Exercise modulates platelet-nasopharyngeal carcinoma cell aggregation and subsequent tissue factor and matrix metalloproteinase activities

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Wang J-S, Chang C-Y, Chow S-E, Chen Y-W, Yang C-M. Exercise modulates platelet-nasopharyngeal carcinoma cell aggregation and subsequent tissue factor and matrix metalloproteinase activities. J Appl Physiol 103: 763–770, 2007. First published May 10, 2007; doi:10.1152/japplphysiol.00165.2007.—Interaction between platelet and carcinoma cell contributes to pathogenesis of cancer-related thrombosis and metastasis. This study investigated whether physical exercise affects platelet-nasopharyngeal carcinoma cell (NPC) interaction and platelet-promoted tissue factor (TF) and matrix metalloproteinase (MMP) activities of NPC. Thirty sedentary men performed on three occasions moderate-intensity exercise [MIE, 60% maximal oxygen consumption (VO2max) for 40 min] and high-intensity exercise (HIE, up to VO2max), with and without warm-up exercise (WUE, 60% VO2max for 20 min) on a bicycle ergometer. Before and immediately after exercise, platelet-NPC aggregation, the TF, MMP-2 and MMP-9 expressions and activities, and TF pathway inhibitor (TFPI) and tissue inhibitor of MMP-1 levels of NPC and platelet were measured. The results of this study demonstrated that HIE enhanced platelet-NPC aggregation in the presence of fibrinogen and was accompanied by increased platelet-promoted TF activity, expression of NPC, decreased platelet-promoted MMP-2 and MMP-9 activities, and TFPI release of NPC, whereas these alterations to HIE on platelet-NPC interactions were ameliorated by WUE pretreatment. Conversely, MIE reduced the formation of platelet-NPC aggregates, but did not change the TF, TFPI, MMP-2, MMP-9, tissue inhibitor of MMP activities, and/or levels of NPC mediated by platelet. It is concluded that HIE may enhance aggregation and coagulation and reduce MMP bioactivity related to platelet-NPC interactions, by raising the binding affinity to fibrinogen and TF activity and expression and lowering TFPI release and MMP-2 and -9 activities. These effects on HIE diminish after WUE. However, MIE minimizes the risk of thrombosis induced by platelet-NPC interactions.

METHODS

Subjects. The Ethics Committee of Chang Gung Memorial Hospital reviewed and approved the protocol for this study. Each subject

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provided informed consent. Thirty healthy, sedentary men were enrolled in the experiment. The physical characteristics of these subjects, expressed as means ± SE, were as follows: age 23.2 ± 2.4 yr; height 172.0 ± 0.4 cm; and body weight 75.2 ± 6.1 kg. Subjects had not engaged in regular physical activity for at least 1 yr before the study. All subjects were nonsmokers, did not use medication/vitamins, were free from infection and cardiopulmonary risks, and abstained from all medication for at least 2 wk before the study. The subjects fasted for at least 8 h before the study and were instructed to refrain from exercise for at least 24 h before blood sampling. All subjects arrived at the testing center at 9:00 AM to avoid possible diurnal influence.

Exercise and blood collection protocol. Subjects performed three exercise protocols on a bicycle ergometer (Corival 400, Lode) in the laboratory for 3 days. The first protocol comprised 2 min of unloaded pedaling; the workload then was raised incrementally by 20–30 W every 3 min until a subject reached exhaustion (HIE). This exercise test determined a subject’s maximal oxygen consumption (Vo2max), as previously described (34). The mean Vo2max was 34.1 ± 5.2 ml·min⁻¹·kg⁻¹. For the second protocol, subjects rode the bicycle at 60% (MIE) of the predetermined Vo2max for 40 min. For the third test, subjects initially rode at 60% Vo2max for 20 min, then recovered at a sitting position for 30 min, and finally engaged in HIE (WUE-HIE). Each exercise protocol was performed over 2-wk intervals to ensure complete recovery between trials.

