Metastasized Non-Small Cell Lung Carcinoma Suppresses Exercise-Induced Thrombin Generation

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**Background:** Physical exercise has been proved to regulate coagulation in haemostatic system. Blood coagulation disorders often associate with cancer progression, but the significance of haemostatic equilibrium remains unclear. This study investigated whether exercise intensities influence tumor cells-induced endogenous thrombin potential (ETP) and membrane-bound tissue factor (TF) expression.

**Methods and Results:** Seven male subjects underwent strenuous and moderate (i.e., exhausted and 60% oxygen consumption) exercise on a bicycle ergometer in two different occasions. Before and immediately after exercise, blood samples were taken for measuring thrombin generation (TG) and tumor cells TF expression by Calibrated Automated Thrombinography and flow cytometry. Our results were summarized as below: (1) total and peak TG levels in plasma increased following strenuous exercise and remained unchanged following moderate exercise; (2) metastasized non-small cell lung carcinoma (H460) induced lower membrane bound-TF expression and generated smaller ETP compared with nasopharyngeal carcinoma (NPC076); and (3) although H460 suppressed severe exercise-promoted TG in plasma, NPC076 did not influence the enhanced TG by severe exercise.

**Conclusions:** Metastasized H460 lung carcinoma suppresses enhanced intrinsic coagulation by vigorous exercise. Our observation provides a physiological basis for the physical exercise-modulated mechanisms between activation of blood coagulation and progression of cancer.

**Key Words:** exercise intensity, coagulation, thrombin, and tissue factor
Introduction

Thrombosis and Malignancy

Since the first observation by Armand Trousseau in 1865, venous thromboembolism (VTE) has been considered as the common complication of malignancies. The association of thrombosis and cancer has a prevalence rate of 10-20% [1,2]. On one hand, thrombotic phenomena relate to malignancy indicate a special condition of the blood predisposed to spontaneous coagulation without inflammatory reactions existence; on the other hand, the possibility between clotting mechanism and development of metastases provide a clinical evidence to supply greater thrombin and platelet-tumor interactions [3]. In the paradigm of malignant clotting system, hypercoagulability facilitates the aggressive biology of cancer and initiates a “vicious cycle” of tumor burden.

There are two pathways leading to thrombin generation [12]. The intrinsic cascade is initiated when contact is made between blood and exposed endothelial surfaces. The extrinsic pathway is initiated upon vascular injury, which leads to exposure of tissue factor. Although they are initiated by different mechanisms, the two unite on a common pathway that leads to blood clot formation. Tissue factor (TF) also plays a central role in the cancer-related coagulome [4-6]. Distinct tumor cells have variable procoagulant possibilities. Nasopharyngeal carcinoma (NPC) is prevalent in southeastern China and Taiwan. This cancer compromises haemostatic issues in the clinical stage and is notorious for its highly metastatic nature [7]. In contrast with NPC, non-small-cell-lung-carcinoma (NSCLC) with higher procoagulant activity is still controversial [8,9]. According to these observations, haemostatic potentials might rely on different histologies and microenvironments of respiratory tract cancer cells.
Platelets play the part of hematology, and the role of platelets in tumor progression and metastasis has been recognized previously, but the mechanism of their action remains unclear. The association between cancer and thromboembolism have a dual meaning: Tumors not only activate the coagulation system, but, activate blood coagulation proteins vice versa. Molecular effects of platelet activation and fibrin formation might also promote its incorporation into microthrombi that reside in the vasculature. When 1968 Gasic et al showed that experimental pulmonary metastasis is reduced by platelet depletion and restored by supplementation of platelets, there has been much research supporting the concept of enhancement of cancer metastasis by platelets \cite{10,11}. Details of the correlations between platelets activity and tumor cells coagulability require for further investigations.

