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Activity of *Ginkgo biloba* Extract and Quercetin on Thrombomodulin Expression and Tissue-type Plasminogen Activator Secretion by Human Umbilical Vein Endothelial Cells

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Objective In order to investigate the pharmacological properties of *Ginkgo biloba* extract (GBE) on improving blood circulation, the regulating action of GBE and quercetin (a main flavonoid ingredient in GBE) on thrombomodulin (TM) expression and tissue-type plasminogen activator (t-PA) secretion was studied. **Methods** Using flow cytometer and gel image system respectively, we evaluated the TM expression and the t-PA secretion by human umbilical vein endothelial cells (HUVECs) *in vitro*. **Results** The increase of TM expression on HUVECs surface was induced by GBE rather than quercetin in a dose- and time-dependent manner. Both GBE and quercetin increased the t-PA release significantly. **Conclusion** The effect of GBE on improving blood circulation may be partly attributed to its promoting TM expression and t-PA secretion by endothelial cells, and quercetin participated in the effect of GBE on t-PA secretion. However, the action of GBE on increasing TM expression needs further study.

Key words: *Ginkgo biloba* extract; Quercetin; Thrombomodulin; Tissue-type plasminogen activator

INTRODUCTION

Ginkgo biloba extract (GBE) has been used as a commonly prescribed drug in traditional Chinese medicine for several thousand years. GBE is still used in clinics in Europe to alleviate symptoms associated with blood circulation disorder. It has been suggested to have the pharmacological actions of antioxidants, peripheral and coronary vasodilation, enhancement in coronary blood flow, and inhibition of thrombus formation and antiplatelet activities[1].

Thrombomodulin (TM) is a high-affinity receptor for thrombin present on the surface of endothelial cells. By means of forming a complex with thrombin, TM protein remodels the procoagulant activity of thrombin and acts as a cofactor for the thrombin-catalyzed activation of circulation protein C (PC)[2]. Activated protein C (APC) counteracts the coagulation cascade by proteolytic degradation of the coagulation factors Va and VIIIa necessary for blood coagulation[3]. Recently, it has been found that TM may down-regulate fibrinolysis by activating thrombin-activatable fibrinolysis inhibitor (TAFI), but its physiological significance is still obscure[4]. TAFI is suggested to have anti-inflammatory properties. TM overexpression can inhibit arterial thrombus in animal model[5]. At present, little is known about whether or not GBE or quercetin can modulate the expression of TM on the surface of human umbilical vein endothelial cells (HUVECs).

Endothelial cells regulate the hemostasis and fibrinolysis activity by expression and secretion of several important factors, including both tissue-type plasminogen activators (t-PA, anticoagulant) and plasminogen activator inhibitors (PAI, procoagulant)[6]. The t-PA converts plasminogen to plasmin, which lyses the fibrin. Moreover, single-chain form of t-PA dissolves fibrin directly[7]. Though ZHAO *et al.*[8] have reported that quercetin up-regulates the t-PA secretion by thrombin-induced HUVECs, effect of GBE on the t-PA secretion by HUVECs has not been sufficiently studied.

In this study, we evaluated the effect of GBE and quercetin (a main flavonoid ingredient in GBE) on TM expression on the surface of HUVECs *in vitro* by flow cytometer (FCM), and examined the efffect of

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GBE and quercetin on t-PA secretion by HUVECs using gel image system.

MATERIALS AND METHODS

Ginkgo biloba Extract and Quercetin

The GBE powder containing 12.4% quercetin, 24.2% flavonoids and 9.4% terpenes was kindly provided by Kangenbei Pharmaceuticals Co., Ltd (China). The compositions of flavonoids and terpenes in GBE were similar to EGB761™[9] used in France and Germany. The quercetin (98%) was purchased from Sigma (USA).

Human Umbilical Vein Endothelial Cells

Human umbilicus was obtained from Zhejiang Obstetrical and Gynecological Hospital with the consent of the local ethic committee. Vascular endothelial cells were harvested from human umbilical vein using a protocol described elsewhere[10]. Briefly, human umbilical vein was flushed with phosphate buffered saline (PBS) containing 0.25% trypsin (Sagon, China) and incubated for 15-20 min at 37℃. Next, the vein was washed with PBS. Cells were harvested from the collection and incubated in DMEM (Gibco, USA) (1×10^5 cells/mL) supplemented with 20% defined fetal calf serum (Hangzhou Sijiqing Biological Engineering, China), 100 units/mL penicillin and 100 μg/mL streptomycin.

