagulation drugs | 20 |
| 2.8 Assays for anti-Xa, prothrombin time, activated partial thromboplastin time and platelet depletion measurement | 21 |
| 2.9 Assay for interaction of tumor cell emboli with platelets and fibrin clots <i>in vivo</i> | 21 |
| 2.10 Treatment of mice for NK depletion experiments | 22 |
| 2.11 Evaluation of the role of B and T lymphocytes in postoperative tumor metastases | 23 |
| 2.12 Statistical analysis | 23 |
| 3. Results | 24 |
| 3.1 Surgical stress results in increased pulmonary metastases and perioperative anticoagulation attenuates this effect | 24 |
| 3.2 Decreased clearance or sustained adherence of tumor cells is seen at early time points in mice subjected to surgical stress | 26 |
| 3.3 Peritumoral clot formation is increased following surgery and inhibited by anticoagulation with LMWH | 26 |
| 3.4 Natural-Killer cells and not B and T lymphocytes are important in the formation of postoperative tumor metastases | 27 |
| 4. Discussion | 55 |
| 5. References | 67 |
| 6. Contribution of Collaborators | 75 |
| 7. Curriculum Vitae | 76 |

LIST OF ABBREVIATIONS

aPTT - Activated partial thromboplastin time

ATU - Antithrombin units

α -MEM - α -minimal essential medium

CMFDA - Chloromethyl fluorescein diacetate

FBS - Fetal bovine serum

FXa - Activated Factor X

FVIIa - Activated Factor VII

GM-CSF - Granulocyte macrophage - colony stimulating factor

IFN α - Interferon α

IFN γ - Interferon γ

IL - Interleukin

IU - International units

IV - Intravenously

JNK - c-Jun N-terminal kinase

LLN - Laparotomy and left nephrectomy

LMWH - Low molecular weight heparin

LPLH - Laparotomy and partial left hepatectomy

MHC - Major histocompatibility complex

NK - Natural-killer cell

NKG2D - Natural-killer group 2 member D immunoreceptor

NKG2DL - Natural-killer group 2 member D immunoreceptor ligand

PAR-2 - Protease activated receptor-2

PBS - Phosphate buffered saline

PDGF - Platelet derived growth factor

PT - Prothrombin time

PTEN - Phosphatase and tensin homolog

SCID - Severe combined immunodeficiency disease

SEM - Standard error of the mean

TCE - Tumor cell emboli

TF - Tissue factor

TFPI - Tissue factor pathway inhibitor

TGF β - Transforming growth factor β

TNF α - Tumor necrosis factor α

VEGF - Vascular endothelial growth factor

VTE - Venous thromboembolic

LIST OF FIGURES

| | |
|--|-------|
| Figure 1. Schematic for the establishment of the surgical stress model of experimental metastases | 29-30 |
| Figure 2. Surgical stress increases tumor metastases | 31-32 |
| Figure 3. Establishment of the duration of hypercoagulable state in mice following surgery | 33-34 |
| Figure 4. Correlation of the hypercoagulable state in mice following surgery to postoperative tumor metastases | 35-36 |
| Figure 5. Perioperative anticoagulation significantly attenuates the increased number of lung metastases seen following surgery | 37-38 |
| Figure 6. Decreased clearance or sustained adherence of tumor cells is seen between 4-12h in surgically stressed animals | 39-40 |
| Figure 7. Surgery performed after tumor cell clearance does not promote cancer metastases | 41-42 |
| Figure 8. Surgery increases platelet clot formation around tumor cell emboli at 4h | 43-44 |
| Figure 9. Surgery increases fibrin deposition around tumor cell emboli at 4h | 45-46 |
| Figure 10. NK cells are important in postoperative tumor cell metastases in the surgical stress model | 47-48 |
| Figure 11. B and T cells are not important in postoperative tumor cell metastases in the surgical stress model | 49-50 |
| Figure 12. Working model of postoperative hypercoagulability and cancer metastases | 51-52 |

LIST OF TABLES

Table 1. Confirmation of anticoagulation at the time of surgery using an appropriate test for specific agent

53-54

1. Introduction

1.1 Cancer metastases

The major challenge in the treatment of cancer remains the control of metastatic spread. Cancer metastases is a highly complex multistep process that involves detachment of tumor cells from the primary tumor, intravasation into the bloodstream, evasion of innate immune surveillance, adhesion to the vascular endothelium of distant organs and extravasation into those tissues (1). Cancer metastases remains a major cause of morbidity and mortality and, as a result, prevention of metastatic disease is a major goal in the control of cancer. Various modalities of treatment ranging from chemotherapy, radiation therapy and surgery are indicated in the management of different types of tumors and at different stages in the disease progression. Advances in our understanding of the metastatic process will continue to provide improved therapeutic targets for this highly complex and heterogeneous disease entity.

1.2 Risk factors for progression of metastases during the perioperative period

Several lines of evidence suggest that surgery results in immune suppression. Several aspects of surgery are implicated in this suppression including anesthetic and analgesic drugs, hypothermia, tissue damage, blood loss, blood transfusion and nociception. It is caused by an intricate array of local and systemic physiological responses leading to changes in cytokine profiles and neuroendocrine responses (2). Natural-Killer (NK) cells are an important component of the innate immune system which possess the ability to both directly lyse target cells and produce immunoregulatory cytokines. Many preclinical reports have indicated that NK cells play an important role in the control of metastatic spread of cancer through their ability to eliminate circulating tumor cells (3). Evidence in humans also

suggests that NK activity might be important in controlling metastatic disease and that patients with advanced metastatic disease often have abnormalities in NK cell function and/or NK cell numbers (4). It is also well established that surgical stress results in NK cell dysfunction (5-7) that may ultimately lead to augmentation of tumor metastases (7-9).

In addition to immune suppression, several non-immunological risk factors might promote metastases immediately after surgery. Firstly, the surgical procedure almost always disrupts the neoplasm and its vascularization leading to release of tumor cells into the circulation (10). Secondly, the presence of the primary tumor is believed to induce the release of factors that limit angiogenesis, such as angiostatins preventing micrometastases from growing beyond a critical size. Removal of the primary tumor eliminates this inhibition and facilitates the development of metastases (11). Thirdly, abundant growth factors that promote healing are released following surgery and have been suspected to promote the development of metastases in local and remote sites (12). More importantly, these processes along with immune suppression may act in synergy to render a patient vulnerable to the subsequent development of metastases.

1.3 Surgical stress promotes cancer metastases

Response of a human body to different stimuli is termed as a "stress response" (13). Injury, diseases and medications can potentially trigger this response (13). In particular, surgical procedures can lead to profound physiological changes in hemodynamics, endocrine and immune function (13-15). Surgical stress response is, therefore, a complex physiological phenomenon that correlates with the magnitude of tissue damage. It is characterized by local and systemic proliferation of various mediators including pro-inflammatory cytokines such as Interleukin (IL)-6 and IL-8, complement factors, proteins of the coagulation system, acute

phase proteins, neuroendocrine mediators and by accumulation of immune-competent cells (16). Apart from the type and invasiveness of the surgical procedure, several other factors can affect the intensity of the surgical stress response including the patient's pre-existing pathologies, genetic predisposition, anesthetic and pain management, all of which can adversely influence the surgical stress response by compromising the patient's homeostatic compensatory mechanisms (16).

Surgical stress has been shown to promote the development of cancer metastases in a number of animal models where the magnitude of surgical stress is directly proportional to the number of metastatic deposits seen in the animal (8, 17-19). However, mechanisms that are responsible for this are poorly understood.

1.4 Surgery activates coagulation pathways resulting in a hypercoagulable state

There is a well established link between surgical stress and activation of hemostatic and coagulation pathways. Surgery is known to promote a pro-coagulant state. The incidence of arterial and venous thromboembolic (VTE) complications such as deep vein thrombosis, pulmonary embolism, stroke and myocardial infarction are significantly increased during the postoperative period (20). Anticoagulant prophylaxis is, therefore, recommended for cancer patients undergoing major surgery because these patients are at a higher risk for postoperative thrombosis (21). Risk factors thought to contribute to thrombosis comprise the so called "Virchow's triad" which consists of alterations in normal blood flow (turbulence and/or stasis), injuries to the vascular endothelium (trauma, shear stress or hypertension) as well as alterations in the constitution of blood (hypercoagulability). On a cellular level, well recognized mechanisms that are responsible for postoperative clotting include:

1) initiation of the extrinsic coagulation cascade by sub-endothelial exposure of tissue factor and subsequent binding with Factor VII leading to an activated tissue factor and Factor VII complex that then results in activation of Factor X leading to conversion of prothrombin into thrombin (22).

2) platelet activation directly by collagen or by thrombin binding of protease activated receptor 1 and 4 (23) and platelet aggregation directly via binding of catecholamine such as epinephrine to α -2 adrenergic receptor or indirectly via thrombin binding of GPIIb/IIIa receptor. Activated platelets translocate P-selectin from their α -granules to platelet plasma membrane and at the same time also release soluble P-selectin (24).

3) deposition of fibrin clots secondary to thrombin generation (22) or platelet derived soluble P-selectin (24).

1.5 Hemostatic disturbances associated with malignancy

The association between cancer and venous thromboembolic events is well recognized. Cancer patients are at an increased risk of developing thromboembolic complications such as deep vein thrombosis (25), pulmonary embolism (25), or syndromes that resemble low grade disseminated intravascular coagulation (26) or manifest as mainly laboratory perturbations (26-28). Up to 90% of patients with metastatic disease are affected by some form of coagulopathy (29). This presents a need for effective long term thromboprophylaxis (30) since the ongoing coagulopathy may become a component of the ongoing malignant disease process with potential impact on morbidity and survival irrespective of the thrombotic complications.

1.6 Activation of coagulation pathways have been implicated in cancer metastases

Several coagulation associated mechanisms have been shown to be important in the promotion of cancer metastases. Of these, roles played by tissue factor, fibrin clots and platelet activation and aggregation have been intensely investigated.

a) Role of tissue factor in cancer metastases

Tissue factor (TF) plays an important role in cancer metastases. It is a 47-kD transmembrane cellular receptor for activated factor VII (22) and thus, acts as a principal regulator of the coagulation cascade (22). It consists of 263 amino acid residues, of which 219 amino acids comprise the extracellular region. It also contains a 23 amino acid hydrophobic transmembrane region and a C-terminal intracellular tail of 21 amino acids (31). The intracellular part of TF also contains two putative phosphorylation sites suggesting a role for this protein in intracellular processes (31). Upon binding of activated factor VII (FVIIa), a coagulation factor circulating at low levels in the blood, the complex of TF/FVII initiates the extrinsic coagulation pathway; the TF/FVIIa complex proteolytically cleaves factor X to activated factor X, which in turn converts prothrombin to thrombin; thrombin then subsequently converts fibrinogen to fibrin resulting in the formation of blood clots (31). The TF/FVIIa complex also cleaves protease activated receptor-2 (PAR-2) and thus, contributes to non-hemostatic roles of TF pathway in cancer and inflammation (32). PAR-2 signaling has been shown to stimulate breast cancer cell migration dependent on IL-8, regulate angiogenesis, and/or play immunomodulatory role through upregulation of angiogenic regulators and cytokines (33). While TF/FVIIa binary complex signaling via PAR-2 predominates in shaping the tumor microenvironment for optimal growth, the ternary complex (TF/FVIIa/FXa) regulates coagulation pathways involving thrombin and become

the determining mechanisms as tumors progress to metastatic disease. Inhibiting thrombin has been shown to profoundly reduce spontaneous tumor metastases (34). Evidence in the literature suggests that the extracellular domain is responsible for the metastatic potential of tumor cells. A monoclonal antibody targeting TF initiated coagulation (5G9) involving the ternary complex was effective in blocking hematogenous metastases where it led to rapid loss of viable tumor cells at metastatic sites and profoundly attenuated late stage metastatic disease where as a monoclonal antibody (10H10) directed at blocking downstream signaling involving PAR-2 had no effect suggesting that TF/FVIIa signaling via PAR-2 does not contribute to the initial survival of arrested tumor cells in the experimental lung metastatic model (33, 35). Moreover, cells expressing TF lacking the cytoplasmic tail exhibited the same metastatic potential as the cells expressing wild type TF in a murine model of pulmonary metastases (36). Thus, tumor cells rely on TF to accomplish environment specific tasks (use TF signaling to induce the angiogenic switch and TF coagulation to accomplish successful metastatic homing). This evidence implies that appropriate targeting of the TF pathway can prevent tumor progression both in early and late stages of the disease.

Several studies have documented that TF expression in primary tumors is considered to be an independent risk factor for metastases (37, 38). It has also been shown that TF levels in metastatic cells is 1000-fold higher than in non-metastatic cells (35). Oncogenic mutations of K-ras, upregulation of oncogenic epidermal growth factor receptors or loss of tumor suppressors p53 and phosphatase and tensin homolog (PTEN) result in constitutive upregulation of TF, which can be further amplified by hypoxia in certain cancers (39). Progression of cancer from noninvasive to invasive disease is also critically dependent on hypoxia-induced expression of vascular endothelial growth factor (VEGF) that induces TF

expression in the host compartment (40). In advanced cancer, tumor associated macrophages, endothelial cells and myofibroblasts contribute to the pool of TF expressed in the tumor microenvironment (41). Increased metastatic potential conferred by TF appears to be mechanistically coupled to distal coagulation system components namely thrombin and fibrin clot production, as well as platelet activation and aggregation. Mice with genetic defects in each of these components of the coagulation system (prothrombin deficiency, fibrinogen deficiency and severe platelet deficiency) develop rare or no metastases when challenged with wild type TF cells (36). Similarly, its expression on tumor cells is associated with a worse prognosis with more invasive tumors demonstrating a higher level of TF expression in both human and experimental models (31, 37, 42).

Moreover, evidence now suggests that oncogenic pathways stimulate the release of TF-containing microvesicles from cancer cells in the circulation which might permit intercellular trafficking of this receptor (43, 44). This could be due to stimulation of the oncogene-dependent cellular vesiculation process (45) as well as enrichment of microvesicles in TF content possibly due to upregulation in cancer cells (46). In light of this, blood levels of TF might reflect parameters such as tumor burden, extent of malignant transformation and risk of cancer coagulopathy. In fact, elevated content of TF containing microparticles has been found in blood from patients with advanced cancers (25). Additionally, circulating TF could also be found in a soluble form - as a cleavage product of the full-length molecule or a splice variant consisting of the soluble TF ectodomain (47-49), however, the biological roles of these different isoforms remain elusive.

b) Role of fibrin in cancer metastases

Fibrin plays an important role in the promotion of tumor metastases. Anti-fibrinolytic agents (agents that prevent dissolution of fibrin clots) such as aprotinin result in enhancement of metastases by forming clots around the tumor cell emboli in a murine model of experimental metastases (50). Studies using fibrinogen deficient mice show a significant attenuation in metastases in both implanted and spontaneous models (51, 52). Fibrin along with platelets appear to form aggregates around tumor cell emboli and this seems to be causally linked to the metastatic potential of tumor cells (50, 53, 54). Several mechanisms might play an important role in the establishment of micrometastases following fibrin coating of tumor cells including facilitating tumor stroma formation by promoting the migration of transformed cells, endothelial cells and fibroblasts (55, 56) supporting effective neovascularization (57) as well as protecting tumor cells from immune mediated destruction (3, 54, 58).

c) Role of platelets in cancer metastases

Several studies support the view that thrombin-mediated platelet activation may play a role in tumor metastases. The elimination of circulating platelets with anti-platelet antibodies has been shown to result in significant attenuation in metastases using several transplantable murine tumor models (59, 60). Competitive inhibition of the key platelet integrin, α IIB β 3, either pharmacologically or with antibodies to β 3 also diminished metastatic potential (61, 62). Pharmacological inhibitors of platelet activation have been shown to attenuate the metastatic potential of circulating tumor cells (63, 64). Studies in transgenic animals have further lent support to the conclusion that platelet function constitutes a major determinant of metastatic success. Genetic loss of the integrin β 3 subunit

in mice has been shown to be associated with decreased metastases (65). Mice with a quantitative platelet defect (NF-E2 deficient mice) or a defect in thrombin mediated platelet activation (PAR-4 deficient mice) show profoundly reduced metastatic potential (66). Similarly, mice with defects in $G\alpha_q$, which confers a defect in platelet thrombus formation, develop metastases rarely following intravenous tumor challenge (54).