El-Sayed MS, et al; 2004
**Exercise and Coagulation**

Physical exercise has been proved to regulate coagulation in haemostatic system. Several questions related to exercise intensities and platelets activities, particularly platelet aggregation/ functions and blood clotting systems, remain unanswered and need to investigate beyond. Based on research concluded to date, blood is hypercoagulable following strenuous exercise mainly due to an increase in coagulation factor VIII (FVIII) with no parallel alterations of other clotting factors \[^{12}\]. Earlier evidence suggests that the increase in FVIII may either be due to activation within the circulation, or to the release of stored or freshly synthesized FVIII. Weiss et al found that even the thrombin generation and fibrin formation significantly increased followed by maximal treadmill running, there was no exercise-induced increment in blood mononuclear cells tissue factor expression. This may indicates that exercise-induced hypercoagulability occurs via intrinsic pathway. Thrombin-antithrombin complex (TAT) and prothrombin fragments 1+2 (F1+2) have been utilized as markers of blood coagulation activation in exercise as well. Significantly increased TAT has been observed following long-distance running and post-maximal incremental cycling. However, not only the coagulation but fibrinolysis activities are increased following strenuous exercise simultaneously \[^{13-16}\].

Even blood haemostasis of varied exercise protocols had been studied before, discrepancies of hematological parameters still presence. In addition to normal physiological condition, the effects of exercise on these markers of blood coagulation and fibrinolysis in cancer patients remain speculative. The benefits of physical activities on blood haemostasis should be further examined and available studies should be replicated.
**Materials and Methods**

**Cancer Cell Lines and Cell Characteristics**

Human nasopharyngeal carcinoma-derived cell line NPC-076 was kindly provided by Dr. J-K Chen (Chang-Gung University, Taiwan)\cite{17,18}. Two lung cancer cell lines of human NSCLC were purchased from Bioresource Collection and Research Center, which are adenocarcinoma (A549) and large cell carcinoma (NCI-H460) respectively. Cells were maintained in Dulbecco’s modification of Eagle’s minimum essential medium/Nutrient Mix F-12 (at 1:1, v/v) or in RPMI-1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 5-10% FBS and antibiotics (100U/ml penicillin and 100µg/ml streptomycin). The characteristics of the cancer cells are shown in Table 1.

**Participants**

Seven healthy, sedentary young men (age 21.0±0.7 yr) participated in this study. None of the subjects was a smoker, has cardiopulmonary/ infectious risks, or has taken any medication/ vitamins 2 weeks preceding the graded exercise test. They also had not taken part in regular physical activity for at least 1 year prior to the study. The aim, details, and procedures were explained to the subjects previous to informed consent acquirement. This study was reviewed and approved by the Ethics Committee of Chang Gung Memorial Hospital. Anthropometric and maximal exercise performance data were summarized in Table 2.

**Experimental Procedures**

All subjects were asked to fast at least 8 hrs and abstain from physical activity for 24 hrs before the test. A stepwise-incremental bicycle ergometer (Corival 400, Lode B.V.Co., Zernikepark, Netherlands) protocol was used to determined individual maximal oxygen consumption ($\dot{VO}_2$)\cite{19}. After 2 min unloaded pedaling of warm-up,
the workload was increased progressively by 20 to 30 watts every 3 min until exhaustion (i.e. strenuous exercise, SE). The average \( \dot{V}O_2 \), 59.1±6.5 ml/kg/min, was determined using PowerLab/8M analyzer (ADInstruments Pty Ltd, Castle Hill, NSW, Australia). Subjects took the second exercise for 40 min at intensity of 60% \( \dot{V}O_2 \) (i.e. moderate exercise, ME) at the same time of the day two weeks later to ensure complete recovery between trials.

Blood samples were collected from the antecubital vein before (at rest) and immediately after exercise in 3.12% sodium citrate buffer (at 9:1, v/v). Platelet-rich plasma (PRP, which is adjusted to 2x10⁸/ml) and platelet-poor-plasma (PPP) were prepared respectively for thrombin generation (TG), tumor cells tissue factor (TF) expression and hemostatic variables analyses. Blood cell counts were measured with machinery cell counter (KX-21, Sysmex Co., Kobe, Japan).

**Preparation of FVIII-depleted plasma**

Resting and post-exercise PPP were incubated with purified IgG2a anti-FVIII:C antibodies from American Diagnostica Inc. (ADI-ESH4, Greenwich, CT, USA) with 4µg/ml for 30min at 37°C.

**Automated Measurements of Thrombin Generation**

Measurement of TG was performed using Calibrated Automated Thrombinography (CAT) system described by Hemker et al. [20,21] in a Fluoroscan Ascent® fluorometer (Thermolabsystems OY, Helsinki, Finland) equipped with a dispenser. For each experiment, a fresh mixture of Fluo-Buffer (contains HEPES, pH 7.35, and calcium chloride) and fluorogenic Fluo-Substrate-DMSO solution (at 40:1, v/v) was referred to as FluCa.

