

Effect of quercetin on adhesion of platelets to microvascular endothelial cells *in vitro*¹

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KEY WORDS vascular endothelium; quercetin; adhesions; enzyme-linked immunosorbent assay; blood platelets

ABSTRACT

AIM: To study the effect of quercetin (Que) on the adhesion of platelets to microvascular endothelial cells (MVEC) isolated from human skin. **METHODS:** [³H]-adenine-labeled platelets were incubated with MVEC. Effect of Que on platelet endothelial cell adhesion molecular (PECAM) expression on MVEC was also evaluated using enzyme-linked immunosorbent assay (ELISA). **RESULTS:** Adhesion of platelet to MVEC reached to maximum at about 30 min. Que inhibited the adhesion of platelets to MVEC in a concentration-dependent manner. Que 5 μmol/L did not show any significant inhibition. When the concentration of Que increased to 10, 20, and 40 μmol/L, the inhibition rate increased to 10.5%, 20.0%, and 42.2%, respectively. Pre-incubation of Que (10-40 μmol/L) with labeled platelets for 30 min also inhibited the adhesion but Que 5 μmol/L did not. The inhibition rate of Que 10, 20, and 40 μmol/L was 18.2%, 29.8%, and 65.3% respectively. Expression of PECAM on the endothelial cells was decreased in a concentration-dependent manner when MVEC were treated with Que (10-40 μmol/L) for 12 h but Que 5 μmol/L did not significantly affect the expression. **CONCLUSION:** Que could inhibit the adhesion of platelets to MVEC. This effect may be related to decreased expression of PECAM on MVEC.

INTRODUCTION

The morphological and functional studies of cardio-

vascular disorders identified endothelial cell (EC) injury and dysfunction as present early and continued to be manifest throughout the disease course. Endothelium is now considered to be a widely distributed organ of considerable biological potential that performs distinctive and highly specialized biological functions that vary in different organs^[1]. The interaction between platelets and endothelial cells plays a crucial role in the dysfunction and pathogenesis of cardiovascular diseases. When a blood vessel is injured, adhesion of circulating platelets to the site of damage happened rapidly. This action induced serious responses that are related to occurrence and development of disease^[2]. Repair of endothelial cell dysfunction is very important to maintain the normal vascular tone and biological function. The understanding and illustration of endothelial dysfunction will aim to new direction to the treatment of cardiovascular diseases. Quercetin (Que, 3,3',5,7-pentahydroxy flavone) is a natural bioflavonoid. Our previous study showed that Que decreased the blood pressure, vascular resistance, platelet aggregation, and had an antiarrhythmic effect^[3-5]. The purpose of this study was to investigate the effect of Que on the adhesion of human platelets to isolated microvascular endothelial cells (MVEC) and its possible mechanism.

MATERIALS AND METHODS

Culture of MVEC MVEC was commercially purchased from Clonetics (San Diego, USA) and grown in MCDB 131 medium supplemented with 5% fetal calf serum, endothelial cell growth supplement 30 mg/L, epidermal growth factor 10 μg/L, hydrocortisone 10 mg/L, heparin and antibiotics 10 kU/L. The medium was changed every 2-3 d until subculture. For subculture, the cells were washed twice with PBS (in g/L: NaCl 8, KCl 0.2, Na₂HPO₄ 1.44, KH₂PO₄ 0.24) without calcium and magnesium. The cells were digested with 0.25% trypsin-0.01% edetic acid (dissolved in PBS, pH 7.4) for 3-5 min until the cells

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were detached from the dish. The digestion was stopped by adding the same volume of medium containing 10 % fetal calf serum and centrifuged at $300 \times g$ for 10 min. The pellets were re-suspended with medium and seeded on culture flask or plate for experiment.

Isolation and labeling of platelets Fresh blood from normal human donor was drawn into centrifuge tube containing 0.38 % sodium citrate. Platelet-rich plasma was prepared by centrifugation at $200 \times g$ for 10 min. The platelets were then labeled by incubation with [^3H]-adenine 10 MBq/L for 30 min at 37 °C. Unlabeled isotope was washed three times with Tyrode's buffer with apyrase 10 U/L. Labeled platelets were adjusted to 1×10^{10} /L and ready for the adhesion test.

Platelet adhesion assay Adhesion of platelet to MVEC was measured as previously described with modification^[6,7]. MVEC were cultured in 96-well plate until about 80 % confluence. Que was added to the MVEC and incubated as desired. Before labeled platelets were added, MVEC were washed three times with Tyrode's buffer, and platelets were suspended in Tyrode's buffer at a concentration of 1×10^{10} /L, then labeled platelets (100 μL per well) were added and incubated for 30 min. After washed out un-incorporated platelets, each well was treated with 3 % NaNO_3 100 μL (dissolved in 0.1 % NaOH) for 20 min. Lysates were collected and radioactivity of each well was determined by Beckman liquid scintillation spectroscopy. The data were shown as TBq.