Upon activation, platelets express P-selectin on their plasma membrane which can bind to sialylated fucosylated mucins such as CD162 and sialyl Lewis a/x expressed by tumor cells (67, 68). In clinical studies, expression of sialylated fucosylated mucins by tumor cells is associated with a worse prognosis and a higher clinical stage of disease (69, 70). Removal of these mucins from tumor cells inhibits platelet-tumor cell interactions and attenuates metastases in a murine model (71). Similarly, P-selectin deficiency in mice also results in a similar effect with reduced platelet-tumor cell interactions and a dramatic decrease in pulmonary tumor metastases (71).

Platelets could affect metastatic potential through multiple mechanisms. Platelet granules contain a number of different cellular growth factors (PDGF, VEGF, matrix proteins such as fibronectin, inflammatory mediators such as platelet factor-4, IL-8, macrophage inflammatory protein 1 α) that might influence tumor cell behavior and stroma formation (72-74). Platelets might contribute to the physical interaction between circulating tumor cells and vascular endothelial cells by supporting stable adhesion to the endothelium and/or transmigration of tumor cells out of the vasculature. Local platelet activation could also promote migration of inflammatory cells, enhancing tumor stroma formation. Tumor cell-associated platelets could also prevent interactions between tumor cells and innate immune cells facilitating metastases (75).

In human studies, thrombocytosis is an independent predictor of a poor prognosis in cancer patients (76, 77). Moreover, postoperative use of the platelet inhibitor, acetylsalicylic acid has demonstrated improved cancer specific survival in a recently published cohort study (78).

1.7 Role of NK cells in tumor surveillance

NK cells are an important component of the innate immune system capable of lysing target cells without prior sensitization (79). NK cells are activated in response to cytokines (soluble mediators in the regulation of immune defense controlling differentiation, proliferation and cytokine production of different cells) such as IFN α , IL-2, IL-12, IL-15 and IL-18 (80). Upon activation they themselves produce cytokines such as IFN- γ , TNF- α , IL-3, 5, 8, 10, 13 and GM-CSF to regulate other cells of the immune system and for autocrine regulation (80). For instance, IL-2 and IL-15 induce proliferation of NK cells; IL-12 and IFN- γ stimulate NK cell cytokine production (81). NK cells are cytotoxic; they contain small granules in their cytoplasm which consist of proteins such as perforin and proteases known as granzymes (82). Upon release in close proximity to a cell slated for killing, perforin forms pores in the cell membrane of the target cell, creating an aqueous channel through which the granzymes and associated molecules can enter, inducing either apoptosis or osmotic cell lysis (82).

NK cells play a crucial role in the immunosurveillance of tumors by recognizing and eliminating tumor cells (83). Their reactivity is guided by the principles of "missing-self" and "induced-self" which imply that cells with low or absent expression of MHC class I (missing-self) and/or a stress-induced expression of ligands for activating NK receptors (induced-self) are preferentially recognized and eliminated (84-86). Thus, a balance between

activating and inhibitory signals mediated by various receptors determines whether NK cell responses are initiated or not. Binding of the tumor associated ligands to activating NK cell receptors is not well characterized except for the MHC class I-related molecules which bind to the activating homodimeric C-type lectin-like natural killer group 2 member D (NKG2D) immunoreceptor on NK cells (87). NKG2D ligands (NKG2DL) are generally not expressed on healthy tissues (88) but are inducibly expressed on cells associated with malignant transformation (89). Expression of these ligands renders cells susceptible to NK cell reactivity despite expression of MHC class I and potently stimulates NK cell cytotoxicity and cytokine secretion (87). Furthermore, NKG2D has been shown to provide protection from spontaneous tumors *in vivo* and in animals deficient of NKG2D have demonstrated a key role of this receptor in immune surveillance (90-92). It has also been shown that tumor cells escape from NKG2D mediated antitumor immunity by reduction of NKG2DL expression on their surface and/or by "silencing" NKG2D on cytotoxic lymphocytes by release of soluble NKG2DL by tumor cells which causes systemically diminished NKG2D expression (93-95).

Numerous models of experimental and spontaneous metastases have demonstrated the antimetastatic function of NK cells (83, 96). Likewise, human studies have also found a correlation between levels of NK activity and susceptibility to several different types of cancer. For instance, patients displaying lower levels of NK activity (for instance, X-linked lymphoproliferative syndrome, Chediak-Higashi syndrome) have been shown to demonstrate a higher incidence of cancer (97). Higher levels of NK activity at the time of tumor removal have been associated with good prognosis following excision of various types of cancers (98, 99).

1.8 Linking activation of coagulation pathways and NK cells in tumor metastases

Substantial evidence now exists in the literature that coagulation factors facilitate hematogenous dissemination of tumor cells (55, 100, 101). As described above, the role of platelets and fibrin has been intensely investigated and it has been shown that fibrinogen and platelets are both necessary (54) and facilitate each other (102) in protecting tumor cells from NK cytotoxicity. Stronger adhesion between tumor cells and platelets was found to occur after tumor cells were incubated with fibrinogen suggesting that fibrinogen can enhance the adhesion between tumor cells and platelets in vessels by acting as a molecular bridge (102). They further showed that among the integrin ligands of fibrinogen expressed on the surface of B16F10 melanoma cells and platelets, $\beta 3$ integrin on the surface of platelets and B16F10 tumor cells is more important than $\beta 1$ integrin in mediating the interaction between tumor cells and platelets (102). Thrombin or platelets were required for the role of fibrinogen in blocking NK cytotoxicity (102). Along the same lines, treatment with anticoagulants make tumor cells more vulnerable to the cytotoxic action of NK cells (3).

Mechanistic studies suggest that platelets, along with fibrin, appear to form aggregates around tumor cell emboli and this appears to be related to the metastatic potential of the tumor cells (53, 103). 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 (104, 105), release of stored proangiogenic factors and mitogenic factors (53) or protecting tumor cells from NK cell mediated immune destruction (3, 8, 36, 54, 58, 75).

1.9 Role of anticoagulants in attenuating metastatic disease

As mentioned previously, much of our understanding of the role of coagulation in the development of metastatic disease comes from studies of anticoagulants. A number of different anticoagulants including hirudin (specific thrombin inhibitor) (58, 106) and warfarin (Factor II, VII, IX and X inhibitor) (58) have been studied in this context of which heparins and their enzymatic derivative low-molecular-weight-heparins (LMWH) have been extensively investigated.

Heparin is a glycosoaminoglycan containing various polymeric units and, therefore, has widely different molecular weight components (107). LMWH is synthesized by partial hydrolysis or enzymatic degradation of standard heparin and has a narrow spectrum of smaller polymeric units (107). Antimetastatic properties of heparin/LMWH have been a focus of interest for decades. Multiple potential mechanisms are implicated in the anticancer effect of these drugs including:

- 1)** Inhibition of Factor X activation and thereby acting as an inhibitor of thrombin and fibrin production (107),
- 2)** Capacity to release tissue factor pathway inhibitor (TFPI) from the vascular endothelium and in turn inhibit TF signaling (33),
- 3)** Interacting with a variety of vascular growth factors released from the tumor cells or the endothelium including VEGF (108),
- 4)** Interfering with selectin- or integrin-mediated adhesion of tumor cells, platelets and leukocytes to the endothelium (109).

In terms of clinical studies, a recently published meta-analysis established that the use of prophylactic LMWH, in addition to standard therapy, was associated with an

improvement in survival in patients with solid malignancies with a 40% reduction in the odds of death at 2 years (110). These studies were quite heterogeneous in terms of the dose and duration of LMWH, differences in tumor type, stage and overall patient prognosis making it difficult to establish any definitive recommendations regarding the use of LMWH in cancer patients with no history of venous thromboembolism. This highlights the need for further studies focusing on mechanisms and optimal regimens of LMWH in cancer patients.

Evidence is even more scarce regarding the effect of administering perioperative anticoagulation on subsequent cancer survival. A randomized study published by Kingston, R et al (111) involving about 600 patients examined the effect of razoxane, a topoisomerase inhibitor, versus placebo following curative surgery for colorectal cancer. They failed to establish the efficacy of razoxane in this study, however, a retrospective post-hoc analysis showed that use of perioperative unfractionated heparin was associated with an improvement in the 5-year overall survival. Although the results of the study were interesting, it was limited by the retrospective nature of the analysis. At the same time, the dosing and duration of heparin was not standardized and treatment groups differed because there was significant variability in the practice patterns among surgeons and centers. This further speaks to the need for prospective clinical trials in establishing the efficacy for the perioperative use of LMWH to improve cancer-specific survival following surgical resection of the tumor.

1.10 Rationale and Study Objectives

Despite the gaps in our understanding of the potential mechanisms crucial to the development of postoperative metastatic disease, surgical resection remains a mainstay of therapy for patients with localized solid malignancies. In spite of complete resection, many

patients develop metastatic recurrence and ultimately die of their disease. Understanding the mechanisms that play key roles in the promotion of cancer metastases following surgery will allow us to design more effective cancer therapeutic strategies for application in the postoperative setting.

Interestingly, as described above, the mechanisms that are responsible for generating a hypercoagulable state following surgery are the same mechanisms that have also been implicated in the development of metastatic disease. At the same time, the link between postoperative hypercoagulability and the significant increase in experimental metastases seen following surgery has not been previously explored. We, therefore, **hypothesized** that **the hypercoagulable state that results from surgical stress might also be responsible for the enhanced development of cancer metastases seen in the postoperative period.**

The overall goal of our study was to define and characterize the interaction between postoperative hypercoagulability and the increase in metastases observed following surgery. Our approach included establishing a reproducible murine model of surgical stress and experimental metastases to investigate the involvement of the coagulation-based mechanisms in the development of tumor metastases in the postoperative period. The **objectives** set forth for the study are as follows:

- 1)** To determine if the use of perioperative anticoagulation could attenuate the development of postoperative metastases and thereby provide evidence for involvement of the coagulation system in our model.

2) To investigate the early fate of tumor cells and to determine whether surgery affects the initial arrest or sustained survival/decreased clearance of micrometastases, or the establishment and growth of macrometastases in our murine surgical model.

3) From the mechanistic point of view, to explore the association between tumor cell emboli and peritumoral clot (fibrin and platelet clots) formation in the postoperative period as one of the mechanisms by which surgery promotes the enhancement of tumor metastases seen in the postoperative period.

4) To evaluate the role of NK cells in determining the fate of tumor cells in the postoperative period.

2. Materials and Methods

2.1 Reagents – Cell labeling dyes Vybrant DiD and green fluorescent chloromethyl fluorescein diacetate (CMFDA) were purchased from Molecular Probes, Invitrogen, OR, USA. Human plasma fibrinogen Alexa-Fluor647 was obtained from Invitrogen, OR, USA. *In vivo* platelet labeling antibody, DyLight 488, and platelet depletion antibody, anti-mouse GPIIb/IIIa were from Emfret Analytics, Germany. Rabbit anti-mouse/rat asialo GM1 polyclonal antibody and non-immune rabbit IgG were purchased from Cedarlane Labs, Hornby, ON, Canada. The following drugs were bought from The Ottawa Hospital Pharmacy: Tinzaparin Sodium (Innohep 10,000 anti-Xa IU/ml) from Leo Pharma, Denmark; Dalteparin (5000 anti-Xa IU/0.2 ml from Pharmacia) and Warfarin 5 mg tablets. Recombinant hirudin (Iprivask) (15 mg desirudin per vial which is equivalent to 300,000 anti-thrombin units) was from Canyon Pharmaceuticals Inc, MD, USA. Actin FS, Calcium Chloride 0.025M and Thromborel S reagents were purchased from Siemens Healthcare Diagnostics, Mississauga, ON, Canada.

2.2 Cell lines – The mouse colon carcinoma cell line, CT26LacZ was purchased from American Type Culture Collection (ATCC, USA). Cells were maintained as monolayer cultures in DMEM/high glucose medium supplemented with 10% Fetal Bovine Serum (FBS). The B16F10LacZ melanoma cell line was obtained from Dr K Graham, London Regional Cancer Program, London, ON, Canada. Cells were maintained in α -Minimal Essential Medium (α -MEM) supplemented with 10% FBS. All cells were incubated at 37°C with 5% CO₂. Before use, cells were washed with Phosphate buffered saline (PBS), released by incubation with 0.05% trypsin for 3 min at 37°C before suspending in PBS for CT26LacZ and α -MEM without serum for B16F10LacZ for intravenous injection in mice.

300,000 cells were injected in a 0.1 ml volume via lateral tail vein per mouse. Cell viability and counts were done using Vi-cell XR cell viability analyzer (Beckman Coulter, USA). Viability of the injected cells was consistently >95%. All media and additives were from HyClone Labs (Logan, Utah, USA).

2.3 Mice – Six- to eight-week old specific-pathogen-free female Balb/c, C57Bl/6 and Fox chase severe combined immunodeficiency disease (SCID) mice were purchased from Charles River Laboratories, Quebec, Canada. Animals were housed in pathogen-free conditions at the Animal Care facility at the University of Ottawa, fed standard chow and water *ad libitum* and maintained on a 12-hour light/dark cycle. They were allowed to acclimate in the vivarium for a minimum of one week following arrival before beginning experiments. All studies were approved by the Animal Care Committee at the University of Ottawa.

2.4 Establishment of murine surgical stress model – Mice were subjected to 2.5% Isoflurane (Baxter Corp, Mississauga, ON, Canada) 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 then every 8h for two days postoperatively. Surgical stress was induced in Balb/c mice by a laparotomy and partial left hepatectomy (LPLH) preceded by an intravenous challenge of CT26LacZ cells to establish pulmonary metastases. The operative procedure included making a 3-cm midline abdominal incision (laparotomy) to access the abdominal cavity. The left lobe of the liver was then identified, tied off with a 5-0 Polysorb suture (Tyco Healthcare, CT, USA) and resected distally. After achieving adequate hemostasis, the abdomen was closed in two layers

(continuous suture with 5-0 polysorb for the muscle layer and staples for the skin). Animals were euthanized at either 3 or 8 days following tumor cell injection and their harvested lungs were stained with X-gal (Bioshop, Burlington, Canada) as described previously (50) to visualize pulmonary metastases. The total number of surface visible metastases was determined on the largest lung lobe (left lobe) using a stereomicroscope (Leica Microsystems, Switzerland). It was found that the total number of lung metastases in all five lung lobes correlated well with the metastases in the largest lung lobe (data not shown) and therefore, the largest lobe (left lobe) counts were used for the study. The experiment was repeated in C57Bl/6 mice using the syngeneic B16F10LacZ cell line to show that the effects are not cell- or mouse-strain-specific.

Using the above described methodology, experiments were conducted using laparotomy and left nephrectomy (left kidney resection – LLN) as a means to induce surgical stress in Balb/c mice with the following modifications. After accessing the abdominal cavity by laparotomy, the left kidney was identified upon externalizing the overlying bowels. Bowels were kept moist throughout the procedure using a saline soaked gauze. The hilum of the kidney was identified, clamped and then tied off using a 5-0 polysorb suture. This was followed by the resection of the kidney and delivery of the bowels back into the abdomen. The abdomen was then closed as described above. Animals were sacrificed at 3 days and their lung tumor burden was quantified.

