Thromboelastography Delineates Hypercoagulability in an Immunocompetent Murine Model of Metastatic Pancreatic Cancer

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Abstract

Introduction: Pancreatic cancer has the highest risk for venous thrombosis of all gastrointestinal malignancies. Although there are recent consensus guidelines for anticoagulation in cancer patients, the implementation of prophylaxis anticoagulation is still sub optimal. Current diagnostic tests are unreliable in predicting cancer related hypercoagulability, leading to interest in examining the kinetics of clot formation by thromboelastography. We hypothesize that thromboelastography will characterize hypercoagulability in a metastatic murine model of pancreatic adenocarcinoma.

Methods: C57/BL6 mice, age 7-9 weeks, underwent splenic inoculation with 2.5×10⁵ Pan02 murine pancreatic adenocarcinoma cells. At necropsy, (7 weeks) blood was collected with citrate (1:10 ratio) and TEG was obtained on Thrombelastograph® Analyzer, TEG was compared between mice with cancer and control mice. Data were analyzed using non-parametric methods because our n=5.

Results: Mice with cancer were found to have significantly higher Maximum Amplitude (MA) and G than control mice. Median MA was 60.6 (IQR: 59.4-62) mm in control mice compared to 74.2 (IQR 71.2-76) mm in mice with cancer. The median G in control mice was 7.7 (IQR 7.3-8.2) dyne/sec² where as the mean G in mice with cancer was 14.4 (IQR 12.3-15.8) dyne/sec². The MA reflects clot strength and G is the clot firmness. Thus indicating a hypercoaguable state in mice with cancer.

Conclusions: Thromboelastography identifies hypercoagulability in an immunocompetent murine metastatic pancreatic cancer model. Further, as thromboelastography can identify abnormalities in blood coagulation, specific patient guided anti-coagulation treatment may be possible.

Keywords: Pancreatic cancer; Anticoagulation; Venous thrombotic events; Thromboelastography; Murine model

Introduction

Pancreatic cancer is the 4th leading cause of cancer death. Of all gastrointestinal malignancies, pancreatic cancer has the highest risk for venous thrombosis, with reported incidences ranging from 17% to 57% [1]. Venous thrombotic Events (VTE) are associated with poor outcomes in all cancer patients. VTE and malignancies have been linked since Trousseau described the association in pancreas cancer patients in the 1870s [2]. Despite over a century of research, cancer associated VTE continues to be a significant health issue, with over 400,000 cases diagnosed annually in the United States alone, resulting in a cost of $8 billion. Surgery also further increases the risk for VTE. VTEs are not only the leading cause of death at 30 days after cancer surgery, but they also predict decreased survival during the first year for all types of cancer [3-6], thus VTE prevention is of great interest.

Although there are recent consensus guidelines for anticoagulation in cancer patients, the implementation of prophylaxis anticoagulation is still sub optimal, despite studies that have recommended routine postdischarge VTE prophylaxis in high-risk patients [7,8]. Instead, it appears that cancer related venous thromboembolism remains under diagnosed and under treated, with one study reporting that less than 1% of patients were on thrombosis prophylaxis [9]. Routine diagnostic tests (INR, PTT, PT, and platelet count) are often unreliable in predicting cancer related hypercoagulability [10].

Thromboelastography (TEG) uses real time analysis to examine the kinetics of clot formation from the time fibrin strands are formed until fibrinolysis [11,12]. It has been found to identify hypercoagulability, which was not identified by PT or aPTT in trauma patients [13]. TEG works by placing a blood sample in an oscillating heated cup and then lowering a suspended pin into the blood sample. As the blood forms fibrin strands of a clot, there is a link between the cup and the pin. This connection is recorded in the thromboelastograph, and these readings are translated into TEG parameters. Examination of these parameters allows determination of abnormal clot formation; identifying both platelets and plasma mediated clotting factors [14].

Hypercoaguability per TEG parameters is indicated by decreased R time, and increased angle, G and MA. Studies have shown that TEG is able to identify hypercoagulability in patients with pulmonary, breast, gynecological and colon cancer; however the studies have not specifically examined pancreatic cancer nor metastatic disease [10,11,14,15]. Metastatic disease is of particular interest as patients are treated with chemotherapy and often have indwelling central lines, both of which further increase the risk of VTE.

Thus we hypothesize that TEG will identify hypercoagulability in mice with metastatic pancreatic cancer. As VTE prophylaxis has
significant potential side effects, the purpose of this work is to develop a preclinical model that would allow for accurate determination of risk benefit ratios of VTE prophylaxis.

**Methods and Materials**

**Chemicals and reagents**

RPMI 1640 medium, fetal bovine serum and penicillin-streptomycin for cell culture were purchased from Invitrogen (Grand Island, NY). HEPEs buffered saline solution, trypsin-EDTA, and trypsin neutralizing solution were purchased from Lonza (Walkersville, MD).

