The inhibition of coagulation activity by warfarin down-regulates lung metastasis.

*Asanuma, K; **Akita, N; **Hayashi, T; ***Yoshida, K; **Okamoto, T; *Matsumine, A; *Uchida, A
Depts. of *Orthopedic Surgery and **Molecular pathology of Mie University
TEL: +81-59-231-5022; Fax: +81-59-231-5211;
kasanum@gmail.com

INTRODUCTION:
Many tumor cells such as lung cancer cells, melanoma cells and osteosarcoma cells elicit procoagulant activity by transmembrane tissue factor, which activates factor X with factor VII, leading to the generation of thrombin and fibrin. It is well documented thrombin stimulated tumor cell mitogenesis[1] and further tumor cell adhesion to platelets [2, 3] endothelial cells [4] and extracellular matrix proteins [3]. The cooperation of these thrombin functions contributes to the experimental finding that thrombin-pretreated B16 tumor cells showed up to 156-fold increase in pulmonary metastasis in mice compared to that of sham-treated cells in vivo [2]. On the other hand, fibrin plays an important role in sustained adhesion and survival of tumor cells within the lung. Furthermore, the development of lung metastasis was strongly diminished in fibrinogene-deficient mice [5].

As shown above, there are considerable evidences that coagulation factors such as thrombin and fibrin play a critical role in tumor metastasis. Although various anticoagulant agents were used clinically and anti-metastatic effect of the agents was reported by many authors, the agents have not been used for anti-metastasis in clinical case. Warfarin, a major oral anticoagulant agent, inactivates blood coagulation activity by interfering with the hepatic synthesis of vitamin K dependent clotting factors II, VII, IX, and X. The purpose of this study was to survey the effect of warfarin on coagulation activity in mice and to identify the involvement between the degree of coagulation activity and tumor growth or lung metastasis in vivo.

METHODS:
Warfarin administration: Warfarin potassium was provided from Eisai Co., Ltd. Five-week-old female C57BL/6 mice were given warfarin solution (0, 200, 250 or 300μg of warfarin /100ml of water).

Measurement of PT (prothrombin time) and INR (international normalized ratio): Two days after warfarin administration, 360μl of blood was taken from the heart with a syringe containing 40μl of 3.8% sodium citrate under diethyl ether anesthesia, and plasma was separated by centrifugation. PT and INR were measured using Thromboplastin C Plus and CA (SYSMEX).

Time course of PT and INR: Two days after warfarin administration, blood was taken as above in a time course (0, 6, 12 hours) and PT and INR were measured.

Tumor Cell injection into tail vein: Two days after warfarin treatment, 16×10⁵ B16F10 (5.0×10⁵/200μl) was injected into tail vein. Two weeks after warfarin administration, mice were sacrificed and lungs were harvested. The number of metastatic foci was counted.

Subcutaneous transplantation of tumor cells: Mice were received subcutaneous injection of B16F10 (5.0×10⁵/200μl). After cell injection, warfarin administration for two weeks was started. Once a tumor was visible, the volume was estimated by the formula: volume=(L×W)²/6 (L=longest diameter, W=shortest diameter) [5].

Statistical Analysis: ANOVA and Fisher’s PLSD as a post hoc test, or the Mann-Whitney test.

RESULTS:
PT and INR: Warfarin administration resulted in a significant prolongation of PT and INR in a dose-dependent manner (INR: 0.85 at control, 2.04 at 200μg/100 ml, 5.81 at 250μg/100 ml and 9.52 at 300μg/100 ml of warfarin). While INR results indicated no significant difference at 200μg/100 ml, PT was delayed significantly. In the time course study, warfarin administration by drinking water inhibited coagulation activity continuously (data not shown).

Lung metastasis: Warfarin reduced lung metastasis in a dose dependent manner (-58.8% at 200μg/100ml, -89.3% at 250μg/100ml, -98.6% at 300μg/100ml vs. control, P<0.001).

Subcutaneous transplantation: Although warfarin administration reduced the average of tumor volume, there were no significant difference (-23.6% at 200μg/100ml, -45.9% at 250μg/100ml, -36.9% at 300μg/100ml vs. control). Survival rate at two weeks time point was 67% (control), 75% (200μg/100ml) and 25% (250μg, 300μg/100ml).

DISCUSSION: Our study indicated that warfarin inactivated blood coagulation activity. Because warfarin is oral anti-coagulant agent with a long half-life, it was succeeded to making continuous low coagulation activity model in mice. Lung metastasis was decreased by warfarin in a dose dependent manner. Although at the highest dose of warfarin (300μg/100ml), the average of INR prolongation was 9.5 and lung metastasis was drastically inhibited (-98.6% vs. control), this high dose at which high risk of bleeding tendency was predicted was not available clinically. In a clinical situation, coagulation activity was controlled within the INR target of 2.0-3.5. In our data, 200μg/100 ml of warfarin was corresponding to INR 2.0 and 58% reduction of lung metastasis compared to control. At least 58% reduction of lung metastasis was expected in the available range of INR from 2.0 to 3.5 in a clinical situation. Tumor cell proliferation may be affected by thrombin mediated by thrombin receptor (PAR-1) on the B16F10 [1, 6]. The expected suppression of tumor growth by anti-coagulant agent was not demonstrated significantly. Anticoagulation therapy for in vivo-grown tumor increased bleeding tendency, resulting in low survival rate (25% at 250μg, 300μg/100ml).

These results lead us the suggestion that anticoagulation therapy is available after tumor resection or before tumor growth. Although in the current study it was demonstrated that the inactivation of coagulation activity by warfarin inhibited lung metastasis, it is still unclear how long the inactivation of coagulation activity is needed for anti-metastatic effect in clinical situation. This needs further study. Because warfarin administration in drinking water resulted in continuous inactivation, this model may be utilized to study anti-metastatic effect of anticoagulant agent using various tumor cells such as osteosarcoma cells and lung cancer cells as a translational research.