2.5 Plasma activated Factor X measurement – To quantify the duration of hypercoagulable state in mouse, activated Factor X (FXa) levels were measured in the plasma from Balb/c mice subjected to surgical stress (LPLH) for varying durations (30 min, 4h, 12h or 48h). Non-surgery Balb/c mice were used as controls. At endpoint, blood was

collected via cardiac puncture using 3.8% sodium citrate as an anticoagulant in 1:9 ratio (1 part sodium citrate and 9 parts venous blood). Samples were centrifuged at 2000g for 20 min to collect plasma which was stored at -20°C until analysis using Coatest kit (Chromogenix, Lexington, MA, USA) according to manufacturer's instructions. The measured absorbance at 405 nm is known to be directly proportional to the amount of FXa.

2.6 Measurement of plasma soluble p-selectin levels - Another marker of hypercoagulable state, soluble p-selectin (24), was used to confirm the duration of the hypercoagulable state in Balb/c mice subjected to surgical stress (LPLH) for varying durations (30 min, 4h, 12h or 48h). Non-surgery mice were used as controls. At endpoint, the blood collection and processing was carried out as described above in section 2.5. Mouse soluble p-selectin levels were measured using an ELISA kit (R&D Systems Inc., MN, USA) according to manufacturer's instructions.

2.7 Treatment of mice with anticoagulation drugs – Balb/c mice were treated with two low-molecular-weight-heparins, tinzaparin [21.96 IU 15-30 min prior to surgery and then 7.32 IU daily until endpoint (112)], and dalteparin [1U/g 15-30 min prior to surgery and then daily until endpoint (113)]; specific thrombin-inhibitor (recombinant hirudin/Iprivask - 2300 ATU/mouse dosed every 4h beginning 2h after surgery until endpoint (106)]; Vitamin-K-dependent factors II, VII, IX and X inhibitor warfarin [0.000266 g in sterile water administered 3d and 1d prior to surgery and 1d post surgery (114)] or anti-platelet GPIIb/IIIa [2ug/g body weight in PBS 2h post surgery and 1h prior to tumor cell injection]. All agents were given in 0.1 ml volume subcutaneously except dalteparin in 0.2 ml volume and anti-platelet antibody given intravenously.

2.8 Assays for anti-Xa, prothrombin time, activated partial thromboplastin time and platelet depletion measurement – Plasma anti-Xa activity level was determined post tinzaparin and dalteparin treatment using a photometric assay utilizing COATEST Heparin kit (Chromogenix, Lexington, MA, USA). Activated partial thromboplastin time (aPTT) after hirudin treatment was measured using Actin FS at 30 min, 2h and 4h post first injection and 30 min post second injection as per protocols from the manufacturer. Thromborel S was used to measure prothrombin time (PT) post warfarin treatment as per manufacturer's instructions. Untreated Balb/c mice were used as controls for the respective experiments. Blood was collected as per protocol for FXa described above in section 2.5. Platelet depletion was confirmed by quantifying platelets from blood smears prepared from mice with or without antibody treatment.

2.9 Assay for interaction of tumor cell emboli with platelets and fibrin clots *in vivo* – To evaluate the interaction between tumor cells and peritumoral clots (fibrin and platelets), CT26LacZ cells were fluorescently labeled *ex vivo* using either DiD dye (5 μ l per ml of cell suspension) or CMFDA dye (10 μ M) according to the manufacturer's instructions for labeling of cells in suspension. DiD dye labels phospholipid membranes of the cell. CMFDA dye passes freely across the cell membrane where the chloromethyl group gets acted upon by cellular glutathione-S-transferase to form an adduct that is retained within the cell. This product is however, non-fluorescent and requires cytosolic esterases to cleave off an acetate to produce the green fluorescent product. Cells remain fluorescent for at least 72h after incubation with fresh media at 37°C and through at least 4 cell divisions. 300,000 CT26LacZ cells labeled with DiD dye were then injected intravenously (IV) in Balb/c mice followed by IV injection of DyLight 488 platelet labeling antibody (2 μ g/mouse) and partial

hepatectomy to induce surgical stress, with or without pretreatment with 21.96 IU of tinzaparin subcutaneously. DyLight 488 labeling antibody is raised against the GPIIb β subunit of the murine platelet/megakaryocyte specific GPIIb-V-IX complex. It effectively and stably labels mouse platelets *in vivo*. According to the manufacturer's statements as well as consulting the references provided in the product sheet (115), at the dose utilized in the study, it is non-cytotoxic and does not interfere with platelet adhesion or aggregation *in vivo*. The animals were then euthanized at 4h post cell injection and their lungs were harvested for visualization of fluorescently labeled platelets (green) with tumor cell emboli (red) under the fluorescent microscope (Carl Zeiss AxioCam HR, Germany). 5-10 sections of the lung from each animal were then imaged and the percentage of tumor cell emboli (TCE) associated with platelets was quantified. Similarly, for fibrinogen studies, 300,000 CT26LacZ cells labeled with CMFDA dye were injected IV in Balb/c mice followed by IV injection of Alexa-Fluor647 fibrinogen (0.12 mg/mouse) as previously described (50). This was followed by partial hepatectomy with or without 21.96 IU of tinzaparin pretreatment. Mice were euthanized at 4h post cell inoculation and their lungs were harvested and imaged as described above to determine the percentage of TCE (green) associated with fibrin (red) under the fluorescent microscope.

2.10 Treatment of mice for NK depletion experiments – NK cells were immunologically depleted using rabbit anti-mouse asialo GM1 polyclonal antibody (25 μ l of reconstituted antibody in 50 μ l total volume per mouse) given intravenously 4 days and 1 day prior to tumor cell injection as well as 2 days post surgery. This regimen of dosing every 72h was in accordance with the recommendations from the manufacturer. This has been confirmed in our lab where it has been shown that a single 25 μ l IV injection of this NK depleting

antibody in Balb/c mouse results in minimal killing (0.8%) against YAC-1 cells (NK sensitive cell line) by spleen cells taken 3 days after a single injection. Therefore, the current treatment regimen of single IV injection of the antibody every 3 days was adopted. Corresponding control animals received equivalent amount of non-immune rabbit IgG (25 µl of reconstituted antibody in 50 µl of total volume per mouse) (36). Animals were subjected to surgical stress via LPLH preceded by an intravenous injection of tumor cells to establish pulmonary metastases. They were sacrificed at 3 days post cell injection and their lung tumor burden was quantified using a stereomicroscope.

2.11 Evaluation of the role of B and T lymphocytes in postoperative tumor metastases

- To evaluate the role of B and T cells in postoperative tumor metastases in our surgical model, experiments were conducted in transgenic animals deficient of B and T cells (SCID mice). Mice received 300,000 CT26LacZ cells intravenously just prior to subjecting them to surgical stress (LPLH) and their lung tumor burden was quantified at 3 days post cell injection.

2.12 Statistical analysis – Data was analyzed using Graph Pad Prism 4.0 statistical software using one-way analysis of variance for comparison between means from three or more groups followed by post-hoc analysis using Bonferroni's test. Student's t-test was used for comparing means between two groups. Probability values less than 0.05 were considered significant.

3. Results

3.1 Surgical stress results in increased pulmonary metastases and perioperative

anticoagulation attenuates this effect. Surgical stress was induced in immune-competent Balb/c mice by a laparotomy and a partial left hepatectomy preceded by a metastatic challenge of CT26LacZ colon cancer cells (Figure 1). As seen in a representative lung photograph from a mouse 8 days following laparotomy and partial left hepatectomy (Figure 2A), surgical stress resulted in a dramatic increase in tumor metastases compared to the animal that did not undergo surgery. Similar results were obtained at an earlier endpoint of 3 days when compared to the non-surgery control (Figure 2B) or mice that received anesthesia only (Figure 2D).

Using the same experimental approach, the surgical stress model was repeated employing B16F10LacZ melanoma cell line syngeneic to C57Bl/6 mice. Surgical stress also significantly increased the number of lung metastatic deposits seen at 3 days in these mice, further suggesting that the prometastatic effect seen following surgery is not mouse-strain or cell-type specific (Figure 2C). 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 seen at 3 days to a similar degree as laparotomy and partial left hepatectomy (Figure 2D).

To define the association between the procoagulant and prometastatic effects of surgery, we measured the activation of the clotting cascade following surgery by measuring plasma Factor Xa (FXa). A significantly higher increase in the percentage of FXa when compared to non-surgery controls was seen 4h post surgery (Figure 3B). Another marker of the procoagulant state, soluble p-selectin level [although a marker of platelet and endothelial

cell activation, now regarded as a marker of an overall high procoagulant state (24)] was also found to be significantly higher 4h and 12h following surgery compared with non-surgery controls (Figure 3C). This suggests that the activation of the coagulation cascade lasts for at least 12h post surgery in mice. We further correlated these findings with experimental metastases and performed surgery 30 min, 4h, 12h or 48h prior to tumor cell injection and quantified lung tumor burden at 3 days following tumor cell challenge (Figure 4A). Surgery performed 30 min and 4h prior to cell inoculation resulted in significantly higher increase in lung metastases while surgery performed 12h and 48h prior to cell inoculation did not demonstrate a statistically significant difference when compared to non-surgery controls (Figure 4B). This suggests that the hypercoagulable state resulting from surgical stress resulting in increased tumor metastases persists for at least 4h post surgery in Balb/c mice. Of note, the 12h data (Figure 4B), has consistently been associated with high variability in data points and one possible explanation for this might relate to 12h being the transition point where the statistically significant effect seen prior to 12h is now progressing towards normalcy (compared to non-surgery controls).

To further determine the contribution of coagulation-based mechanisms in cancer metastases in our surgical model, five different anticoagulant agents - tinzaparin, dalteparin, hirudin, warfarin and anti-platelet GPIIb/IIIa were utilized. Anticoagulation was confirmed at the time of surgery using an appropriate test for the specific agent (Table 1). Perioperative administration of all five anticoagulants resulted in a significant attenuation of the pulmonary metastases (Figure 5A and B) demonstrating that coagulation-mediated pathways are involved in the development of tumor metastases following surgery in our murine model.

3.2 Decreased clearance or sustained adherence of tumor cells is seen at early time points in mice subjected to surgical stress. To investigate the early fate of tumor cells, mice were euthanized at different time points (10 min, 4h and 12h) following cell inoculation with or without surgical stress (LPLH) and perioperative treatment with LMWH (tinzaparin) and their lung tumor burden was quantified (Figure 6A). At 10 min and 4h post cell inoculation, no significant differences were seen between the groups; however, at 12h, significantly more tumor metastases were seen in mice that were subjected to surgical stress which was not observed when surgically stressed mice were pretreated with tinzaparin (Figure 6B and C). This suggests that surgery impedes the clearance or promotes the survival of tumor cells at 12h and that anticoagulation with LMWH in surgically stressed animals can promote clearance of tumor cells or impede their survival.

To further explore this phenomenon, mice were subjected to surgical stress at different time points (0h, 4h, 12h or 24h) post cell injection and were euthanized 3 days after the lung challenge (Figure 7A). Surgery performed immediately, 4h and 12h post cell inoculation resulted in significantly higher lung metastases when compared with surgery performed at 24h post cell injection or non-surgery controls (Figure 7B). This indicates that surgery performed after tumor cell clearance does not promote the development of metastatic disease.

3.3 Peritumoral clot formation is increased following surgery and inhibited by anticoagulation with LMWH. Previous studies have suggested that clot formation around TCE is associated with sustained adherence or decreased clearance of metastases (3, 58, 75). To determine if this mechanism plays a role in the postoperative period, association of fibrin and platelet clots with TCE in the lung at an early time point (4h) was evaluated following

surgery (Figure 8A and 9A). Increased platelet clot formation around TCE was seen in animals that underwent surgery but not seen in the mice that were pretreated with LMWH. More specifically, a 2-fold increase in the percent of TCE associated with platelet clots was seen in animals that underwent surgery compared with non-surgery controls (Figure 8B and C). Likewise, surgery also increased fibrin deposition around TCE with a 3-fold increase in the percent of TCE associated with fibrin clots compared with non-surgery controls (Figure 9B and C). This was not seen in the animals that were pretreated with LMWH.

3.4 Natural-Killer cells and not B and T lymphocytes are important in the formation of postoperative tumor metastases. Previous studies have demonstrated that NK cells play an important role in clearing circulating tumor cells in the vasculature (83, 96). To determine if this mechanism is operating in the postoperative period in our surgical model of experimental metastases, the surgical stress experiment was repeated upon depleting NK cells from Balb/c mice (Figure 10A). If NK cells are key players in the prometastatic effect of surgery, then these effects should not be evident in NK depleted mice. If, however, factors other than NK cells are sufficient to mediate these effects, then these effects should still be evident in NK depleted animals. Depletion of NK cells caused a 2-fold increase in the lung tumor burden in non-surgery controls (no surgery, NK intact compared with no surgery, NK depleted) demonstrating the high sensitivity of this measure to NK cell activity (Figure 10B). In animals with intact NK cells, a significant increase in lung tumor metastases was seen, where as in mice depleted of NK cells, this prometastatic effect of surgery (LPLH) was no longer evident (Figure 10B) suggesting that NK cells play a central role in the formation of lung tumor metastases following surgery. At the same time, the lack of a further increase in metastases in the surgically stressed animals depleted of NK cells

compared with non-surgery controls depleted of NK cells argues against the presence of another mechanism playing a significant role.

In addition, we also evaluated the role of B and T lymphocytes in our surgical stress model and performed surgery (LPLH) in transgenic mice deficient of B and T cells (SCID) and quantified the lung tumor burden at 3 days post cell inoculation (Figure 11A). A significant increase in pulmonary tumor metastases was observed in animals subjected to surgical stress (Figure 11B) suggesting that B and T cells are not the primary mediators of the metastases enhancing effects of surgery in our model.

Figure 1 Schematic for the establishment of the surgical stress model of experimental metastases. Surgical stress was induced in Balb/c mice by a laparotomy and a partial left hepatectomy which was preceded by an intravenous challenge of CT26LacZ colon cancer cells (lung challenge) with and without perioperative treatment with LMWH, tinzaparin. Animals were euthanized at either day 3 or day 8 following cell injection and their lungs were harvested and stained with X-gal to visualize the pulmonary metastases.