At rest and immediately following selected exercise tests, blood samples from a forearm vein were obtained to measure hematologic parameters and platelet-NPC interactions. Blood cell counts were analyzed using a Sysmex SF-3000 cell counter.

NPC culture and platelet isolation. Dr. J.-K. Chen (Department of Physiology, Chang Gung University, Taiwan) kindly provided the human NPC cell line (NPC 076). Cells were cultured in a DMEM/F-12 medium (Sigma), supplemented with 10% vol/vol fetal bovine serum (Gibco) and 100 IU/ml penicillin-streptomycin (Sigma) in an atmosphere of 5% CO2 at 37°C. Platelet-rich plasma was prepared by centrifugation at 120 g for 10 min at room temperature. Four milliliters of platelet-rich plasma were then mixed with 8 ml of HBSS immediately following exposure to static and shear conditions. The fluorescence from 5,000 events, representing the CD42b-PE-labeled platelets bound to BCECF-labeled NPCs, were then determined by a FACScan flow cytometer (Becton Dickinson). In brief, the NPCs (G2 gate) were gated separately from the platelets (G1 gate) on the basis of forward/sideward scatter, then the PE-stained events found in the G2 gate were expressed as the percentage of definition platelet-NPC aggregates (Fig. 1).

Expressions or activities of TF, TFPI, MMP-2, MMP-9, and TIMP-1. A 0.4 μM pore, 12-mm-diameter tissue culture insert (Transwell, Costar) was coated with 1 mg/ml fibronogen (Sigma), and 200-μl platelets (5 × 10⁸ cells/ml) were then added to the insert and kept there for 20 min at 37°C to allow the platelets to settle on the fibronogen-coated surface. This insert, with or without adhered platelets, was then placed into a chamber containing 400 μl NPCs (1 × 10⁸ cells/ml) or the NPC-free DMEM/F-12 medium (Sigma) for 4 h at 37°C. Platelet suspension was incubated with a saturating concentration of monoclonal anti-human CD42 antibody conjugated with phycoerythrin (PE) (CD42b-PE) (eBioscience), and NPC suspension was stained with 2 μM BCECF-AM (Molecular Probes) in darkness for 20 min at 37°C, and then washed with HBSS. Platelets labeled with CD42b-PE (2.5 × 10⁶ cells/ml) and NPCs labeled with BCECF (1 × 10⁷ cells/ml) were mixed into HBSS with 1 mg/ml human albumin (Sigma), 1 mg/ml human fibrinogen (Sigma), or 1 mg/ml human fibrinogen (Sigma), and sheared at control levels of shear stress (0 and 5 dyn/cm²) at 37°C for 5 or 10 min using a rotational viscometer (CAP 2000, Brookfield), as described previously (38). In some experiments, 1 μM PMA was added to the platelet-NPC mixture for 5 min at 37°C under protein-free and static conditions, as positive control samples. These cell mixtures were transferred into polypropylene tubes containing 2% formaldehyde in HBSS immediately following exposure to static and shear conditions. The fluorescence from 5,000 events, representing the CD42b-PE-labeled platelets bound to BCECF-labeled NPCs, were then determined by a FACScan flow cytometer (Becton Dickinson). In brief, the NPCs (G2 gate) were gated separately from the platelets (G1 gate) on the basis of forward/sideward scatter, then the PE-stained events found in the G2 gate were expressed as the percentage of definition platelet-NPC aggregates (Fig. 1).