Detection of TM

After growing to confluence, HUVECs were incubated with GBE or quercetin at 37℃ in 5% CO_2 air in 24-well plates. For assay of TM, the cells were prepared as previously described[11]. In brief, cells were harvested, washed twice with PBS buffer, and resuspended in 100 microliters PBS containing 0.1% NaN_3 (Sigma, USA) and 1% fetal calf serum. Next, cells in PBS were incubated with 10 microliters of human FITC-anti-TM antibody (Lot 584F30130A, Chemicon, USA) for 30 minutes at 4℃ in the dark. Finally, the cells were washed, centrifuged and resuspended in PBS containing 0.1% NaN_3 and 1% fetal calf serum. The expression of TM on cell surface was examined by FCM (FACSort, BD, USA) using CELL QUEST™ software through FL1 channel. For instrutment setting, unstained cells were prepared.

Assay of t-PA

As t-PA secreted by HUVECs can dissolve fibrin directly[7], it was examined as previously described[12]. Twenty-five μL of 1 mol/L $CaCl_2$ (Zhejiang Lianhua Company, China) was added to a mixture of 100 μL of 5 u/mL human α-thrombin (Lot 32k7602, Sigma, USA) were and 2.4 mL of 12 mg/mL human fibrinogen (Lot 072k7606, Sigma, USA). Instantly, 70 μL final mixture was added to each well of 96-well plates (NUNC, Denmark) and incubated for 3 hours at 37℃ for forming fibrin clot. Next, the 100 μL harvested HUVECs (5×10^5 cells/mL) was seeded on the surface of fibrin and grew to confluence. Then GBE or quercetin was added to the supernatant of HUVECs without bleb formation. After the cells were incubated with GBE or quercetin for different times, the well optical intensity (INT) representing the extent of fibrin dissolution was detected with Gel Doc™ 2000 gel image system (Bio-Rad, USA) using Quantity One™ software (Menu: volume→Tool: 96-well volume assay) (n=5).

As t-PA could dissolve fibrin directly, the extent of fibrin dissolution indicated the amount of t-PA secreted by HUVECs and was calculated from the following equation.

$$\text{Dissolution index \%} = (\text{INT}/\text{INT}_0)\ \%$$

The INT is the optical intensity of each well at different time points, and the INT_0 is the basal optical intensity of each well at zero time point. The dissolution index only represented the extent of fibrin dissolution induced by t-PA.

Statistical Analysis

Data were expressed as $\bar{x} \pm s$. Statistical significance was detected by using Student's t test. A P value <0.05 was considered statistically significant.

RESULTS

At the concentrations of 10, 30, 50, and 100 μg/mL of GBE for 24 hours, a dose-dependent elevation of TM expression on HUVEC surface was observed with a significant increase at 30, 50, and 100 μg/mL of GBE (Fig. 1a). However, at the concentrations of 1.27, 3.8, 6.33, and 12.65 μg/mL of quercetin, similar to that of it contained in 10, 30, 50, and 100 μg/mL of GBE, there was no significant effect on the TM expression (Fig. 1b).

When confluent HUVECs were incubated with 50 μg/mL of GBE for different times, a time-dependent enhancement of TM expression was observed (Fig. 2a). After incubation for 24, 36, and 48 hours, TM expression increased 72.21%, 79.33%, and 54.94%, respectively with the most significant increase occurring after 36-hour incubation; compared with control at zero time, the increases

were significant (P<0.01). When HUVECs were incubated with 6.33 μg/mL quercetin for different times, no significant change of TM expression occurred (Fig. 2b).

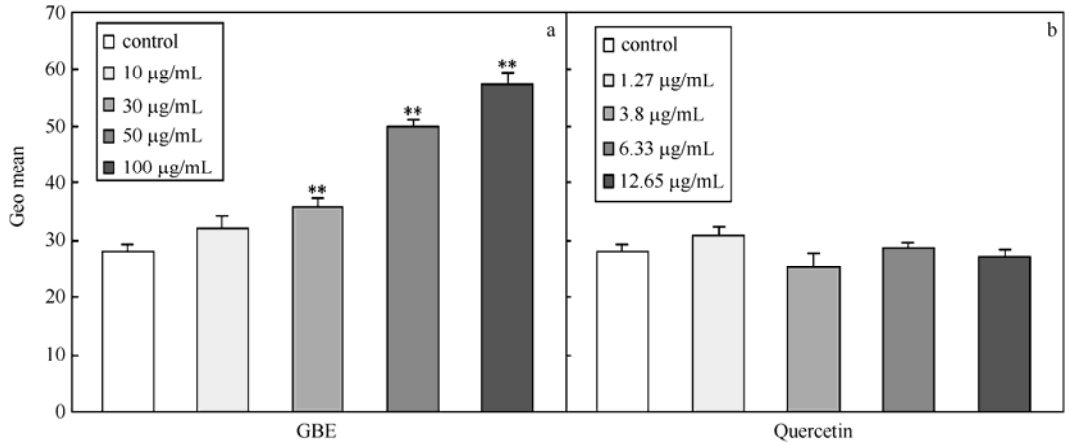

FIG. 1. Effect of GBE (a) and quercetin (b) on TM expression with various dosages. Each column represents $\bar{x} \pm s$ (triplicate samples). $^{**}P$<0.01 compared with the value of control.