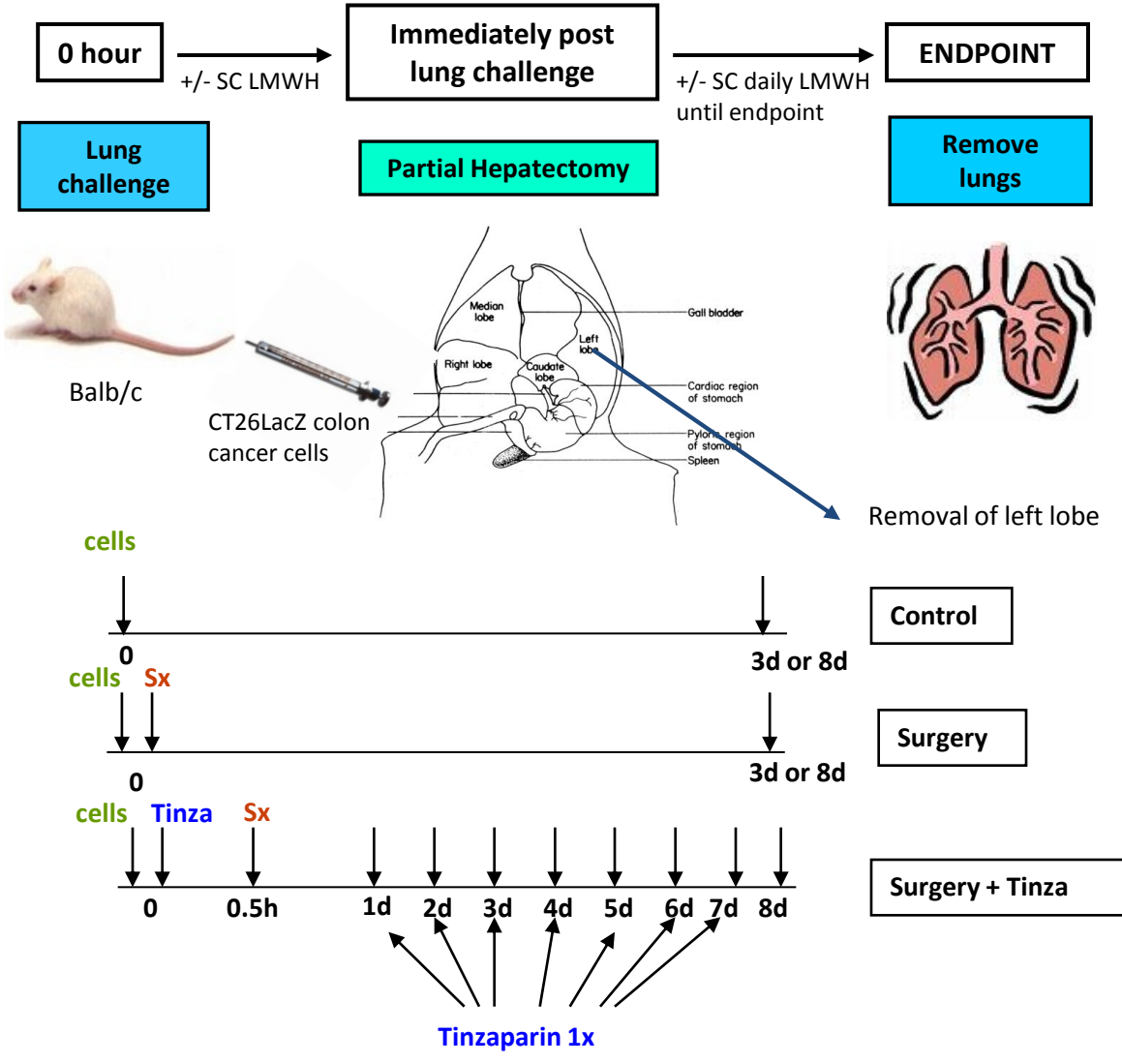


Figure 2 Surgical stress increases tumor metastases. A, Photographs of lungs from non-surgery control (left) and an animal that underwent laparotomy and partial left hepatectomy (LPLH) (right) at 8 days. B, Quantification of lung surface metastases [counts represented by mean \pm standard error of the mean (SEM)] from surgically stressed Balb/c mice (n=9) and controls (n=10) at 3 days. Graph represents data compiled from two independent experiments. C, Quantification of lung surface metastases (mean counts \pm SEM) from surgically stressed C57Bl/6 mice (n=10) and controls (n=12) at 3 days. Graph represents data compiled from two independent experiments. D, Quantification of lung surface metastases (mean counts \pm SEM) in Balb/c mice that underwent LPLH (n=9) or laparotomy and left nephrectomy (LLN) (n=4) at 3 days. Data compiled from two independent experiments is shown. *p<0.001, **p<0.01 compared with controls.

A Control Surgery (Partial Hepatectomy)

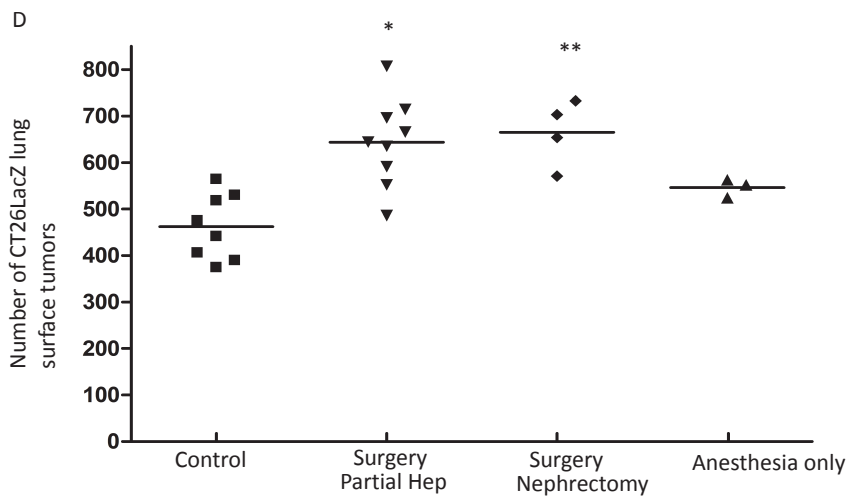
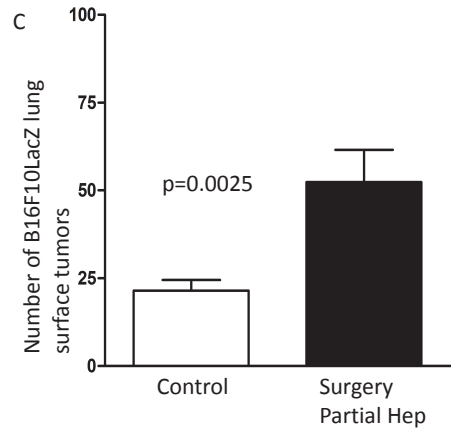
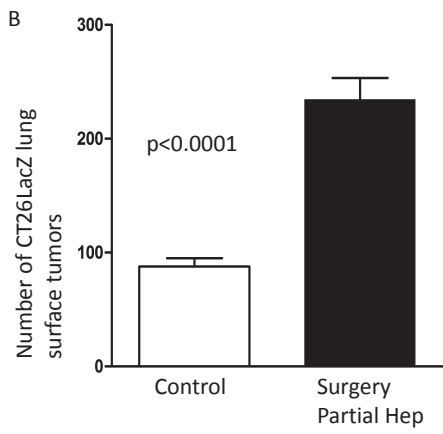


Figure 3 Establishment of the duration of hypercoagulable state in mice following surgery. A, Schematic of the experimental design. Balb/c mice were subjected to surgical stress (LPLH) for 30 min, 4h, 12h or 48h and their blood was collected via cardiac puncture at these respective endpoints. Blood was processed as described in the Materials and Methods section to obtain plasma used for measurement of FXa and soluble p-selectin levels. B, Percent increase in plasma FXa from Balb/c mice subjected to surgical stress (LPLH) for 30 min, 4h, 12h or 48h with n=5 per group compared to non-surgery controls. *p=0.039 when absorbance values were compared to non-surgery controls. C, Percent increase in plasma soluble p-selectin levels from Balb/c mice subjected to surgical stress (LPLH) for 30 min, 4h, 12h or 48h with n=5 per group compared to non-surgery controls. *p<0.001 when concentration (ng/ml) was compared with non-surgery controls. The raw data obtained from FXa (absorbance values) and soluble p-selectin (concentration values) were converted to percent increase compared with non-surgery controls to allow for comparisons to be made between the two modalities of characterizing the hypercoagulable state (Factor Xa and soluble p-selectin).

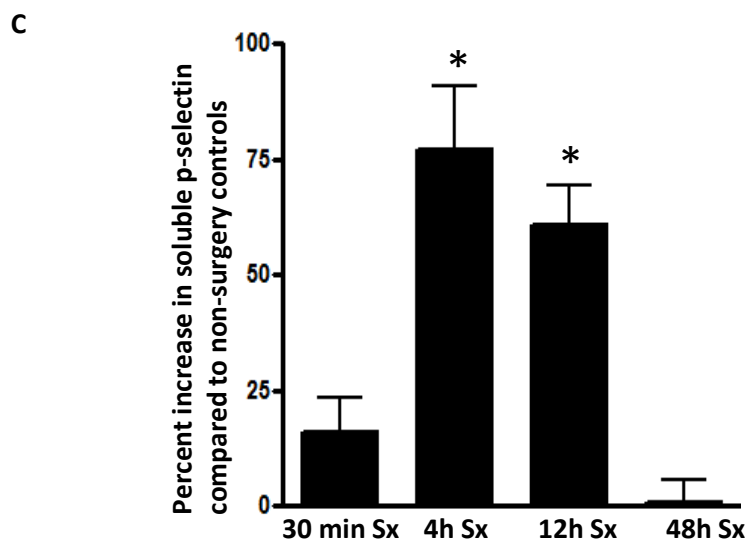
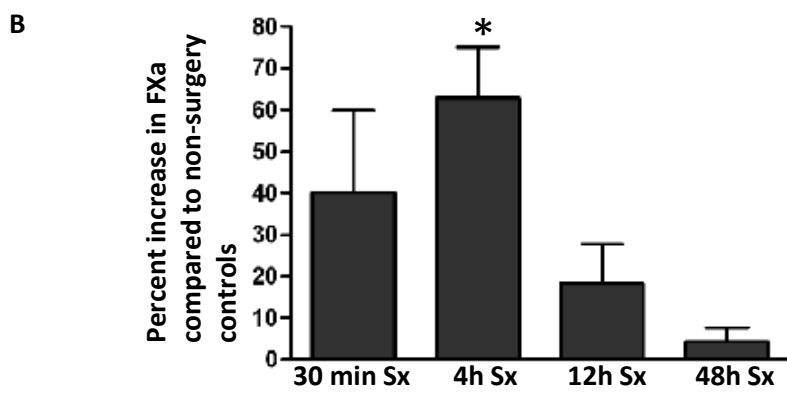
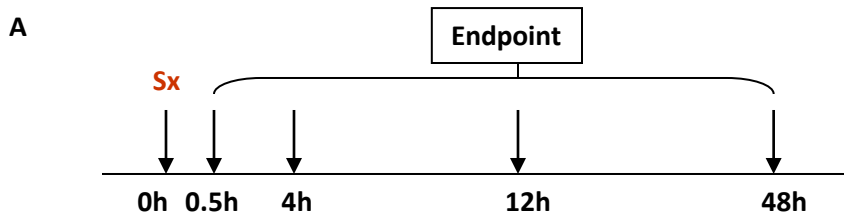
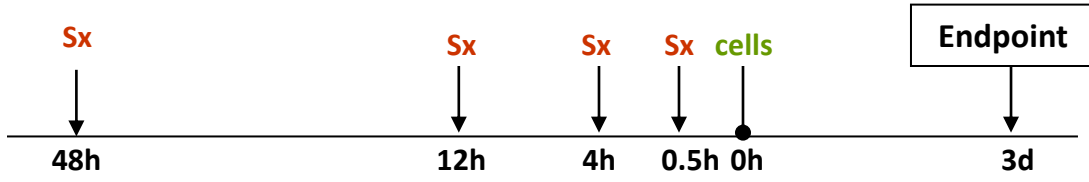


Figure 4 Correlation of the hypercoagulable state in mice following surgery to postoperative tumor metastases. A, Schematic of the experimental set up. Balb/c mice were subjected to surgical stress (LPLH) 30 min, 4h, 12h or 48h prior to tumor cell injection and were euthanized 3 days following the metastatic challenge. Lung tumor burden was quantified and converted to percent increase in tumor counts compared to non-surgery controls. B, Percent increase in lung surface metastases in Balb/c mice subjected to surgical stress 30 min (n=8), 4h (n=5), 12h (n=9) or 48h (n=4) prior to tumor cell injection and euthanized 3 days post tumor cell injection, compared to non-surgery controls. Data pooled from two independent experiments is shown. *p=0.008, **p=0.013 when counts of lung surface metastases are compared to non-surgery controls. Two separate experiments involving different time points (one involving time points 30 min, 4h and 12h for surgery along with non-surgery control; second involving 48h surgery along with non-surgery control) are depicted on the same graph and therefore, for accurate comparisons between the different time points, data is represented as percent increase in lung surface metastases compared to respective non-surgery controls.

A



B

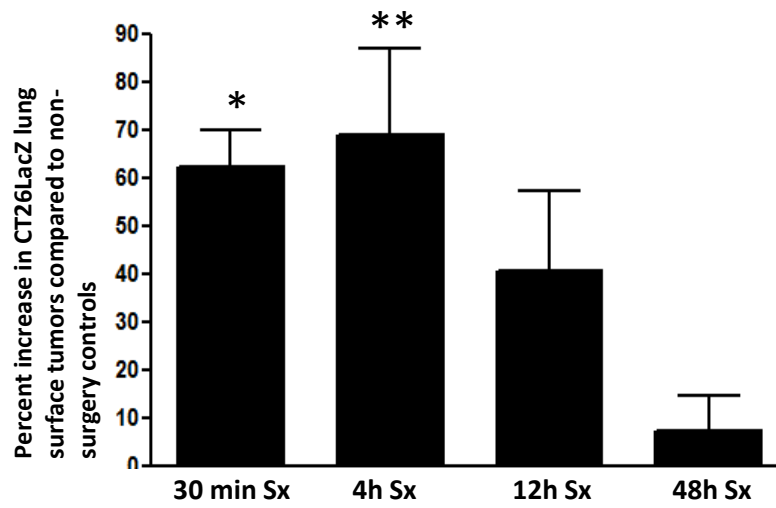


Figure 5 Perioperative anticoagulation significantly attenuates the increased number of lung metastases seen following surgery. A, Quantification of lung tumor metastases at 3 days from surgically stressed animals treated with perioperative anticoagulants. Experiment included 4-5 mice/group. * $p < 0.05$ compared to non-surgery control; ** $p < 0.01$, *** $p < 0.001$ compared to surgery group. B, Quantification of lung tumor metastases (mean counts \pm SEM) at 3 days from surgically stressed mice with and without platelet depletion. Pooled data from two independent experiments is highlighted with $n=8-11$ per group. * $p < 0.001$ compared to non-surgery mice with intact platelets. tinzap=tinzaparin; daltep=dalteparin.