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Fig. 1. Flow cytometric analysis of platelet-nasopharyngeal carcinoma (NPC) aggregation in the absence (A–D) and presence (E–H) of fibrinogen under shear flow condition. Platelets (Plts) labeled with CD42b-phycoerythrin (PE) (2.5 × 10⁶ cells/ml) and NPCs labeled with BCECF (1 × 10⁷ cells/ml) were mixed into HBSS without and with 1 mg/ml human fibrinogen and then sheared at 5 dyn/cm² of shear stress at 37°C for 5 min using a rotational viscometer. The NPC population (G2 gate) was gated separately from the platelet population (G1 gate) on the basis of forward (FSC) and sideward scatter (SSC) (A and E), and then the PE-stained events found in the G2 gate (D and H) were expressed as the percentage of definition platelet-NPC aggregates.
Table 1. Comparisons of blood cell counts following MIE, HIE, and WUE-HIE

<table>
<thead>
<tr>
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<th>R</th>
<th>E</th>
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<tbody>
<tr>
<td>RBC, 10⁶/µl</td>
<td>5.27±0.51</td>
<td>5.40±0.30</td>
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<tr>
<td>MIE</td>
<td>5.25±0.34</td>
<td>5.51±0.44*</td>
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<tr>
<td>HIE</td>
<td>5.17±0.53</td>
<td>5.57±0.30*</td>
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<tr>
<td>WUE-HIE</td>
<td>6.30±0.53</td>
<td>7.51±0.73*</td>
</tr>
<tr>
<td>Leukocyte, 10⁹/µl</td>
<td>6.40±0.84</td>
<td>10.41±1.25*</td>
</tr>
<tr>
<td>MIE</td>
<td>6.54±0.97</td>
<td>9.85±1.09*</td>
</tr>
<tr>
<td>HIE</td>
<td>206±8</td>
<td>257±20*</td>
</tr>
<tr>
<td>WUE-HIE</td>
<td>217±20</td>
<td>271±26*</td>
</tr>
<tr>
<td>Platelet, 10⁹/µl</td>
<td>202±14</td>
<td>264±16*</td>
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Values are means ± SE; n = 30. R, rest; E, immediately after exercise; RBC, red blood cells; MIE, moderate-intensity exercise; HIE, high-intensity exercise; WUE-HIE, HIE with warm-up exercise. *P < 0.05, R vs. E.

37°C on an orbital shaker (shake rate set at 100 rpm). The NPC suspension was then transferred into the polypropylene tube and centrifuged at 1,600 g for 10 min at 4°C to divide into two parts: one for the cells and the other for the culture supernatants.

The cells were fixed by 2% formaldehyde (Sigma) in HBSS; then treated with mouse anti-human TF monoclonal antibody (Chemican), mouse anti-human MMP-2 monoclonal antibody (Chemican), or mouse anti-human MMP-9 monoclonal antibody (Chemican) in HBSS with 1% BSA; incubated for 16 h at 25°C in developing buffers (50 mM Tris, 40 mM HCl, 200 mM NaCl, 5 mM CaCl₂, and 0.2% Brij 35) (Sigma) on a rotary shaker. After incubation, the gels were stained in 30% methanol (Sigma), 10% acetic acid (Sigma), and 0.5% (wt/vol) Coomassie brilliant blue (Sigma) for 1 h followed by destaining. Mixed human MMP-2 (NeoMarkers) and MMP-9 (NeoMarkers) were applied as positive controls. Gelatinolytic activity was manifested as horizontal white bands on a blue background.

Changes of TF and MMP-2/-9 activities as well as TIMP-1 and TFPI contents of NPC by platelet were calculated by utilizing the following formula: (TF activity, MMP-2/-9 activity, TIMP-1 content, or TFPI content of platelet-NPC coincubation divided by TF activity, MMP-2/-9 activity, TIMP-1 content, or TFPI content of platelet alone plus NPC alone) minus 1 multiplied by 100.

**Statistics.** Data are expressed as means ± SE. StatView IV statistical software packages were employed for data analysis. Comparisons of cell counts, platelet-NPC aggregation, and TF, TFPI, MMP-2, MMP-9, and TIMP-1 expressions or activities at rest and immediately after various exercise tests were analyzed by repeated-measure ANOVA and Tukey’s multiple-range test. The level of statistical significance was set at P < 0.05.