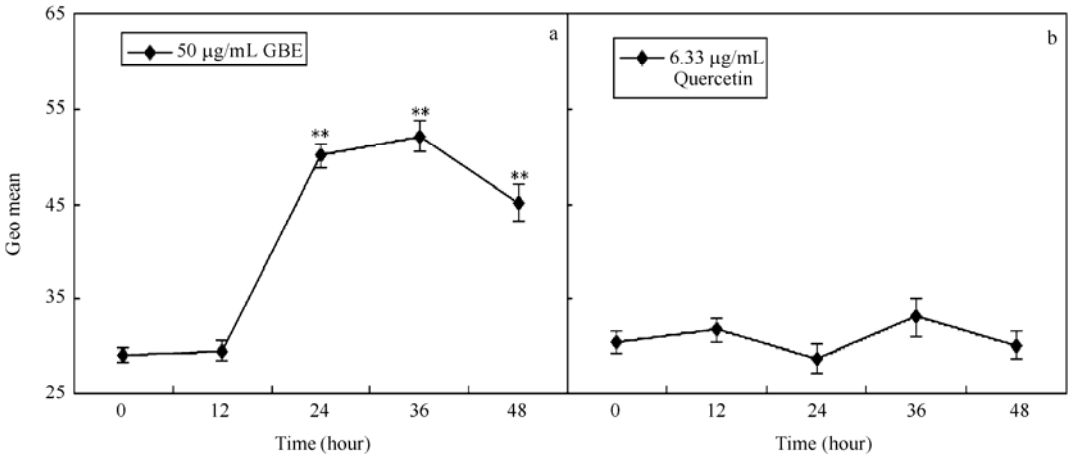

FIG. 2. Effect of GBE (a) and quercetin (b) on TM expression for various interval. Values represent $\bar{x} \pm s$ (triplicate samples). $^{**}P$<0.01 compared with control at zero time.

A dose-dependent increase of t-PA secretion was observed with significant increases noted at 30, 50, and 100 μg/mL of GBE (Fig. 3a). Fig. 3b shows that when HUVECs were incubated with quercetin at 3.8-12.65 μg/mL for 4 days, t-PA secretion increased significantly.

When HUVECs were incubated with 50 μg/mL of GBE (Fig. 4a) or 6.33 μg/mL of quercetin (Fig. 4b) for different times, a time-dependent elevation of t-PA secretion occurred.

DISCUSSION

As a gatekeeper between blood and tissues, human endothelial cells maintain blood flow and prevent thrombus formation by, expression of TM protein, and secretion of t-PA[6].

The TM-protein C pathway is a major anti-thrombotic mechanism present in endothelial cells[13]. The thrombin functions as recruiting platelets and clotting fibrinogen. Excess thrombin moves downstream and interacts with TM, catalysing the activation of protein C. Activated protein C interacts with protein S on endothelial cell or platelet membranes, and then proteolytically inactivates a coagulation co-factor Va, an important co-factor in the formation of thrombus. In addition, activated protein C inactivates another coagulation co-factor VIIIa in a complicated manner. When thrombin binds to TM, it no longer clots fibrinogen or activates