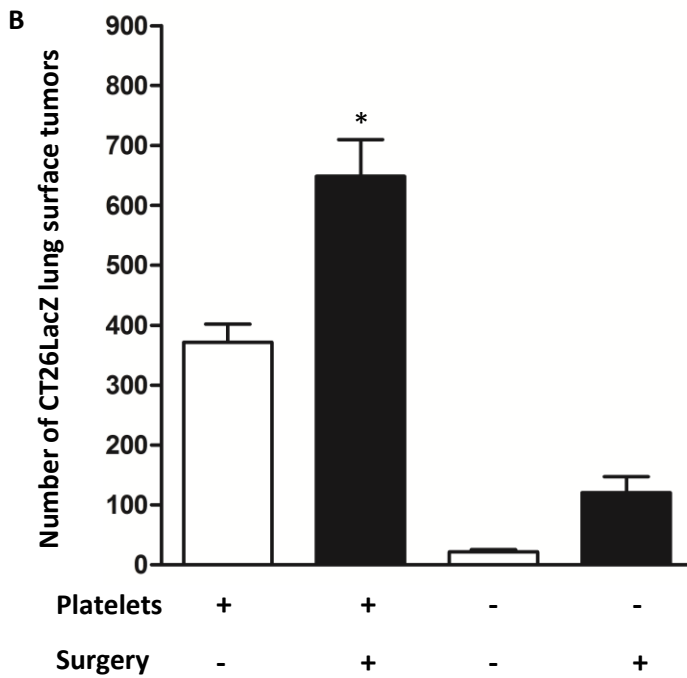
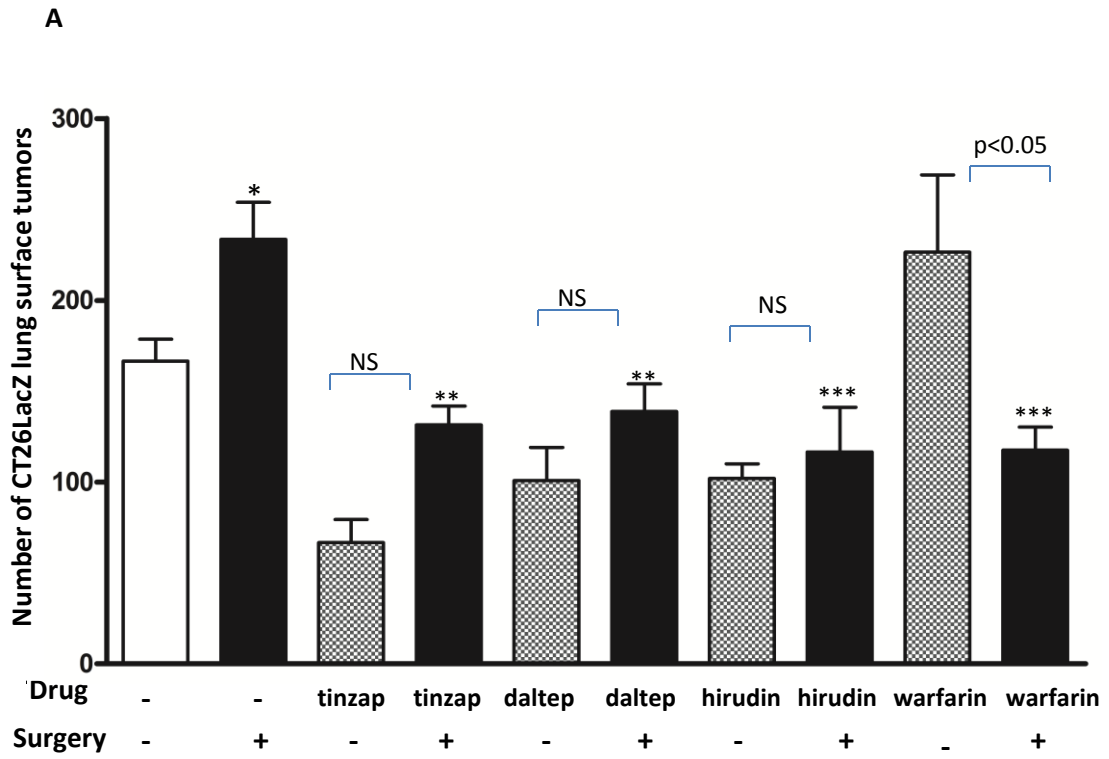


Figure 6 Decreased clearance or sustained adherence of tumor cells is seen between 4-12h in surgically stressed animals. A, Schematic of the experimental set up. Balb/c mice were subjected to surgical stress (LPLH) preceded by injection of CT26LacZ cells with and without perioperative anticoagulation with LMWH and were sacrificed 10 min, 4h or 12h following tumor cell injection. Lung tumor burden was quantified at these endpoints. B, Representative sections of tumor laden lungs from Balb/c mice that underwent no surgery (*first row*), surgical stress (*middle row*) and pretreatment with tinzaparin prior to surgical stress (*third row*) and euthanized at 10 min, 4h or 12h post tumor cell injection. Original magnification, 12.5x. C, Quantification of lung tumor burden (mean counts \pm SEM) from 12h group (n=3/group). *p<0.01 compared to control. **p<0.001 compared to surgery group.

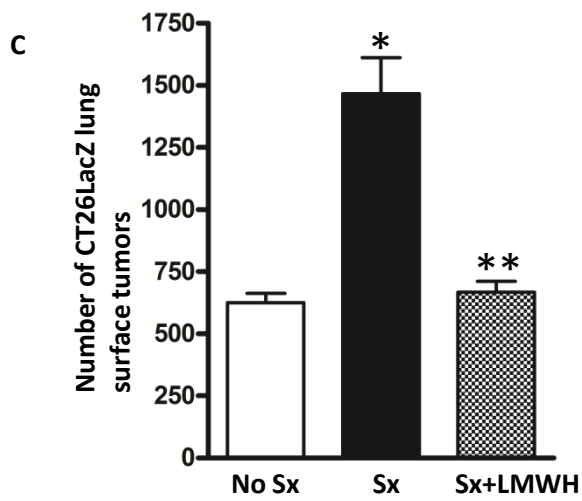
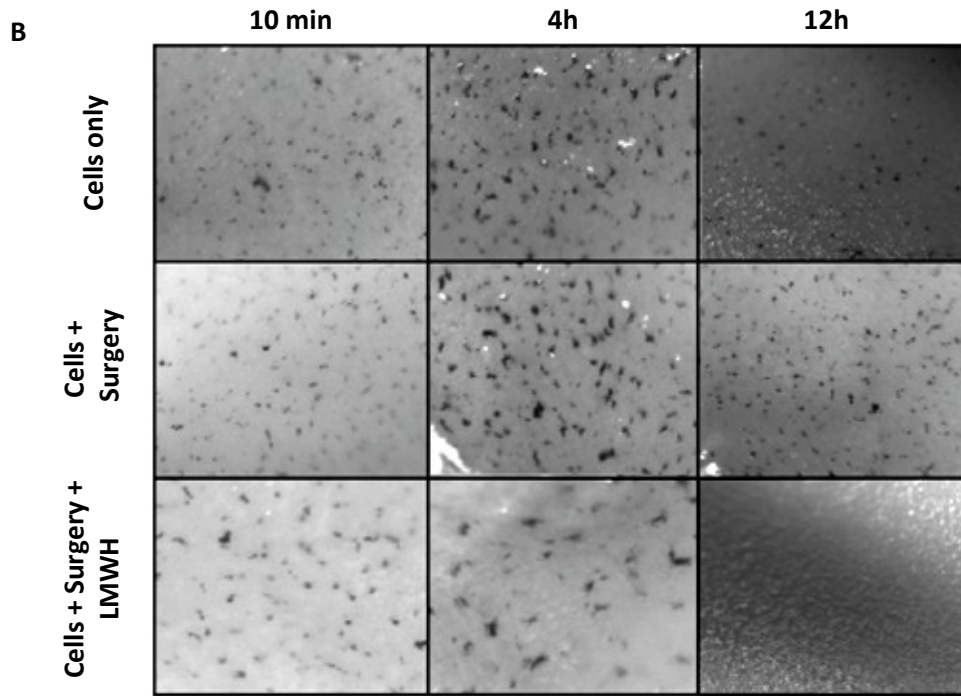
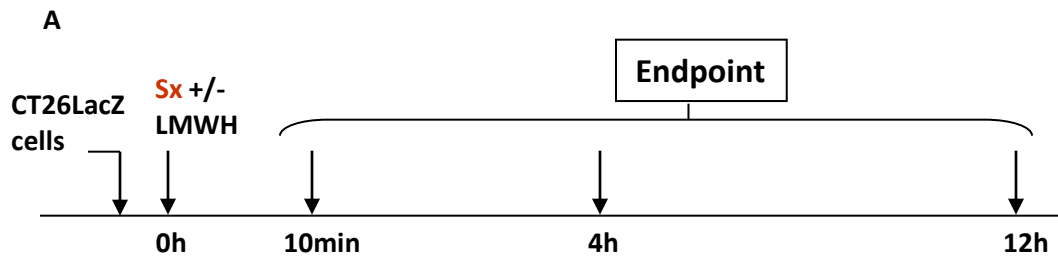
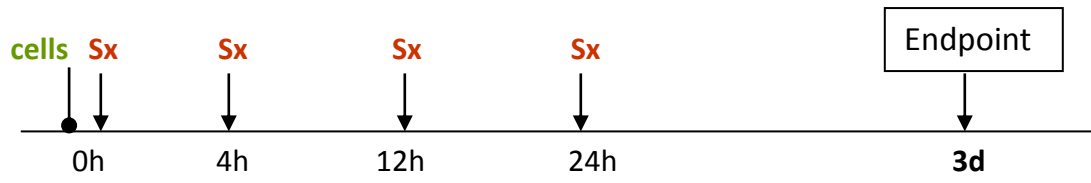


Figure 7 Surgery performed after tumor cell clearance does not promote cancer metastases. A, Schematic representation of the experimental set up. Balb/c mice were subjected to surgical stress (LPLH) immediately, 4h, 12h or 24h following tumor cell injection and were sacrificed 3 days following tumor cell injection. Lungs were harvested for evaluation of pulmonary metastases. B, Quantification of lung tumor metastases (mean counts \pm SEM) from mice subjected to surgical stress immediately (n=11), 4h (n=10), 12h (n=9) or 24h (n=5) after tumor cell injection and euthanized 3 days post tumor cell injection. Non-surgery mice served as controls (n=10). Pooled data from two independent experiments is shown. *p<0.001 compared to non-surgery controls.

A



B

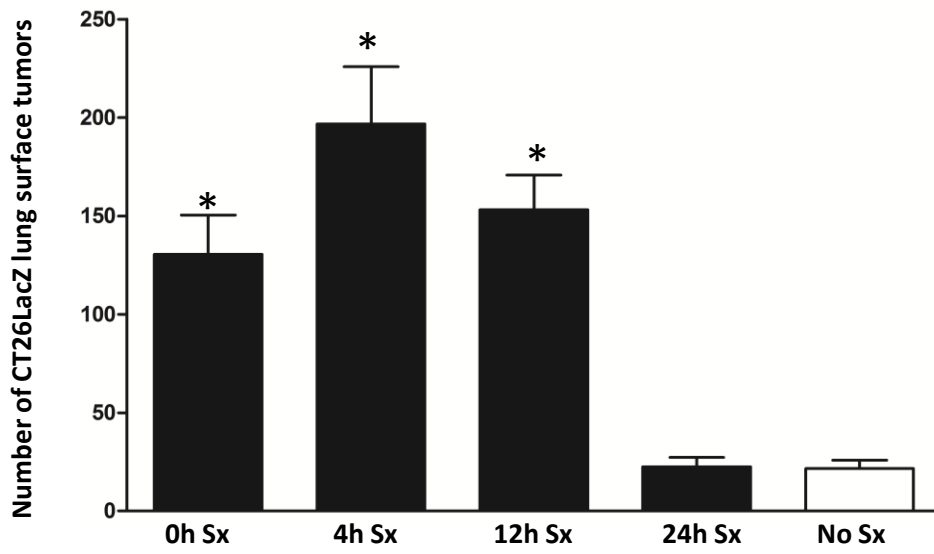


Figure 8 Surgery increases platelet clot formation around tumor cell emboli at 4h. A, Schematic of the experimental set up. Balb/c mice were injected with DiD labeled CT26LacZ cells followed by intravenous administration of DyLight488 platelet labeling antibody, surgery (LPLH) with and without treatment with LMWH and euthanized at 4h following the intervention. Lungs were harvested at endpoint to evaluate the association of tumor cell emboli with platelet clots. B, Representative fluorescence pictures from lungs of mice that underwent no surgery, surgery or treatment with tinzaparin prior to surgery and euthanized 4h post tumor cell injection. *Column one* shows DiD (red) labeled tumor cells while *column two* shows DyLight488 (green) labeled platelets from the same lung sections. Merged pictures from the same sections are depicted in *column three*. Original magnification, 10x. C, Between five to ten sections of each lung from each mouse was imaged and percentage of tumor cell emboli associated with platelet clots was determined per high power field from the merged pictures which was then converted into fold increase compared with non-surgery controls. 4-5 mice were used per group. *p=0.0004 compared to control. **p<0.0001 compared to surgery group.

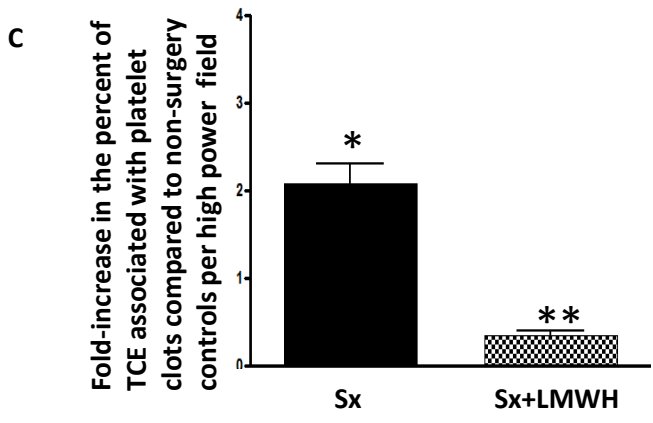
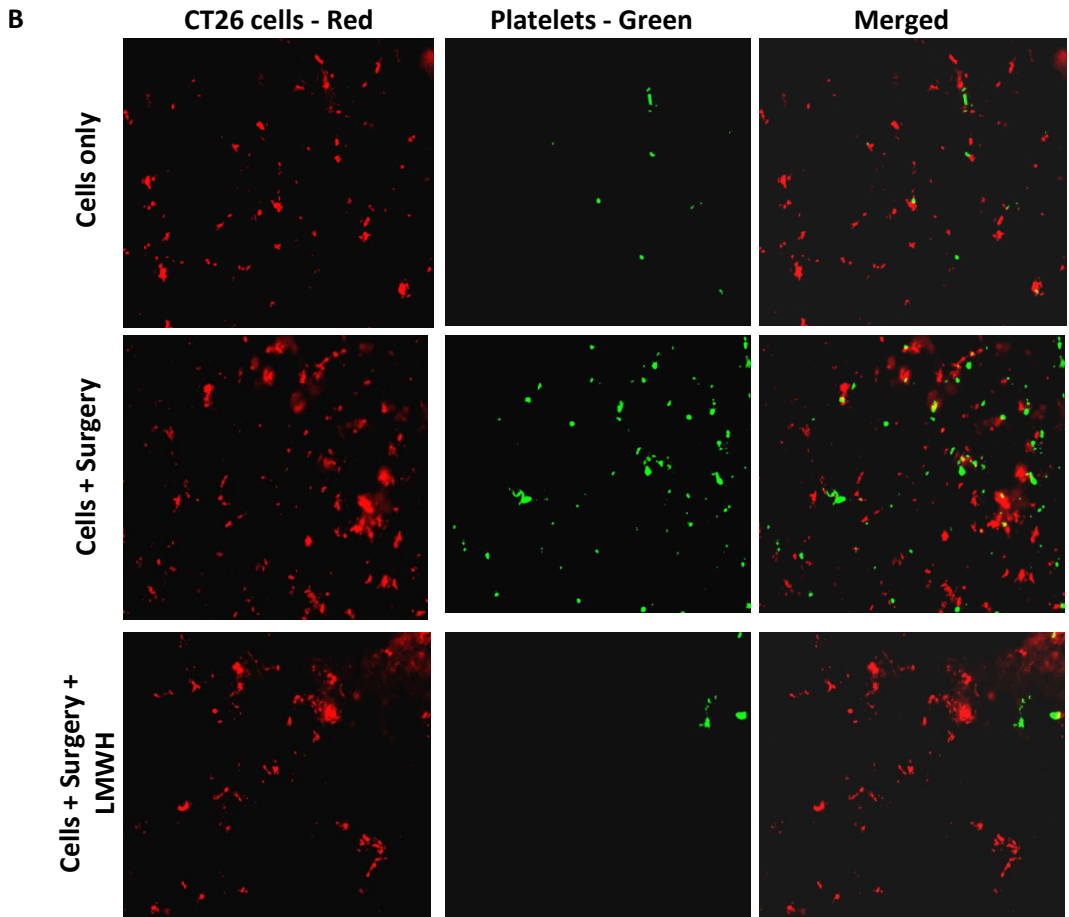
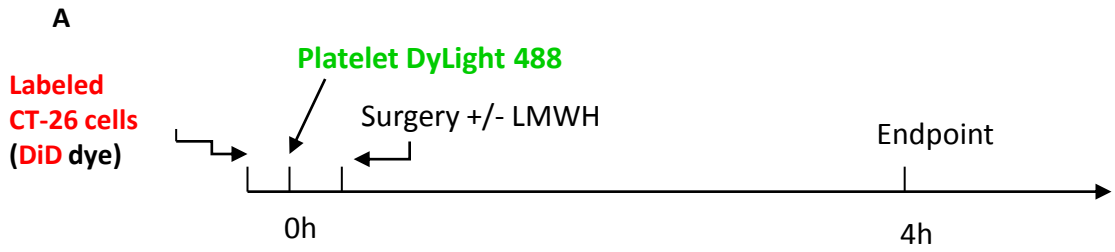


Figure 9 Surgery increases fibrin deposition around tumor cell emboli at 4h. A, Schematic of the experimental set up. Balb/c mice were injected with CMFDA labeled CT26LacZ cells followed by AlexaFluor647-conjugated fibrinogen intravenously, underwent surgery (LPLH) with and without treatment with LMWH and sacrificed 4h post intervention. Lungs were harvested to evaluate the association of tumor cell emboli with fibrin clots. B, Representative fluorescence pictures from lungs of mice that underwent no surgery, surgery or treatment with tinzaparin prior to surgery and euthanized 4h post tumor cell injection. *Column one* shows CMFDA (green) labeled tumor cells while *column two* shows AlexaFluor647-conjugated fibrin (red) from the same lung sections. Merged pictures from the same sections are shown in *column three*. Original magnification, 10x. B, Between five to eleven sections of each lung from each mouse was imaged and the percentage of tumor cell emboli associated with fibrin clots was determined per high power field from the merged pictures which was then converted into fold increase compared with non-surgery controls. 5-6 mice were used per group. *p=0.0028 compared to control. **p=0.0003 compared to surgery group.