**RESULTS**

**Platelet-NPC aggregation.** Under protein-free condition, all MIE, HIE, and WUE-HIE did not alter the extent of platelet-
NPC aggregation at static condition or 5 dyn/cm² of shear stress for 5 or 10 min (data not shown). The mixtures of platelet and NPC in the presence of fibrinogen were linked with higher percentages of platelet-NPC aggregation induced by shear stress than those in the presence of albumin or fibronectin (data not shown). Although MIE, HIE, and WUE-HIE increased blood platelet counts by similar degrees (Table 1), the extent of platelet-NPC aggregation promoted by fibrinogen at 5 dyn/cm² of shear stress was depressed by MIE (Fig. 2, B and E) and enhanced by HIE (Fig. 2, C and E). Furthermore, HIE preceded by WUE also significantly decreased the fibrinogen-mediated platelet-NPC aggregation under shear flow condition (Fig. 2, D and E). However, neither albumin nor fibronectin altered the ability of platelets to bind to NPCs, either at rest or immediately after HIE, WUE-HIE, or MIE under static and shear flow conditions (data not shown).

**TF expression and activity in NPC coinubicated with platelet.** HIE increased TF expression on platelets (Fig. 3) and TF activity derived from platelets (Fig. 4A), while these TF expressions and activity were unchanged in response to MIE and WUE-HIE (Figs. 3 and 4A). The expression of TF on NPC rose 4 h after the platelet-adhered semipermeable insert was placed into the NPC chamber (Fig. 4A). Moreover, the solution in NPC coinubicated with platelet also had a higher TF activity than that in NPC alone (Fig. 4A). HIE increased the extent of platelet-promoted TF expression (Fig. 3) and activity (Fig. 4B) of NPC, while neither MIE nor WUE-HIE affected these effects in TF expression (Fig. 3) and activity (Fig. 4).

**MMP-2 and -9 expressions and activities in NPC coinubicated with platelet.** Membrane-bound MMP-2 (Fig. 5A) and MMP-9 (Fig. 5B) expressions on platelets alone and platelet-treated NPCs were unchanged in response to all MIE, HIE, and WUE-HIE. However, HIE lowered the solution’s MMP-2 and -9 activities in NPC coinubicated with platelet (Fig. 6B) and suppressed the extent of platelet-promoted MMP-2 (Fig. 7A) and MMP-9 (Fig. 7B) activities of NPC.

Conversely, these MMP-2 and -9 activities in platelet-NPC coinubication were unchanged in response to MIE and WUE-HIE (Figs. 6 and 7).

**TFPI and TIMP-1 contents in NPC coinubicated with platelet.** Although HIE increased TIMP-1 released from platelet (Fig. 8A), there were not significant changes in the extent of platelet-promoted TIMP-1 release of NPC following this exercise (Fig. 9B). Furthermore, HIE also reduced TFPI concentration (Fig. 9A) in the platelet-NPC coinubication, as well as suppressed the enhancement of NPC-derived TFPI by platelet (Fig. 9B). However, neither TFPI nor TIMP-1 concentrations in the cell mixture significantly changed following WUE-HIE and MIE (Figs. 8 and 9).

### DISCUSSION

This investigation is the first to clearly demonstrate that exercise intensity is an important factor in platelet-NPC aggregation and platelet-mediated TF and MMP bioactivities of NPC. Namely, HIE sensitizes platelet-NPC aggregation, enhances TF-induced coagulation, and suppresses MMP-2- and -9-induced gelatinolysis, accompanied by a decrease in TFPI level and an increase in TIMP-1 level under conditions of platelet-NPC coinubication. Conversely, MIE suppresses NPC-platelet aggregation, which remains unaltered in TF/TFPI and MMPs/TIMP bioactivities in treating NPCs with platelets. However, WUE attenuates enhancements of heterotypic aggregation and TF-induced coagulation and limits reductions of MMP-2- and -9-induced gelatinolysis during HIE performed by sedentary men.