**Murine pancreatic adenocarcinoma culture**

The murine pancreatic adenocarcinoma line, PAN02, was purchased from the Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute (Frederick, MD). Cells were cultured and maintained in RPMI 1640, with 10% FBS, and 1% penicillin/streptomycin. Cells were reconstituted from culture at a concentration of 5x10^6 cells per ml, to provide a tumor inoculation of 2.5x10^3 cells per 50 μL.

**Immunocompetent murine metastatic model of pancreatic adenocarcinoma**

Experiments were performed in C57/BL6 mice (Jackson Laboratories, Bar Harbor, ME). All studies were performed under the guidelines of an approved protocol of the University of Colorado at Denver Institutional Animal Care and Use Committee (IACUC). After acclimation, mice underwent general anesthesia and were inoculated with a sub-capussular splenic injection of 2.5x10^6 PAN02 cells as previously described [16,17], control mice underwent the same surgery with inoculation of saline instead of tumor cells. Mice were followed clinically, weighed three times weekly, and sacrificed seven weeks after injection of tumor cells deterioration per IACUC regulations. At the time of necropsy, blood was collected with citrate (10:1 ratio) and run in ‘Thrombelastograph’® Hemostasis Analyzer by Haemonetics®. N=5 in all groups.

**Thrombelastography**

Whole mouse blood coagulation was determined by citrated native Thrombelastograph® Hemostasis Analyzer by Haemonetics® according to manufacturer’s recommendation and as previously described; Citrated native TEG was used due to prior studies showing this to be the preferred study in rodents, as they are hypercoagulable when compared to humans [18]. Thrombelastography® (TEG) was performed with blood collected from cardiac puncture prior to necropsy, 0.5 mL blood was collected with 50 mL 3.2% sodium citrate and inverted five times to avoid platelet clumping. 340 μL of this citrated whole blood was added to 20 mL of 0.2 M calcium chloride in a disposable plastic TEG cup, with the assay performed on a TEG 5000 Thrombelastograph® Hemostasis Analyzer (Haemonetics, Niles, IL) at 37°C within 15 minutes of blood collection.

All TEG parameters were recorded from standard tracings: reaction time (R, min), coagulation time (K, min), angle (α, degrees), maximum amplitude or clot strength (MA, mm), clot stability (G, dynes/cm²). Standard TEG measurements and interpretations are listed in Table 1. The R-value, which is the time, elapsed from start of the test until the developing clot provides enough resistance to produce 2 mm amplitude reading on the TEG tracing. This represents the initiation phase of enzymatic clotting factors. K measures the time from clotting factor initiation (R) until clot formation reaches amplitude of 20 mm. The angle (α) is formed by the slope of a tangent line traced from the R to the K time measured in degrees. K time and angle (α) denote the rate at which the clot strengthens and is most representative of thrombin cleavage of the fibrinogen into fibrin. The maximum amplitude, or MA, indicates the point at which clot strength reaches its maximum amplitude in mm on the TEG tracing, and reflects the end result of platelet-fibrin interaction via the GPIIb-IIIa receptors. The MA represents the ultimate clot strength and is a a result of the activity of both fibrin and platelet; however, it is thought to be more significantly due to contribution of platelets. G is a calculated measure of total clot strength derived from amplitude (A, mm). G value represents clot stability and has been shown to be the predictive in identifying hypercoagulability various studies [19].

**Results**

The raw data of MA and G are listed (Table 2). Clot strength, as measured by MA and G, was found to be stronger in mice with cancer when compared to control mice. Mice with cancer were found to have significantly higher Maximum Amplitude (MA) and G than control mice with wilcoxon p value=0.0216 for both MA and G. Data were analyzed using non-parametric methods because our n=5. Median MA was 60.6 (IQR: 59.4-62) mm in control mice compared to 74.2 (IQR 71.2-76) mm in mice with cancer. The median G in control mice was 66.8 (IQR 62-70) mm in mice with cancer and 74.2 (IQR 71.2-76) mm in mice with cancer. There was no difference in R time, or time to form clot between mice with cancer and control mice, nor was there a difference in angle. Thus the main difference in cancer mice and control mice is