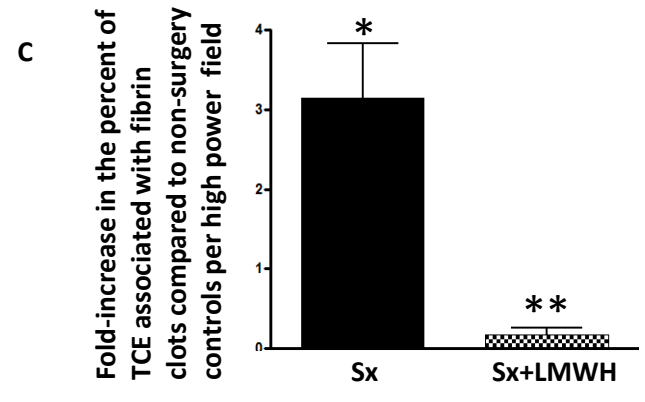
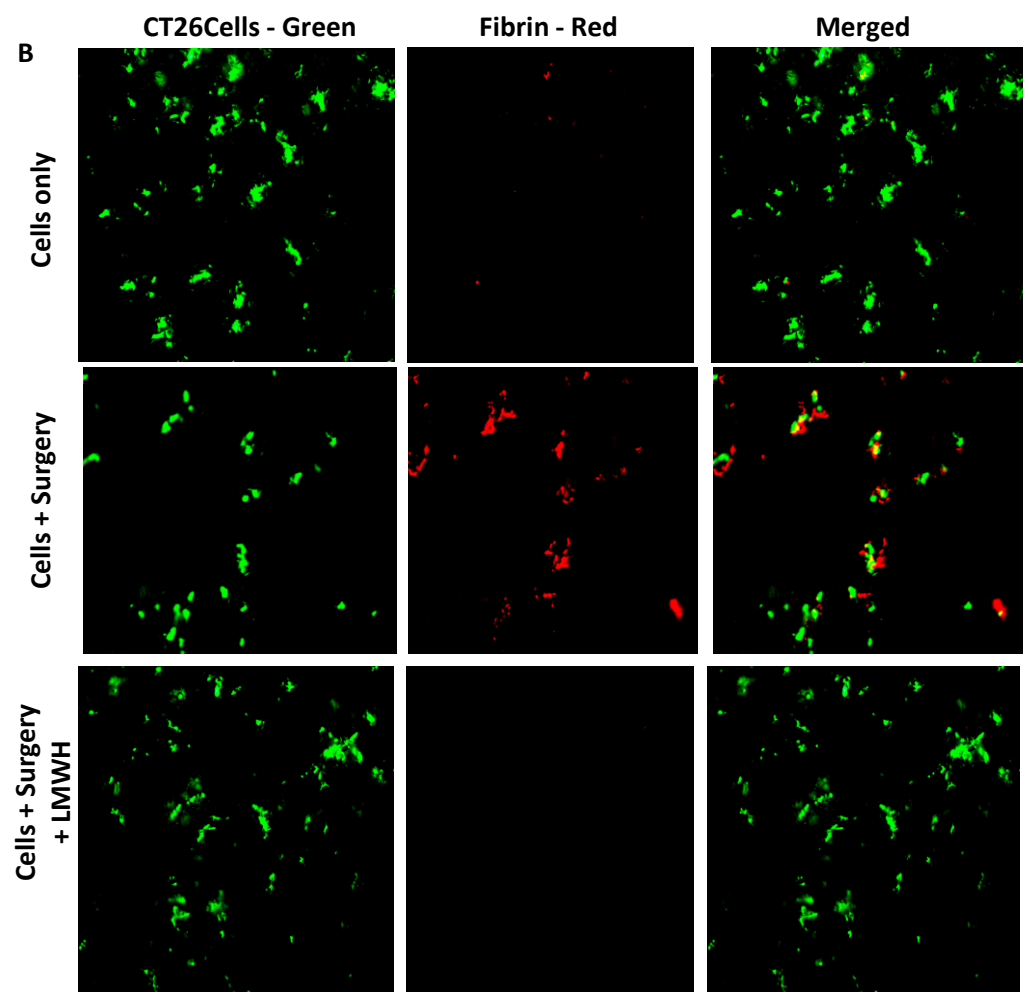
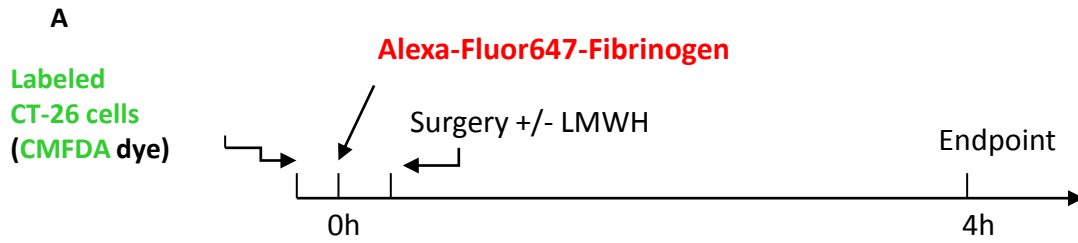


Figure 10 NK cells are important in postoperative tumor cell metastases in the surgical model. A, Schematic representation of the experimental set up. Balb/c mice were treated intravenously with NK depleting antibody (anti-asialo) or control IgG on days -4, -1 and +2, received CT26LacZ tumor cells and underwent surgery (LPLH) on day 0 and were euthanized at day 3 following surgery and tumor cell injection to determine their lung tumor burden. B, Quantification of lung tumor metastases (mean counts \pm SEM) from mice with and without NK depletion in the setting of surgical stress. Pooled data from three independent experiments is shown with n=7-13 per group. *p<0.0001 compared to non-surgery control with intact NK cells. NS=non-significant p value.

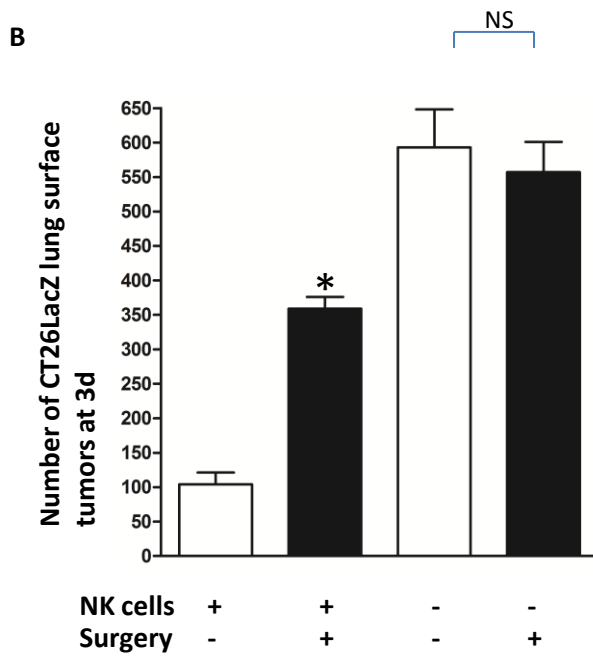
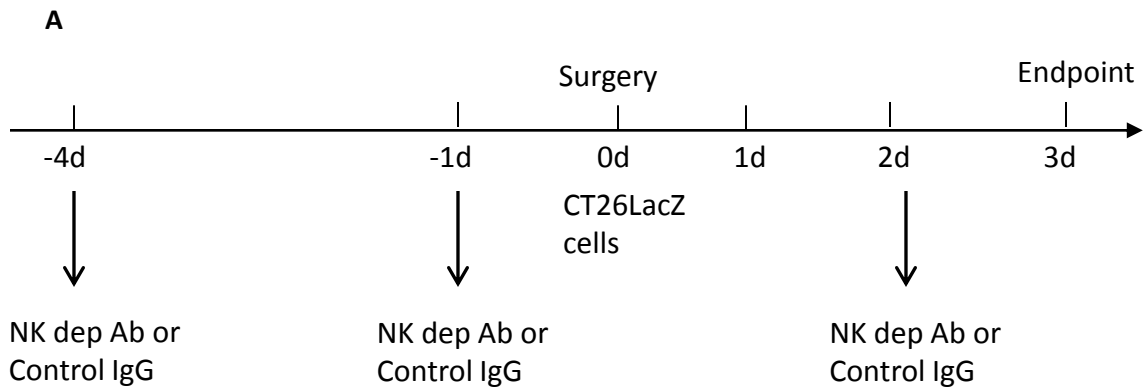
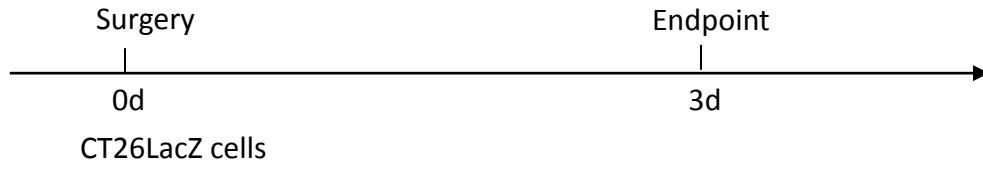


Figure 11 B and T cells are not important in postoperative tumor cell metastases in the surgical stress model. A, Schematic diagram of the experimental design. SCID mice were subjected to surgical stress (LPLH) and injected with CT26LacZ tumor cells intravenously to establish pulmonary metastases and sacrificed at day 3 post surgery and tumor cell injection to evaluate the lung tumor burden. B, Quantification of lung tumor metastases (mean counts \pm SEM) in mice with and without surgical stress. Pooled data from two independent experiments is shown with n=10 per group. *p=0.0004 compared with non-surgery controls.

A



B

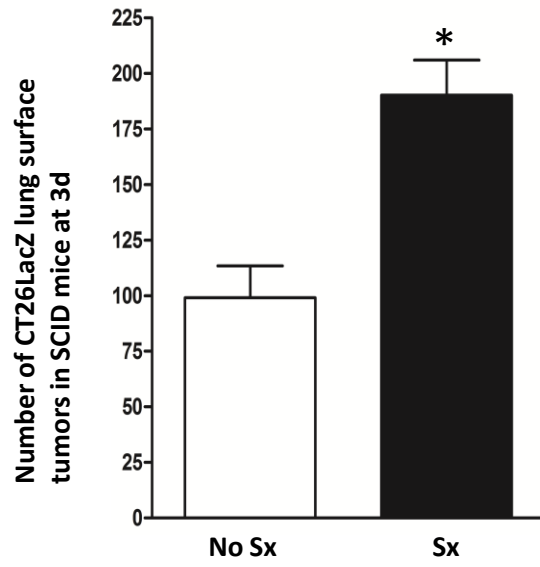


Figure 12 Working model of postoperative hypercoagulability and cancer metastases. Surgical stress/trauma results in the activation of the extrinsic coagulation cascade with activation of Factor X leading to formation of fibrin clots. Platelets are similarly activated by surgical stress with release of soluble P-selectin. These then interact with tumor cell emboli in the circulation, and through potential multiple mechanisms ultimately lead to decreased clearance or increased survival of these tumor cells resulting in the formation of cancer metastases. NK cells are important in the formation of postoperative tumor metastases. Treatment with anticoagulants such as LMWH inhibits activated Factor X resulting in decreased formation of peritumoral clots (involving platelet and fibrin) and leads to increased clearance/decreased survival of tumor cells resulting in attenuation of tumor metastases. The exact mechanistic roles of these major players - fibrin, platelets, tumor cells and NK cells and the interplay between them requires further investigations.

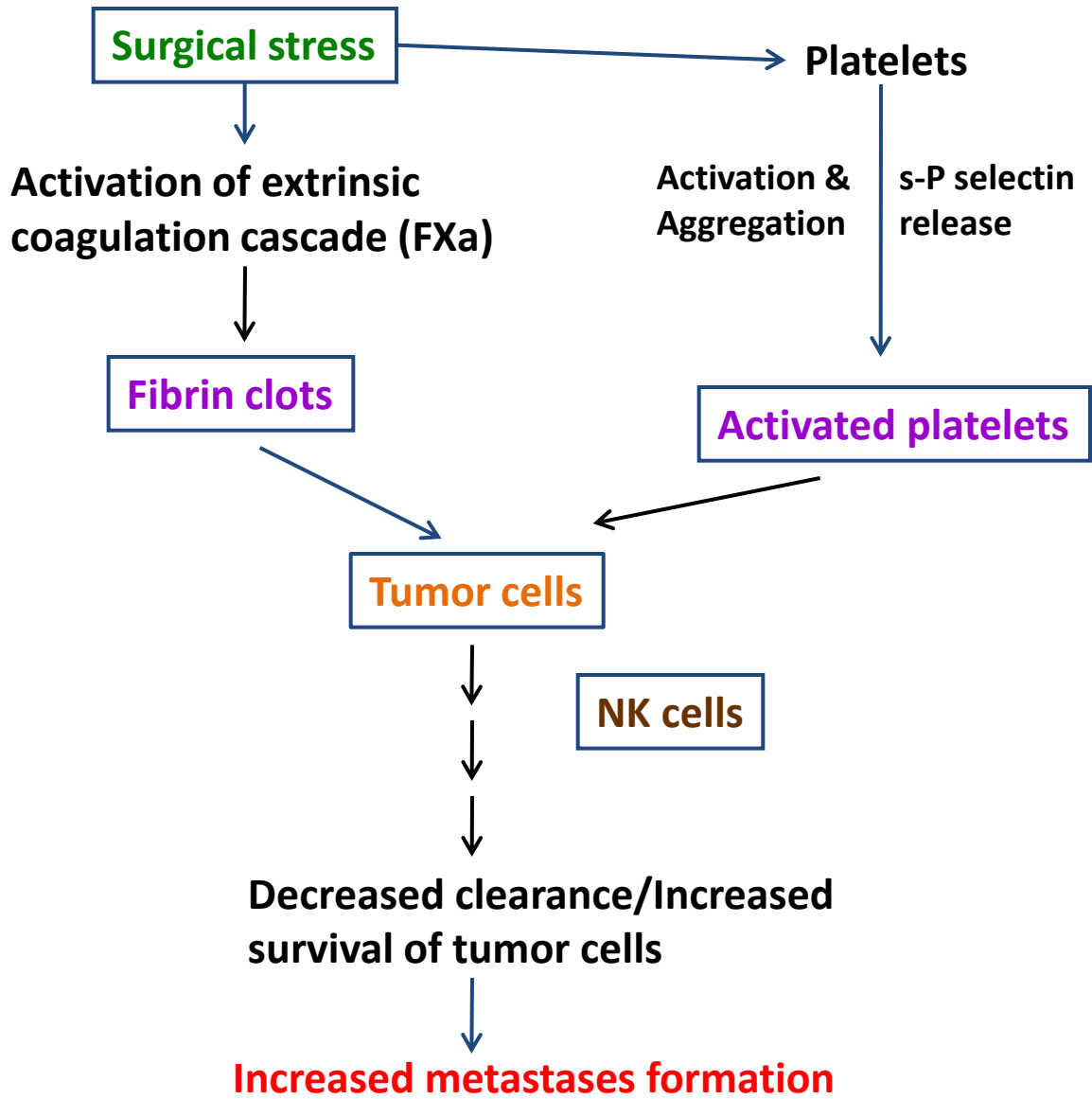


Table 1 Confirmation of anticoagulation at the time of surgery using an appropriate test for specific agent. Tests to confirm anticoagulation in mice treated with various anticoagulants were conducted depending on the anticoagulant drug in question. The appropriate test for the specific agent and the results obtained are highlighted in the table.