Platelet-tumor cell interactions are thought to contribute to metastatic dissemination (2, 8, 18, 27). Related studies have
revealed that a fall in blood platelet concentration and interference with platelet-tumor cell interactions suppressed the spread of cancer, whereas formation of tumor cell emboli consisting of platelets promoted retention of tumor cells in the capillaries, eventually colonizing distant organs (8, 10, 28, 29). Fibrinogen is a significant determinant of the metastatic potential of circulating tumor cells (28), since it can mediate the interaction of tumor cells with platelets by cross-linking gly-
coprotein IIb/IIIa (GPIIb/IIIa) on platelets and integrin αv/β3 on tumor cells under flow conditions (10, 13). Data from previous works by the authors demonstrated that strenuous exercise (80–100% V\textsubscript{O2max}) enhanced the capacity of adhered platelets on fibrinogen-coated surface to withstand physiological shear stress (34–36) and platelet aggregation induced by shear stress (36, 37, 39), possibly by enhancing von Willebrand factor to bind to platelets and subsequent activation of GPIIb/IIIa or expression of P-selectin on platelets. Conversely, moderate exercise (60% V\textsubscript{O2max}) suppressed platelet adhesiveness on fibrinogen-coated surface via downregulating these adhesion molecules on platelets (35, 36, 40). Measurement results in this study displayed that platelet-NPC aggregation mediated by fibrinogen at 5 dyn/cm\textsuperscript{2} of shear stress (mimicking a venous circuit) was enhanced by HIE and suppressed by MIE, suggesting that acute exercise somehow alters the performance of the platelet fibrinogen receptor (36) and subsequently influences the formation of platelet-tumor cell aggregates under physiological blood flow, with reactions depending on the exercise intensities.

Notably, HIE preceded by WUE suppressed the platelet-NPC aggregation mediated by fibrinogen under shear flow condition. This finding was similar to the results from our laboratory’s recent investigation on the impact of different levels of exercise on platelet-leukocyte interactions (38). That is, the WUE attenuated the extent of platelet-leukocyte aggregation induced by HIE via deactivation of adhesion molecules on platelets (38). A previous investigation showed that patients with ischemia heart disease developed a warm-up phenomenon during repeated exercise testing, characterized by a delay in angina pain onset and diminished electrocardiographic evidence of myocardial ischemia; this effect is as a form of preconditioning (30). Ischemic preconditioning can benefit tissue ischemia-reperfusion injury by preserving the mitochondrial redox state, which protects tissue against reperfusion injury caused by leukocyte-derived oxidants (15). Oxidant products derived from leukocytes also enhanced platelet reactivity (31). A recent study demonstrated that strenuous exercise diminished both GSH content and mitochondrial transmembrane potential, whereas moderate exercise improved antioxidative capacity and reduced lipid peroxidation in leukocytes (41). In light of these findings, light-intensity exercise to MIE may limit the enhancement of platelet activation by vigorous exercise, although improving circulatory or cellular antioxidative capacity, thus suppressing platelet interaction with other cells, such as leukocytes and cancer cells. Hence, the WUE could act as a preconditioning effect to perform HIE, thus reducing the risk of platelet-NPC thrombi evoked by severe exercise.

During metastasis, tumor cells have been observed to adhere to microvasculature and activate coagulation via surface-expressed TF, resulting in local fibrin deposition and platelet aggregation (23, 32). Experimental results in this study demonstrated that adhered platelets on fibrinogen-coated surface also raised TF level on NPC membrane and augmented the extent of TF activity in platelet-NPC coincubation, suggesting that activated platelet can also enhance tumor TF expression on NPC and promote procoagulant activity in the microcirculation of platelets and NPCs. The findings seem to explain plausibly why patients with NPC suffered from hypercoagulation and hyperplatelet reactivity in the circulating blood (17, 22).