Platelet-rich plasma-reagent and thrombin calibrator were reconstituted with distilled water. Three different cancer cells (1x10⁶/ml) were suspended separately with resting or post-exercise PPP/PRP as TG measurement conditioned samples. All the
reagents and samples were warmed up to 37°C before starting the experiment. Used 20µl of PRP reagent or 20µl of thrombin calibrator together with 80µl conditioned samples in the round-bottom 96-wells plates (Nunc, Roskilde, Denmark). After automated dispensing 20µl FluCa, the final reaction mixture will contain 0.5pM tissue factor. Fluorescence intensity was detected at wavelengths of 390nm (excitation filter) and 460nm (emission filter). Each well was measured over 20s intervals until thrombin activity against time curve completed (120min duration). Finally we used the Software Thrombinoscope® version 3.0.0.26 (Thrombinoscope BV, Maastricht, The Netherlands) to analyze our results. All experiments were carried out in duplicate. The three most important parameters are the lag time (LT), the peak of thrombin (Peak) and the endogenous thrombin potential (ETP) corresponding to the area under the curve.

**FACS Analysis**

To demonstrate the expression of tissue factor on the surface of target cells, three cancer cell lines incubated with resting and post-exercise PPP or PRP for 60 minutes, and then stained with FITC-conjugated anti-human TF (ADI-4508). Tumor surface thrombomodulin levels under Hank’s buffer saline solution (HBSS, Sigma-Aldrich, St. Louis, MO, USA) were incubated with anti-human TM (ADI-2375). Both cancer cell surface TF and TM were determined and analyzed by FACSscan (Becton Dickinson, San Jose, CA, USA).

**Statistics**

The results were shown as mean ± standard error and analyzed with StatView statistical software version 5.0.1 (SAS Institute Inc., Cary, NC, USA). Repeated measures ANOVA were used to examine total and peak TG levels in plasma and tumor cell surface TF and TM expression followed by two different exercise intensities. Significance was set at the p values less than 0.05.
Results

Thrombin generation (TG) in adult plasma with different exercise intensities

With high tissue factor and phospholipids levels (20pM and 4µM), total thrombin production were augmented significantly after strenuous exercise, but remained unchanged following moderate exercise (Table 4, \( P<0.05 \)). However, if plasma coagulation factor VIII were depleted in advance, neither ETP nor peak height of thrombin increased after strenuous exercise (Fig.1B and 1C, \( P<0.05 \)). The effect of FVIII depletion delayed thrombin generation slope without changing termination curve (Fig. 1A, 1D and 1E, \( P<0.05 \)).

Three cancer cell lines dealt with characteristics as diverse as procoagulants and anticoagulants

To elucidate different cancer cells haemostatic characteristics, membrane-bound TF expression were measured. We found that NPC076 had higher tissue factor level than A549 and H460, but had equivalent thrombomodulin values of the three cell lines (Table 1, \( P<0.05 \)). Under low tissue factor (0.5pM) mixture reaction, NPC076 also had shorter lag time and higher thrombin activity than other two NSCLCs.

On the other hand, A549 had similar extent of ETP to control plasma, which H460 suppressed basic TG level inversely (Fig.2, \( P<0.05 \)). Thrombin generation curve of large cell lung carcinoma (NCI-H460) itself represented a more rapid and narrow form than primary PPP or PRP curve.
Inhibition of exercise-induced TG by metastatic large cell lung carcinoma (NCI-H460)

Both NPC076 and A549 maintained strenuous exercise-induced thrombin formation except H460. Total thrombin generation derived from exercise effects, originated from intrinsic coagulation pathway, was inhibited by H460 through postponing lag time and time to peak height (Fig.3, \(P<0.05\)).