**Table 1:** Thrombelastography Measurements and Interpretation.

<table>
<thead>
<tr>
<th></th>
<th>Decreased</th>
<th>Increased</th>
</tr>
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<tbody>
<tr>
<td>R (min)</td>
<td>Clotting time:</td>
<td>Time to initial fibrin for tracing amplitude to reach 2 mm, enzymatic reaction</td>
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<tr>
<td></td>
<td>Hypercoagulable</td>
<td>Hypocoagulable</td>
</tr>
<tr>
<td></td>
<td>Clotting time:</td>
<td>Factor deficiency, anticoagulant,</td>
</tr>
<tr>
<td>K</td>
<td>Clot Kinetics:</td>
<td>Speed to reach clot strength 20mm amplitude, intrinsic clotting factors, fibrinogen, platelet function</td>
</tr>
<tr>
<td></td>
<td>Hypercoagulable</td>
<td>Hypocoagulable</td>
</tr>
<tr>
<td>Angle</td>
<td>Clot strengthening, Rapidity of fibrin-buildup and clot formation, angle of tracing from r to K value.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hypercoagulable</td>
<td>Hypofibrinogenemia or thrombocytopenia</td>
</tr>
<tr>
<td>MA or G</td>
<td>Overall clot strength, represents maximum dynamics of fibrin and platelet bonding</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hypercoagulable</td>
<td>Platelet abnormalities</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>MA (mm)</th>
<th>G (dynes/cm²)</th>
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<tbody>
<tr>
<td>Control mice</td>
<td>60.6</td>
<td>7.7</td>
</tr>
<tr>
<td></td>
<td>66.8</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>62</td>
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</tr>
<tr>
<td></td>
<td>57.1</td>
<td>6.7</td>
</tr>
<tr>
<td></td>
<td>59.4</td>
<td>7.3</td>
</tr>
<tr>
<td>Cancer Mice</td>
<td>74.2</td>
<td>14.4</td>
</tr>
<tr>
<td></td>
<td>79</td>
<td>18.9</td>
</tr>
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<td></td>
<td>76</td>
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<tr>
<td></td>
<td>64.3</td>
<td>9</td>
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**Table 2:** Raw data.
in clot strength, which may be a reflection of platelet activity (Figures 1 and 2).

Discussion

VTE is a morbid and costly complication after cancer surgery. There is considerable interest in the VTE research, such that in 2008 the Surgeon General issued a "Call to Action" for research to aid in the prevention and treatment of VTE [20]. Cancer has been linked to increased risk of VTE since Trousseau first noted the relationship over a century ago; however, metastatic disease has been recently implicated as a strong predictor of VTE [5]. Research has been ongoing in the search of markers that can predict VTE in cancer patients; however, no one test has consistently identified cancer related hypercoagulability [21]. We thus intend to see if TEG could potentially identify cancer related hypercoagulability.

The role of anticoagulation prophylaxis in cancer patient however is not clear. Although it appears that it should be more aggressively approached [7,8], anticoagulation prophylaxis does have potential serious side effects. TEG appears to be a cogent means to identify and accurately, prophylactically treat pancreas cancer patients based on the TEG component abnormalities. This work, represents, development of a preclinical model, by which patient algorithms can be established.

It is our belief that TEG will prove to be superior in identifying hypercoagulability in pancreas cancer patients, which may be difficult to predict by analysis of traditional anticoagulation tests. Traditional coagulation test (PT/INR and aPTT) are limited [10]. PT and INR tests measure the activity of coagulation factors of the extrinsic clotting system, while aPTT measures the intrinsic clotting factors. TEG, on the other hand, uses whole blood to look at the interactions of the components of blood clot formation, thus giving an overview of the clot quality. In multiple studies, TEG has identified hypercoagulability in various types of surgical patients [14]. R time, or the time until the first detectable clot, can be thought of as the intrinsic and extrinsic coagulation cascades converging on the common pathway of fibrin formation. Meanwhile, K time represents clot kinetics or coagulation time, as it is the speed of the clot formation, which is dependent of fibrinogen. The MA measures maximal clot strength, which is determined by the platelets, while the G value is a measure of clot stability, which is also a platelet function. Kashuk et al., in an analysis of surgical patients in the surgical intensive care unit, calculated that for every 1 dyne/cm² increase in G, the odds of a VTE increased by 25% [19].

With this knowledge, one may look at TEG variables and customize anti-coagulation prophylaxis accordingly. In our study, the cancer and control groups had similar R times, which indicate that they form blood clot at the same speed. R times reflect initial fibrin formation, which is the product of the coagulation cascade. As there is no difference between the R times of mice with cancer and control, it may be argued that the proper anticoagulation should not be heparin, low molecular weight heparin, or warfarin, as these all work to block the coagulation cascade.

Instead, we detected that the differences in coagulability between mice with cancer and control mice were in the MA and G values. These values measure overall clot strength, which are reflections of platelet and fibrin interaction, although platelet activity is thought to be the main determinant in the clot strength. Thus aspirin may be a more appropriate form of anticoagulation. In fact, aspirin has been shown to reduce recurrence of venous thromboembolism in patients diagnosed with DVT who had discontinued anticoagulant treatment, with no apparent increase in the risk of major bleeding [22]. According to our results, pancreatic cancer induces hypercoagulability through platelets as indicated by prolonged MA and G values, by extension, aspirin may be the best DVT prophylaxis for patients with pancreatic cancer.

There are several limitations of this study. Our limited N severely limits our study, which is intended as a pilot study. Furthermore, we recognize the difficulty in translating rodent studies to human, however there have been studies that have found that scope and limitations of TEG in animal studies parallel those in humans [23]. Despite these issues, we purport that the use of TEG in detecting cancer hypercoagulability has potential clinical relevance in determining which type of anticoagulation may be best suited.

The pathogenesis of cancer-associated VTE is multifactorial and complex. Many factors are thought to contribute to the probabilities of VTE in cancer patients, including the type and the stage of cancer, with metastatic disease increasing the likelihood of VTE [24]. In addition to directing therapy, TEG may aid in the identification of the pathophysiology behind cancer-associated VTE.
Acknowledgments

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References