| Drug | Class of drug | Anticoagulant activity assay used | Results (values at times post injection) |
|--|--|---|---|
| Tinzaparin | LMWH | Plasma anti-Xa level | 0.94±0.007 IU/ml at 30 min, levels persisted at 2h. Control mice = -0.08±0.01 IU/ml. |
| Dalteparin | LMWH | Plasma anti-Xa level | 0.805±0.007 IU/ml at 30 min. Control mice = -0.2±0.0 IU/ml. |
| Hirudin | Specific thrombin inhibitor | Activated partial thromboplastin time | 80±21 seconds at 30 min. 43±10 seconds at 2h. 25.5±0.0 seconds at 4h. Control mice = 26±5.6 seconds. |
| Warfarin | Vitamin-K dependent factors (Factor II, VII, IX and X) inhibitor | Prothrombin time | 72±9.6 seconds on day 0. Control mice = 8.8±0.35 seconds. |
| Platelet GPIIb/IIIa specific monoclonal antibody | Platelet depletor | Blood smear platelet quantification per high power field (platelet count) | 84.6±9 with no platelet depletion. 5.9±2.1 with platelet depletion. |

4. Discussion

The present 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 the precipitation of a hypercoagulable state (17, 20). Previous animal studies have also demonstrated that surgery promotes the formation of tumor metastases (8, 17-19). At the same time, mechanisms important in hemostasis and thrombosis have been implicated in the development of cancer metastases (51-53). Based on these observations, we hypothesized that these same mechanisms might also play a crucial role in the augmentation of tumor metastases seen following surgery. Our approach, therefore, was to study coagulation-based mechanisms in a murine model of surgical stress in which experimental pulmonary metastases is achieved through an intravenous challenge of tumor cells.

Using this model, we showed that surgical stress induced by two different means (laparotomy and partial left hepatectomy or laparotomy and left nephrectomy) results in a significant increase in lung tumor metastases compared with non-surgery controls (Figure 2). These results are in line with other reports that also show that surgery indeed results in an increased tumor metastases in different animal models (17-19). We have validated our model using a different cell line (B16F10 melanoma cells) and strain of mouse (C57Bl/6) to show that our results are not mouse strain- or cell-specific. Using our surgery model, we investigated the role of coagulation-based mechanisms that might be responsible for the development of tumor metastases observed following surgery. We first established that the time course of the postoperative hypercoagulable state (as measured by Factor Xa and soluble p-selectin levels) mirrors the postoperative prometastatic state in our mouse model.

Factor Xa levels rise immediately and peak at 4h following surgery before returning to normal by 12h (Figure 3). Similarly, soluble p-selectin levels also peak at 4h and although remain significantly high at 12h compared to non-surgery controls, begin to show a downward trend before normalizing by 48h after surgery (Figure 3). In a corresponding way, the increase in metastases is seen when mice are administered a metastatic challenge up to 4h after surgery, but this effect is not statistically demonstrable when the metastatic challenge is administered at 12h (Figure 4) suggesting that the metastatic success is greatly enhanced when the peak of the hypercoagulable state corresponds to the delivery of the metastatic challenge. We next demonstrated that perioperative anticoagulation, using four different anticoagulant agents (two LMWH – tinzaparin and dalteparin; warfarin and hirudin), attenuated the increase in tumor metastases seen following surgery (Figure 5A). Interestingly, warfarin administration in the absence of surgical stress did not decrease metastases compared to non-surgery controls. A number of factors might have influenced this result. First of all, in most preclinical cancer work where warfarin has been used, it has been administered through the oral route via drinking water (58, 116). Lack of appropriate resources in our animal care facilities to appropriately measure the volume of fluid intake by individual mice precluded us from using this particular route of administration. We, therefore, used a subcutaneous route and regimen previously published where warfarin, dissolved in peanut oil, was administered on day 3 and 1 prior to the metastatic challenge (114). Due to solubility issues of warfarin in peanut oil, we instead used a salt formulation of this drug, dissolved in sterile water and administered subcutaneously on day 3 and 1 prior to the metastatic challenge and surgery as well as day 1 post surgery. Following this regimen (266 μ g/mouse), we obtained a PT of 72 seconds in warfarinized mice compared to 8.8

seconds in control non-warfarinized mice. This was in sharp contrast to the PT obtained by others (120-180 seconds) where they demonstrated warfarin's efficacy in reducing metastases in animal models (58, 116). Certainly, doing surgery in mice with such high PTs would result in a very high morbidity and mortality for these mice complicating the interpretation of the results. We, therefore, adopted the current regimen that resulted in the PT that was fairly high (72 seconds) yet allowed our surgeries to be conducted in a safe manner. Unlike warfarin, other agents (LMWH and hirudin) demonstrated a reduction in tumor metastases in non-surgery mice in line with previously published reports (58, 106, 112, 117). While both LMWH and warfarin inhibit FXa and FIIa, LMWH additionally inhibit the pathway at the initiator site (TF) by enhancing the release of TFPI from the endothelium which has the capacity to significantly inhibit the production of FXa and subsequently FIIa. It can also potently inhibit thrombin-mediated platelet activation and aggregation since thrombin production is significantly decreased upon inhibiting the extrinsic coagulation cascade at the TF level. Moreover, there have also been reports where in certain tumor types, warfarin's efficacy in reducing metastases has not been observed in spite of achieving a PT of >180 seconds. Whether these differences can be attributed to differences in tumor types between the studies is unclear. Furthermore, evidence from the clinical literature also suggests that LMWH offer a survival benefit in cancer patients over warfarin. The first prospective, randomized trial evaluating warfarin in survival of cancer patients was published in the 1980s which showed that warfarin administration significantly affects the mortality in patients with lung cancer (118). Later randomized clinical trials on warfarin patients with other types of cancer provided contradictory results (119). The CLOT study involving 600 patients randomized to receiving either dalteparin (LMWH) 200

IU/kg/day subcutaneously for 5-7 days followed by 6 month of oral warfarin therapy or 200 IU/kg/day subcutaneously for one month followed by 150 IU/kg/day dalteparin subcutaneously in secondary prophylaxis of cancer patients with thrombosis demonstrated that 6-month treatment with dalteparin is more effective than oral anticoagulation in reducing the risk of recurrent VTE in patients with cancer, without increasing the risk of bleeding (120). Similarly, a recent meta-analysis of randomized controlled trials to evaluate the impact of anticoagulants on survival in cancer patients without venous thromboembolic disease demonstrated a significantly improved overall survival with LMWH compared with warfarin (121, 122). Interestingly enough though, in the setting of surgery, warfarin's efficacy in reducing metastases was comparable to other anticoagulants (LMWH and hirudin) (Figure 5A). It is conceivable that in the presence of a hypercoagulable system such as in the setting of surgery, it can dampen the system by inhibiting the activated coagulation factors. Nevertheless, the data still convincingly demonstrates that in surgically stressed mice treated with different anticoagulants (LMWH, hirudin and warfarin), a reduction in the metastases is observed compared with surgically stressed mice not treated with these drugs suggesting that coagulation-based pathways play a key role in postoperative cancer metastases in our surgical stress model.

Likewise, depleting platelets prior to the metastatic challenge also resulted in abrogation of tumor metastases postoperatively (Figure 5B). This further suggests that a coagulation-dependent mechanism involving platelets is also important in promoting cancer metastases in the postoperative period.

There are a number of interrelated mechanism(s) responsible for postoperative clotting. These include: (1) initiation of the extrinsic coagulation cascade by sub-endothelial

exposure of tissue factor and with binding and activation of Factor VII, leading to the activation of Factor X and generation of thrombin (Factor IIa), (2) platelet activation directly by collagen, or via thrombin with binding of P-selectin and degranulation, (3) ultimately leading to the deposition of platelet and fibrin clots secondary to thrombin generation and platelet aggregation. Each of these mechanisms have been implicated in the development of metastases and different anticoagulants target different factors in the cascade. LMWH, such as dalteparin and tinzaparin, by virtue of their ability to directly inhibit Factor Xa and Factor IIa and indirectly inhibit TF by releasing TFPI from the vascular endothelium can dampen the cascade at several steps. Warfarin which inhibits Vitamin-K dependent coagulation factors (Factor II, VII, IX and X) also acts to inhibit the generation of Factor Xa and subsequently the generation of thrombin. Similarly, hirudin is a specific thrombin inhibitor (IIa) as well. Each of these anticoagulants has the ability to attenuate the prometastatic effect of surgery to a similar degree and each converge 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 following surgical stress pointing to a mechanism which is common to both the extrinsic coagulation cascade and platelet activation and aggregation.

Accordingly we aimed to identify the 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 tumor cell emboli prevent the clearance but not the initial arrest of tumor cell emboli (58, 123). We performed a time-course study evaluating the early fate of the tumor cells following surgical stress and treatment with LMWH, tinzaparin as a means to achieve anticoagulation. 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 tumor cell emboli in the surgically stressed animals by 12h post tumor cell injection but this increase was not seen when surgically stressed animals were pretreated with anticoagulation (Figure 6 and 7). No such difference was found at the earlier time point of 10 min, while at 4h, there was a small but insignificant difference. There are two explanations for the changes seen in tumor cell emboli numbers between 4h and 12h; 1) tumor cells are being cleared (cytolysis or 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 tumor cell emboli were associated with platelets and fibrin clots in animals subjected to surgical stress and this was abrogated by treatment with LMWH (Figure 8 and 9). Interestingly, about a 2-fold increase in the percent of tumor cell emboli associated with platelets and a 3-fold increase in the percent of tumor cell emboli associated with fibrin was found in surgically stressed animals compared to non-surgery controls and this number correlates with the fold-increase in metastases that we have observed following surgical stress in this model. Whether this observation is merely an association or represents a cause-and-effect phenomenon requires further investigations 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 (50, 52, 75, 124). Platelet activation and aggregation seen following surgery could result in activation of protease activated receptor signaling pathways leading to up-regulation 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), may release growth factors such as VEGF to promote angiogenesis and promote cell survival (53). Furthermore, thrombin also converts fibrinogen to fibrin. Fibrin deposits have been found in and around various types of tumors providing scaffolding for angiogenesis and possibly protecting them against host defenses (125, 126). As a dimeric molecule with multiple integrin and non-integrin binding motifs, fibrinogen might serve as an important molecular bridge between tumor cells, platelets and endothelial cells promoting stable adhesion and help 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 following surgery (7) and that anticoagulation plays a role in NK cell clearance of tumor metastases (3, 58). It would be interesting to determine if there is a link between surgery, coagulation and NK cell function in postoperative tumor metastases. We, therefore, sought to investigate the role of NK cells in our surgical stress model of experimental metastases. Our approach was to use pharmacological means to deplete NK cells and repeat our surgical stress experiment in that setting. If factors/mechanisms other than NK cells were indeed significant for the prometastatic effect of surgery, then we would expect to see the persistence of a significant increase in the lung tumor burden in surgically stressed mice depleted of NK cells compared to non-surgery mice depleted of NK cells. We found that in comparison to the NK intact group where surgery significantly enhanced metastases compared to non-surgery controls,

this effect was no longer evident in the setting of NK depletion. The lack of further increase in metastases in NK depleted surgically stressed mice compared with NK depleted non-surgery controls suggests that NK cells play an important role in postoperative tumor metastases in our model (Figure 10) and that another equally significant mechanism in promoting metastases enhancing effects of surgery is less likely. We further demonstrated that B and T cells are not the primary mediators of the metastases enhancing effects of surgery since the prometastatic effect of surgery was maintained in mice deficient of B and T cells (Figure 11). With respect to comparing the results obtained from SCID mice (immune-deficient) to immune-competent animals, it must be noted that the SCID mice utilized in this study have a partial Balb/c background and therefore, one would not expect to observe differences between tumor cell seeding in SCIDS compared with Balb/c solely based on the differences in the background of mice.

In light of the above observations regarding NK cells, we then questioned whether surgery was doing something to NK cells. Indeed, clinical and animal data supports that surgery significantly impairs NK cell function (6-9, 127), however, mechanistic details to date have remained elusive. In fact, work from our laboratory shows that surgery significantly impairs NK cell function (Tai, L-H et al, unpublished results) and characterization and investigation of the mechanisms responsible for this postoperative impairment of NK cells is a big focus of our research currently. Future work will involve investigating the important linkages between the components of the coagulation pathways (platelets, fibrin clots) and postoperative NK cell function that might influence cancer metastases. Several studies have alluded to potential mechanisms implicating coagulation and NK cells in cancer metastases. There is evidence to suggest that fibrin (52, 128, 129)

and platelet aggregates (75) associated with micrometastatic tumor cells might present an intractable physical barrier to NK cells limiting their contact with target tumor cells (54). Recent studies have shown that tumor cells prefer to adhere to fibrinogen than to platelets, and fibrinogen enhances the interaction between tumor cells and platelets (102). They, in turn, facilitate each other in protecting tumor cells from NK cell cytotoxicity (102). In fact, receptors such as α m- β 2 on NK cells have been shown to bind immobilized fibrinogen and platelet surface components (130). 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 down-regulating NK cell function (131) making them less likely to lyse potential target cells. In fact, it has been shown that platelet-derived soluble factors, secreted on coating of tumor cells or after stimulation with classic platelet agonists impair NK cell antitumor reactivity resulting in diminished granule mobilization, cytotoxicity and interferon- γ production. The impaired NK cell reactivity was found to be mediated by down-regulation of the activating immunoreceptor NKG2D on NK cells by platelet-derived transforming growth factor- β (TGF- β). It has been shown that platelets produce at least 40 times more active TGF- β than any other cell type (132, 133). Neutralization of TGF- β in the platelet releasate not only prevented NKG2D down-regulation but also restored NK cell antitumor reactivity (134). Moreover, it has been shown that elevated TGF- β 1 secretion and down-modulation of NKG2D underlies impaired NK cytotoxicity in cancer patients. Insight into the mechanisms for reduced TGF- β induced down-regulation of NKG2D have revealed that IL-2/IL-18 combination treatment of TGF- β treated NK cells can prevent the down-modulation of NKG2D via c-Jun N-terminal pathway (JNK) (135). This is particularly interesting since IL-

2 and IL-18 are known to be potent activators of NK cell effector functions (136) and monotherapy with each of these cytokines did not recover NKG2D expression on TGF- β treated NK cell line (135). It was postulated that the combination therapy with IL-2/IL-18 required the regulation of activator protein-1 transcriptional activity through the JNK pathway resulting in increased NKG2D expression on NK cells (135). Finally, these peritumoral clots might limit the capacity of circulating NK cells to lyse target 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) (137). These potential mechanisms are not mutually exclusive, and it is conceivable that a combination of processes may ultimately determine tumor cell metastatic success. Therefore, 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.