Platelet-induced tumor cells surface TF expression

Tumor cells membrane-bound TF expressions were modulated by platelet in exercise intensity-dependent mode, which was confirmed by flow cytometry. Although there was no significant differences between rest and exercise in NPC076, TF level changes among moderate and strenuous exercise regimens achieved apparently in A549 and H460 cancer cell lines (Fig.4, \(P<0.05\)). Strenuous exercise enhanced TF amount of A549 compared with moderate exercise, but remained unchanged in H460.
Summary and Significance

Our results were summarized as below: (1) total and peak TG levels in plasma increased following strenuous exercise and remained unchanged following moderate exercise; (2) metastasized non-small cell lung carcinoma (H460) induced lower membrane bound-TF expression and generated smaller ETP compared with nasopharyngeal carcinoma (NPC076); and (3) although H460 suppressed severe exercise-promoted TG in plasma, NPC076 did not influence the enhanced TG by severe exercise.

Physical exercise has been proved to regulate coagulation system in intensity-dependent manner, but metastasized H460 lung carcinoma suppresses enhanced intrinsic coagulation by vigorous exercise. Our results provide an additional mechanism to modulate exercise-mediated activation of blood coagulation in vitro. To obtain more clear-cut answer, further definite roles of coagulation factors between physical activities and cancer cells are needed to be elucidated.
References


Figure Legends

Figure 1. Effects of Factor VIII on exercise plasma-generated TG. (A) to (D) are four parameters of thrombin generation, (E) shows the graphic curve. LT, lag time (min); ETP, endogenous thrombin potential (nM*min); Peak, peak height of thrombin (nM); and TTP, time to peak (min). SR, rest of strenuous exercise; SE, immediately after strenuous exercise; SR’, rest of strenuous exercise with FVIII-depletion; SE’, immediately after strenuous exercise in FVIII-depletion. *P<0.05, R vs. E; † P<0.05, Control vs. FVIII-depletion.

Figure 2. Thrombin generation variables under PPP or PRP varied in different cancer cell lines. PPP, platelet-poor-plasma; PRP, platelet-rich-plasma; vehicle, PPP or PRP only without incubated with cancer cells. *P<0.05, NPC076 vs. A549 vs. H460 in PPP; † P<0.05, NPC076 vs. A549 vs. H460 in PRP.

Figure 3. Different cancer cell lines modified exercise-mediated TG parameters. *P<0.05, R vs. E in PRP within the same cancer cells.

Figure 4. Changes of platelet-induced tissue factor (TF) expression of NPC076 (A), A549 (B), and H460 (C) between different exercise intensities. M, moderate exercise; S, strenuous exercise; R, rest; E, immediately after exercise. *P<0.05, R vs. E; † P<0.05, M vs. S.
Table 1. Levels of cell surface tissue factor and thrombomodulin expression in cell lines derived from human respiratory tract cancers

<table>
<thead>
<tr>
<th>Characteristics of original tumors</th>
<th>MFI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histology</td>
<td>Source</td>
</tr>
<tr>
<td>Nasopharynx</td>
<td></td>
</tr>
<tr>
<td>NPC-076 Sq Primary lesion</td>
<td>169.45±19.40*</td>
</tr>
<tr>
<td>NSCLC</td>
<td></td>
</tr>
<tr>
<td>A549 Type II/Ad Primary lesion</td>
<td>30.96±5.29</td>
</tr>
<tr>
<td>H460 La Pleural effusion</td>
<td>31.12±4.89</td>
</tr>
</tbody>
</table>

1. NSCLC, non-small-cell lung carcinoma; MFI, mean fluorescence intensity; TF, tissue factor; TM, thrombomodulin; Sq, squamous cell carcinoma; Ad, adenocarcinoma; La, large cell carcinoma.
2. Surface-bound TF and TM expression were measured by flow cytometry.
3. *P < 0.05, NPC076 vs. A549 vs. H460.
### Table 2. Anthropometric and maximal exercise performance

<table>
<thead>
<tr>
<th>N=7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
</tr>
<tr>
<td>Body height (cm)</td>
</tr>
<tr>
<td>Body mass (kg)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
</tr>
<tr>
<td>$V_{E\text{peak}}$ (L/min)</td>
</tr>
<tr>
<td>$V_{O_2\text{peak}}$ (ml/min/kg)</td>
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<tr>
<td>$VCO_2\text{peak}$ (ml/min/kg)</td>
</tr>
<tr>
<td>R</td>
</tr>
<tr>
<td>$HR_{\text{rest}}$ (bpm)</td>
</tr>
<tr>
<td>$HR_{\text{max}}$ (bpm)</td>
</tr>
<tr>
<td>Workload (watt)</td>
</tr>
<tr>
<td>TE (minutes)</td>
</tr>
</tbody>
</table>