Fig. 8. Effects of different exercise regimens on tissue inhibitors of MMP (TIMP)-1 concentrations of platelet alone and platelet-NPC coincubation (A) and changes of TIMP-1 activity of NPC by platelet (B). Values are means ± SE; n = 10. *P < 0.05, R, Plt vs. E, Plt; R, NPC+Plt vs. E, NPC+Plt.

Fig. 9. Effects of different exercise regimens on TF pathway inhibitor (TFPI) concentrations of platelet alone and platelet-NPC coincubation (A) and changes of TFPI activity of NPC by platelet (B). Values are means ± SE; n = 10. *P < 0.05, R, NPC+Plt vs. E, NPC+Plt; Rp vs. Ep.
simultaneously lowered platelet-promoted TFPI released from NPC, thus possibly reducing formation of Xa-TFPI-VIIa-TF complex, which hindered the automatic braking system of the TFPI in TF-induced coagulation, resulting in a hypercoagulable state following this exercise regimen.

MMPs control the processes of tumor invasion and metastasis, while TIMPs form 1:1 noncovalent complexes with MMPs, consequently blocking access of substrates to the MMP catalytic site (16). An inappropriate balance of MMP and TIMP production has been correlated with tumor metastasis (16). According to previous investigations, platelets facilitated invasiveness of tumor cells owing to improved MMP-9 secretion (18, 33). Results from this present study showed that membrane-bound MMP-2 and -9 expressions on platelets alone and platelet-treated NPCs were unchanged in response to the three exercise regimens. However, HIE suppressed MMP-2 and -9 gelatinolytic activities in platelet-NPC coincubation and was accompanied by elevated TIMP level released from platelets, suggesting that HIE may suppress MMP activities of NPC-platelet interaction by primarily increasing platelet-derived TIMP level. In contrast to their anti-MMP activity, TIMPs also have growth stimulatory and antiapoptotic effects to tumor cells, as observed in previous studies (19). Therefore, heavy exercise may promote survival and growth of tumor cells by raising TIMP levels in the microenvironment with tumor cells and activated platelets.

Conversely, these effects on TF/TFPI and MMPs/TIMP remained unchanged in response to MIE. Furthermore, WUE also eliminated the HIE-disturbed homeostasis between TF/TFPI and MMPs/TIMP in platelet interaction with NPC. According to previous investigations, moderate exercise (50–74% \( V_{O2\max} \)) suppressed platelet aggregation, which remained unchanged in the coagulation system, whereas strenuous exercise (>75% \( V_{O2\max} \)) enhanced simultaneously platelet aggregation and coagulation (for a review, see Ref. 36). The different changes between platelet-NPC aggregation and subsequent coagulation following acute exercise were similar to the aforementioned studies concerning platelet function and coagulation mediated by exercise (36). Therefore, it seems that acute exercise may modulate various hemostatic parameters induced by platelet-NPC interactions, with the reactions determined by the exercise intensity. However, the underlying mechanisms of exercise-induced changes on platelet-NPC interactions need to be investigated further.

In conclusion, HIE induced both prothrombotic and anti-MMP bioactivities in platelet-NPC interactions, enhancing the binding affinity to fibrinogen, raising TF expression/activity and TIMP release, and lowering TFPI release and MMP-2/9 activities following this exercise. WUE diminished this effect on HIE. Additionally, MIE minimized the risks of thrombosis induced by platelet and NPC. The first finding may provide a novel exercise regimen for preventing severe exercise-induced, cancer-related thrombosis through WUE pretreatment. The second finding indicates that moderate exercise may be a “safe” exercise dosage for minimizing the risk of cancer-related thrombosis by eliciting beneficial physiological changes. As in numerous other investigations, one restriction of the present work is that the subjects tended to be young and healthy. Hence, further clinical evidence will be necessary to extrapolate the present results to patients with NPC or other hematogenous metastasis.

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