We have established that interfering with the generation of fibrin clots or platelet clots have significantly attenuated lung tumor metastases in our surgical stress model. This strongly suggests the involvement of the coagulation cascade in promoting cancer metastases in the postoperative period. Future experiments will focus on delineating the linkage, if any, between platelets and NK cells as well as between LMWH and NK cells in postoperative cancer metastases. Conducting experiments in the setting of pharmacological inhibition of NK cells using anti-asialo antibody initially to investigate the role of NK cells in the setting of platelet depletion will serve as an important first step to then study the phenomenon in genetically manipulated mice deficient of NK cells (IL-2 receptor γ knock out). These transgenic mice are available commercially and are on a C57 black mice

background and hence, experiments could be conducted using the B16F10LacZ cell line as a means to establish pulmonary metastases. Similarly, the above studies could be repeated in the setting of anticoagulation using LMWH to further investigate the mechanistic details involving LMWH and NK cells in postoperative tumor metastases in our murine surgical stress model. These studies will be crucial in advancing our understanding of the mechanisms that might play an important role in the development of cancer metastases following surgery. At the same time, we are also in the process of establishing a spontaneous model of postoperative tumor metastases using mammary fat pad injections using a breast cancer cell line, 4T1 which spontaneously metastasizes to the lungs. It would, therefore, be very interesting to see if surgical stress in the setting of this implanted tumor in the mammary fat pad enhances tumor metastases.

The present study, therefore, demonstrates that surgical stress promotes the development of cancer metastases via a coagulation-dependent mechanism in a murine model of surgical stress. Additionally, NK cells seem to play an important role in the development of postoperative tumor metastases in this model where as B and T lymphocytes do not appear to have a similar effect over the same time period. Based on 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 involving activation of Factor X leading to formation of fibrin clots. Surgery also results in the activation of platelets with subsequent release of soluble P- selectin in the plasma. These activated platelets and fibrin clots then associate with tumor cell emboli in the circulation and through multiple mechanisms yet to be identified in this model, lead to decreased clearance or increased survival of tumor cells ultimately resulting in the formation of

metastases. Moreover, NK cells seem to play a crucial role in the promotion of postoperative metastases (Figure 12). The exact mechanisms involving these major players (platelets, fibrin(ogen), NK cells and tumor cells) and the precise interplay between them requires further investigation and are being actively pursued in our laboratory.

Observations from this study suggest that therapies designed to inhibit association of tumor cells with platelets/fibrin(ogen) in combination with therapeutic strategies that augment the clearance of tumor cells via innate immune system, could be effective in controlling the spread of metastatic disease. Agents that target specific signal transduction proteins involved in platelet activation or limit platelet aggregation could be particularly attractive candidates since the remainder of the clotting cascade would be left intact. As the complex interplay between these major players is further elucidated, it will enhance our understanding of the mechanisms important in promoting postoperative cancer metastases, raising hopes for the development of better therapies.

Surgery remains the appropriate and necessary means of treatment of most solid malignancies and therefore, understanding the mechanisms responsible for surgery-enhanced tumor metastases is crucial to the design of new adjuvant therapeutic strategies that would prevent tumor recurrence after surgery. This is especially important since the perioperative therapeutic window of opportunity offers promising means of improving patient outcome but is unfortunately underutilized. Based on the results of the current study, 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.

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6. Contribution of Collaborators

Theresa Falls did all the intravenous tail vein injections in mice.

Dr Lee-Hwa Tai provided the graph that appears in Figure 6C.

Curriculum Vitae

Education

- 2009-current **Masters of Science**, Department of Biochemistry, University of Ottawa
Clinician Investigator Program, University of Ottawa
- 2007-current **General Surgery Residency**, University of Ottawa
- 2003-2007 **Doctor of Medicine**, Faculty of Medicine, University of Ottawa
- 2002-2003 **Master's of Science**, Department of Pharmacology, Faculty of Medicine,
University of Toronto (Completed one-year of the 2-year program)
- 1995-1999 **Honors Bachelor's of Science** in Human Biology/Pharmacology with High
Distinction, Faculty of Arts and Science, University of Toronto

Academic Honors

- 2011 PSI Resident Research Prize, University of Ottawa
- 2011 Best Overall Paper/Podium presentation at the Department of Surgery Resident
Research Day - Collins Day
- 2011 Best Podium Presentation at the General Surgery Research Day, The Ottawa
Hospital
- 2011 First Prize in the Oral Presentation in the Masters category at the Research Seminar
Day, Department of Biochemistry, Microbiology and Immunology, University of
Ottawa
- 2010 2010 Best Paper in Basic Science, Canadian Association of General Surgeons
- 2010 Ontario Graduate Scholarship, \$5000.00 per term for three terms
- 2010 Third Prize in the Poster competition in the Masters category at the Biochemistry,
Microbiology and Immunology Graduate Student Research Day
- 2010 University of Ottawa National Excellence Scholarship
Faculty of Graduate and Postdoctoral Studies, Department of Biochemistry
- 2009 Second Prize in the Poster competition in the Masters category at the 9th Annual
Ottawa Hospital Research Institute (OHRI) Research Day
- 2009 University of Ottawa National Excellence Scholarship
Faculty of Graduate and Postdoctoral Studies, Department of Biochemistry

- 2009 Frederick Banting and Charles Best Canada Graduate Scholarship – Master’s Award (CGSMA)
Canadian Institutes of Health Research (CIHR)
\$17,500.00 per year for one year
- 2009 Ontario Graduate Scholarship, \$5000.00 per term for three terms
- 2009 Admission Scholarship for admission into the MSc program in Biochemistry, Faculty of Graduate and Postdoctoral Studies, University of Ottawa, Minimum financial support \$43976.00 over 6 sessions
- 2006 Nominated for the Outstanding Clinical Performance Award in the Internal Medicine Third year Core Rotation, University of Ottawa
- Nominated for the Outstanding Clinical Performance Award in Paediatrics Third year Core Rotation, University of Ottawa
- Nominated for the Outstanding Clinical Performance Award in Surgery Third year Core Rotation, University of Ottawa
- Nominated for the Outstanding Clinical Performance Award in Family Medicine Third year Core Rotation, University of Ottawa
- 2006 Ontario Medical Foundation Student Bursary, Ontario Medical Association and the Faculty of Medicine, University of Ottawa, \$2000.00
- 2006 Summer Research Scholarship Honorarium, Thoracic Surgery Laboratory, Toronto General Hospital, University of Toronto, \$1000.00
- 2005 CIHR Summer Research Scholarship in Medicine, \$5048.00
- 2005 Ontario Thoracic Society Summer Research Scholarship, Ontario Lung Association, \$3000.00
- 2004 Rx&D/CIHR Summer Research Scholarship in Medicine, \$5048.00
- 2004 Summer Undergraduate Research Studentship, Life Sciences Award Institute of Medical Science, University of Toronto, \$2400.00 (declined)
- 2003 Postgraduate Scholarship A (PGS A), Natural Sciences and Engineering Research Council of Canada (NSERC), \$34,600.00 over 24 months
- 2003 Ontario Graduate Scholarship, Government of Ontario, \$15,000.00 over three terms
- 2003 Research Training Competition (RestraComp) Scholarship, The Hospital for Sick Children Research Institute, University of Toronto, \$19,000.00 per annum for two years

Contributions to Research and Development

Manuscript Publications

1. Tang P, Mura M, Seth R, Liu M. Acute Lung injury and cell death: How many ways cells can die? *AJP-Lung Cell Mol Physio*. Invited review. 2008. 294(4):L632-41.
2. Mura M, Andrade CF, Han B, Seth R, Zhang Y, Bai X-H, Waddell TK, Hwang D, Keshavjee S, Liu M. Intestinal ischemia-reperfusion-induced acute lung injury and oncotic cell death in multiple organs. *Shock*. 2007. 28(2):227-238.
3. Mura M, Han B, Andrade CF, Seth R, Hwang D, Waddell TK, Keshavjee S, Liu M. The early responses of VEGF and its receptors during acute lung injury: implication of VEGF in alveolar epithelial cell survival. *Critical Care*. 2006. 10(5):R130.
4. Seth R, Yang S, Choi S, Sabean M, Roberts EA. *In-vitro* assessment of copper-induced toxicity in the human hepatoma line, Hep G2. *Toxicology In Vitro*. 2004. 18:501-509.

Manuscript submitted

Surgical stress promotes the development of cancer metastases by a coagulation-dependent mechanism in a murine model. Seth R, Tai L, Kus A, Falls T, Bell J, Carrier M, Atkins H, Boushey R, Auer R. (Submitted to J of Clin Invest - under review)

Textbook Chapter Invitation

Co-author with Dr E Tsai, Staff neurosurgeon and Dr M Shamji, senior resident, Division of Neurosurgery, Ottawa Civic Hospital for the Neurosurgery textbook entitled Neurosurgery: Tricks of the Trade for the chapter on Cervical Laminoforaminotomy. This textbook is based out of Stanford University and will be a comprehensive overview of the major procedures in neurosurgery.

Conference Presentations

1. **Surgical stress promotes the development of cancer metastases by a coagulation-dependent mechanism.** Seth R, Kus A, Falls T, Bell J, Carrier M, Atkins H, Boushey R, Auer R.
 - Collins Day, Department of Surgery Resident Research Day, Ottawa, May 13, 2011.
 - General Surgery Research Day, Ottawa, April 27, 2011.
 - Department of Biochemistry, Immunology and Microbiology Research Seminar Day, University of Ottawa, February 23, 2011.
 - Canadian Surgery Forum, Quebec City, Quebec, September 2-5, 2010.
 - Canadian Association of General Surgery Resident Retreat, Quebec City, Quebec, September 1, 2010.
 - Collins Day, Department of Surgery Research Day, Heart Institute, Ottawa, May 14, 2010.
 - Canadian Society of Surgical Oncology Annual Scientific Meeting, Fairmont Queen Elizabeth II Hotel, Montreal, Quebec, April 30, 2010.
 - General Surgery Research Day, Ottawa, April 21, 2010.

2. Effect of surgical stress and hypercoagulability in experimental colorectal cancer metastases. Seth R, Mackenzie L, Auer R.

- Canadian Association of General Surgery Resident Retreat, Victoria, BC Sept 8-9, 2009.

3. *In-vitro* assessment of copper toxicity in the human hepatoma cell line Hep G2. Seth R, Roberts E.

- 17th Annual Sheila Sherlock Liver Research Day, Eaton Center Marriott Hotel, Toronto, April 4, 2002.

Poster Presentations

1. Surgical stress promotes the development of cancer metastases by coagulation-dependent inhibition of Natural-Killer cell mediated tumor cell clearance. Seth R, Tai L, Kus A, Falls T, Bell J, Carrier M, Atkins H, Boushey R, Auer R.

- American Association for Cancer Research 102nd Annual Meeting, April 2-6, 2011, Orange County Convention Center, Orlando, Florida.
- 10th Annual Ottawa Hospital Research Institute (OHRI) Research Day, November 18, 2010, Hampton Inn, Ottawa.

2. The effect of surgical stress and hypercoagulability on experimental colorectal cancer metastases. Seth R, Kus A, Falls T, Bell J, Carrier M, Atkins H, Boushey R, Auer R.

- Canadian Society of Clinical Investigation Annual Scientific Meeting, Ottawa, September 20-22, 2010.
- Department of Biochemistry, Microbiology and Immunology Graduate Student Research Day, May 20, 2010.
- Connect for the Cure, Eastern Ontario Cancer Innovation Forum, Sheraton Ottawa, March 11, 2010.
- Ontario Institute of Cancer Research Annual Scientific Meeting, Nottawasaga Inn, Alliston, Ontario, February 28-March 2, 2010.
- 9th Annual The Ottawa Hospital Research Institute (OHRI) Research Day, Hellenic Banquet Centre, Ottawa, November 27, 2009.

3. A novel oncolytic virus that stimulates anti-tumor immunity. Rintoul J, Falls T, Seth R, MacKenzie L, Stanford M, Bell J.

- 9th Annual The Ottawa Hospital Research Institute (OHRI) Research Day, Hellenic Banquet Centre, Ottawa, November 27, 2009.
- Cold Spring Harbour Laboratories, Harnessing immunity to prevent and treat disease. Cold Spring Harbour, New York, November 11-14, 2009.

4. Developing a neo-adjuvant murine surgical cancer model. Kus A, MacKenzie L, Lemay C, Seth R, Parato K, Atkins H, Bell J, Auer R.

- 9th Annual The Ottawa Hospital Research Institute (OHRI) Research Day, Hellenic Banquet Centre, Ottawa, November 27, 2009.

5. Intestinal ischemia-reperfusion-induced acute lung injury and non-apoptotic cell death in multiple organs. Mura M, Andrade CF, Seth R, Bai XH, Han B, Hwang D, Waddell TK, Keshavjee S, Liu M.

- 5th Annual Research Day in Respiratory and Respiratory Physiology, Campbell Conference Facility, Munk Center, University of Toronto, Toronto, June 15, 2005.

6. Systemic injury and cell death in the early phase of non-pulmonary Acute Respiratory Distress Syndrome. Mura M, Andrade CF, Seth R, Han B, Lodyga M, Hwang D, Dos Santos CC, Waddell TK, Keshavjee S and Liu M.

- American Thoracic Society International Conference, San Diego, California, May 20-25, 2005.

7. Intestinal ischemia-reperfusion-induced acute lung injury and non-apoptotic cell death in multiple organs. Mura M, Andrade CF, Seth R, Bai X, Han B, Hwang D, Waddell TK, Keshavjee S and Liu M.

- 31st Gallie Day (Annual Research Day for the Department of Surgery, University of Toronto), The Liberty Grand Entertainment Complex Exhibition Place, Toronto, May 6, 2005.

8. Investigation of multiple organ failure in a murine model of Intestinal ischemia-reperfusion-induced acute lung injury. Seth R, Mura M, Liu M.

- Institute of Medical Science Summer Student Research Day, University of Toronto, August 26, 2004.
- Medical Student Research Day, University of Ottawa, September 22, 2004.

Abstract Publications

1. Surgical stress promotes the development of cancer metastases by coagulation-dependent inhibition of Natural-Killer cell mediated tumor cell clearance. Seth R, Tai L, Kus A, Falls T, Bell J, Carrier M, Atkins H, Boushey R, Auer R. Proceedings of the American Association for Cancer Research 2011.

2. Surgical stress promotes the development of cancer metastases by a coagulation-dependent mechanism. Seth R, Kus A, Falls T, Bell J, Carrier M, Atkins H, Boushey R, Auer R.

- Clin Invest Med, Aug 2010, 33; 4S, p17.
- Can J Surg, Aug 2010, 53(4): S51–S104.

3. Role of oxidative stress in hepatocellular damage in the Jackson toxic milk mouse (tx-j) model of Wilson disease. Roberts EA, Seth R, Yang S. Can J Gastroenterol. 2005; (CASL winter meeting 2005)

4. *In-vitro* assessment of cytotoxicity by metals (Copper, Zinc and Iron) in the human hepatoma cell line, Hep G2. Seth R, Yang S, Roberts EA. Hepatology. 2002; 36: 453A.

Workshops attended

| | |
|------|---|
| 2009 | 4 th Annual Clinical Research Course The Ottawa Hospital Research Institute, Oct 26-29, 2009 |
| 2009 | 3 rd Annual Principles and Practice of Clinical Research Course Delta Hotel Mississauga, ON, Sept 16-18, 2009 |
| 2009 | SAGES Basic Residents' Workshop: Gastrointestinal Endoscopy and Laparoscopy An introduction for Surgeons Endo-surgery Institute, Cincinnati, Ohio, August 13-14, 2009 |

Academic Memberships

- 2010-current **American Association for Cancer Research**, Associate member
- 2006-Current **American College of Surgeons**
- 2003-Current **Canadian Medical Association**
- 2003-Current **Ontario Medical Association**
- 1997-Current **Golden Key International Honor Society**, University of Toronto Chapter
- Membership is by invitation only.
 - Based on academic merit and limited to the top 15 % of bachelor's degree students.