1. BMI (body mass index) equals to body mass divided by the square of height.
2. $V_{E\text{peak}}, V_{O_2\text{peak}},$ and $VCO_2\text{peak}$ indicate “peak ventilation”, “maximal O₂- and CO₂-consumption” separately.
3. R (respiratory exchange ratio) equals to $CO_2\text{produced}/O_2\text{consumed}$.
4. HR represents “heart rate”; TE depicts “time to exhaustion”.

Table 3. Comparisons of blood cell counts between different exercise intensities

<table>
<thead>
<tr>
<th></th>
<th>N=7</th>
<th>R</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RBC (x10^6/µL)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>4.37±0.24</td>
<td>4.90±0.18</td>
<td></td>
</tr>
<tr>
<td>S</td>
<td>4.46±0.19</td>
<td>5.34±0.14*</td>
<td></td>
</tr>
<tr>
<td><strong>WBC (x10^3/µL)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>4.98±0.49</td>
<td>5.74±0.55*</td>
<td></td>
</tr>
<tr>
<td>S</td>
<td>4.82±0.51</td>
<td>9.15±0.62*</td>
<td></td>
</tr>
<tr>
<td><strong>Platelet (x10^3/µL)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>181.00±11.35</td>
<td>212.75±10.00</td>
<td></td>
</tr>
<tr>
<td>S</td>
<td>184.17±5.76</td>
<td>251.83±13.88*</td>
<td></td>
</tr>
</tbody>
</table>

1. R represents “rest”; E indicates “immediately after exercise”.
2. M, moderate intensity; S, strenuous exercise.
3. *P < 0.05, R vs. E.
Table 4. TG measurement in adult pre- and post-exercise plasma with PPP reagent (contains 20pM tissue factor and 4μM phospholipids):

<table>
<thead>
<tr>
<th></th>
<th>N=7</th>
<th>MR</th>
<th>ME</th>
<th>SR</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>LT (min)</td>
<td>2.35 ±0.11</td>
<td>2.33 ±0.14</td>
<td>2.12 ±0.11</td>
<td>2.37 ±0.12*</td>
<td></td>
</tr>
<tr>
<td>ETP (nM*min⁻¹)</td>
<td>1383.65 ±4.96</td>
<td>1360.19 ±7.74</td>
<td>1332.00 ±2.01</td>
<td>1624.96 ±6.66*</td>
<td></td>
</tr>
<tr>
<td>Peak (nM)</td>
<td>255.92 ±3.87</td>
<td>256.69 ±5.99</td>
<td>248.08 ±1.74</td>
<td>313.42 ±5.04*</td>
<td></td>
</tr>
<tr>
<td>TTP (min)</td>
<td>4.88 ±0.14</td>
<td>4.95 ±0.19</td>
<td>4.65 ±0.17</td>
<td>4.65 ±0.14</td>
<td></td>
</tr>
<tr>
<td>StarT (min)</td>
<td>21.95 ±2.32</td>
<td>21.70 ±2.52</td>
<td>21.14 ±2.27</td>
<td>22.14 ±2.52</td>
<td></td>
</tr>
</tbody>
</table>

1. TG, thrombin generation; PPP, platelet-poor-plasma; LT, lag time; ETP, endogenous thrombin potential; Peak, peak height; TTP, time to peak; StarT, start tail.
2. M, moderate exercise; S, strenuous/ exhausted exercise; R, resting; E, immediately after exercise.
3. *P <0.05, R vs. E.
Figure 1.

(A) LT

(B) ETP

(C) Peak

(D) TTP

(E) Thrombin (nM)

Time (min)

Control
anti-FVIII
Figure 2.

(A) LT

(B) ETP

(C) Peak

(D) TTP

(E) StarT
Figure 3.

(A) LT

(B) ETP

(C) Peak

(D) TTP

(E) StarT

Legend:
- PRP
- NPC
- A549
- H460

* denotes significant difference.
Figure 4.

Changes of Tissue Factor Expression (Folds)

(A) NPC076

(B) A549

(C) H460