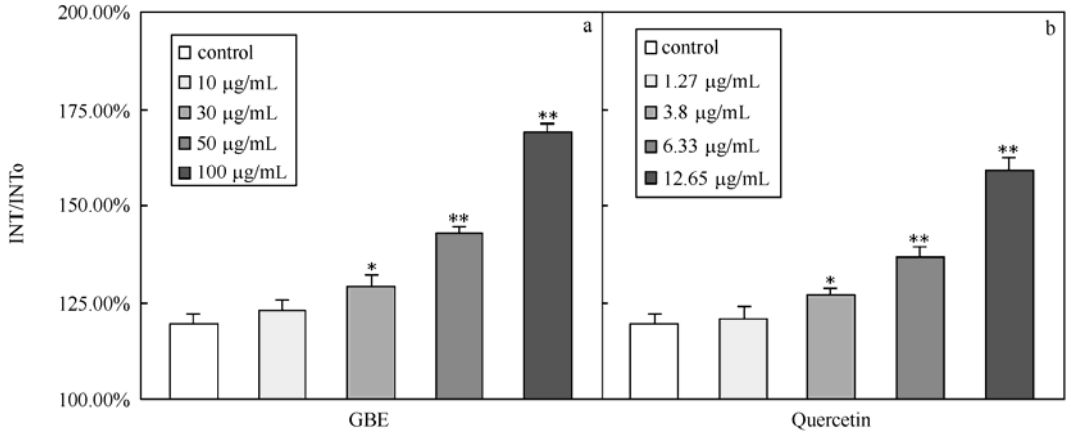

FIG. 3. Effect of different doses of GBE (a) and quercetin (b) on t-PA secretion. Each column represents $\bar{x} \pm s$ (n=5). $^{*}P<0.05$, $^{**}P<0.01$ compared with the control.

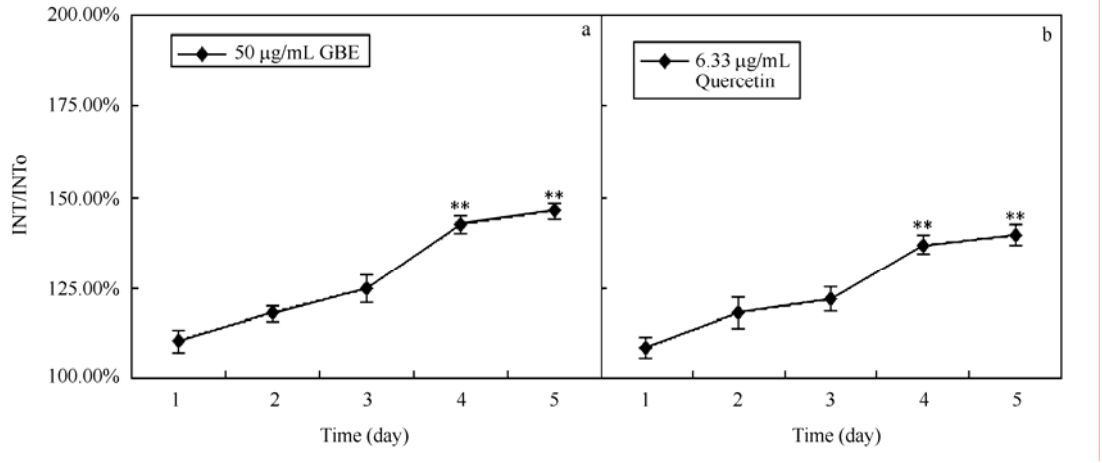

FIG. 4. Effect of GBE (a) and quercetin (b) on t-PA secretion for different time. Values represent $\bar{x} \pm s$ (n=5). $^{**}P<0.01$ compared with the control.

platelets. Particularly focusing on protecting mice from the acute thrombotic challenge, the combined ticlopidine (one of the most widely prescribed antiplatelet drugs) with GBE was more effective than a single ticlopidine[14]. Our data indicate that GBE but not quercetin increases the TM expression on HUVEC surface in a dose-and time-dependent manner, suggesting that GBE can suppresse the formation of thrombun by elevating TM expression (Figs. 1 and 2).

When HUVECs were incubated with GBE or quercetin, the level of t-PA secretion was also evaluated in this study. Endothelial cells regulate the fibrinolytic activity by synthesizing and secreting t-PA. The fibrinolytic activity is primarily dependent on the activation rate of plasminogen activated by t-PA. Cultured HUVECs mainly secrete t-PA rather than u-PA (urokinase-type plasminogen activator), which cannot be analyzed by ELISA in HUVEC cultures[15]. Our data show that both GBE and quercetin up-regulate the t-PA secretion in a dose- and time-dependent manner, and the effect of GBE on the t-PA secretion may be mainly due to the influence of quercetin present in GBE (Figs. 3 and 4). GBE can produce significant increase of $[Ca^{2+}]i$ in cultured rat aorta endothelial cells[16]. Furthermore, following endothelial stimulation with the Ca^{2+} ionophore A23187, the increase of $[Ca^{2+}]i$ in endothelial cells activate the secretion of t-PA[17]. This pathway may explain why GBE and quercetin increase the secretion of t-PA. In the present study, an *in vitro* model was used to examine the secretion of t-PA by HUVECs. This model is efficient, economical and convenient.

In summary, data in this study demonstrate that GBE but not quercetin augments the TM expresssion on the surface of HUVECs. Furthermore, the t-PA secretion by HUVECs is up-regulated by GBE, and this mainly involves quercetin contained in GBE. Evidence from this study suggests that the effect of GBE on improving blood circulation may be partly attributed to its promoting TM expression and t-PA secretion by endothelial cells. Further studies are necessary to reveal the mechanisms.

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