Expression of PECAM on MVEC Expression of platelet endothelial cell adhesion molecular (PECAM) on MVEC was determined by enzyme-linked immunosorbent assay (ELISA). MVEC were cultured on 96-well plate until confluence, and Que was added and incubated as designed, then MVEC was washed and fixed with methanol. Expression of PECAM was measured, and the data were indicated as A (410 nm).

Reagents All reagents were from Sigma (Saint Louis, USA). [^3H]-Adenine was purchased from Amsham (Minneapolis, USA). PECAM monoclonal antibody was from R&D system (Arlington Heights, USA).

Statistics Pooled data were analyzed using Microsoft Excel program. All the values were expressed as $\bar{x} \pm s$ and compared with *t*-test.

RESULTS

Effect of Que on the adhesion of platelet to

MVEC When the platelets were added to MVEC, they adhered to MVEC immediately and reached to maximum at about 30 min (Fig 1). Que was added to confluent MVEC and continued to incubate at 37 °C for 12 h, Que (10–40 $\mu\text{mol/L}$) inhibited the adhesion of platelets to MVEC in a concentration-dependent manner, while Que 5 $\mu\text{mol/L}$ did not show any inhibition. When the concentration increased to 10, 20, and 40 $\mu\text{mol/L}$ separately, the inhibition rate increased to 10.5 %, 20.0 % and 42.2 % respectively. Pre-incubation of labeled platelets with Que for 30 min before adding MVEC also showed a significant decrease of adhesion in a concentration-dependent manner. Que (5–40 $\mu\text{mol/L}$) was added to labeled platelets and incubated for 30 min, and then added to MVEC and incubated for another 30 min, Que 5 $\mu\text{mol/L}$ had no significant effect, but Que 10, 20, and 40 $\mu\text{mol/L}$ decreased the adhesion significantly. The inhibition rate was 18.2 %, 29.8 %, and 65.3 % respectively (Tab 1).

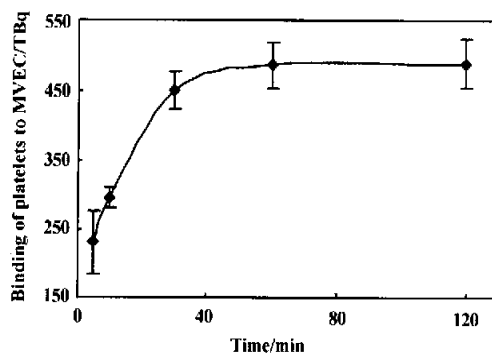


Fig 1. Adhesion of platelets to MVEC in culture at different time points. $n = 4$ experiments. $\bar{x} \pm s$.

Effect of Que on the expression of PECAM on MVEC Que 5–40 $\mu\text{mol/L}$ were incubated with confluent MVEC at 37 °C for 12 h. There was no statistically different expression of PECAM on MVEC between control and Que 5 $\mu\text{mol/L}$ treated group. When the dose of Que increased to 10, 20, and 40 $\mu\text{mol/L}$, the inhibition rate of PECAM expression was 25.0 %, 42.5 %, and 55.5 % respectively (Tab 2).

DISCUSSION

In this study, we measured the effect of Que on the adhesion of platelet to MVEC, and the relationship between platelet adhesion to MVEC and expression of

Tab 1. Effect of quercetin (Que) on the adhesion of platelet to MVEC by liquid scintillation spectroscopy. n = 4 experiments. $\bar{x} \pm s$. ^aP > 0.05, ^bP < 0.05, ^cP < 0.01 vs control.

Groups	Non-pretreated Binding of platelets to MVEC/TBq	Inhibition rate/%	Pretreated with Que Binding of platelets to MVEC/TBq	Inhibition rate/%
Control	449 ± 27	—	435 ± 48	—
Que 5 μmol/L	433 ± 33 ^a	3.6	411 ± 22 ^a	5.5
10 μmol/L	402 ± 54 ^b	10.5	356 ± 18 ^b	18.2
20 μmol/L	359 ± 14 ^b	20.0	305 ± 15 ^b	29.8
40 μmol/L	260 ± 25 ^c	42.2	151 ± 88 ^c	65.3

Tab 2. ELISA assay of PECAM expression on cultured MVEC treated with Que for 12 h. n = 4 experiments. $\bar{x} \pm s$. ^aP > 0.05, ^bP < 0.05, ^cP < 0.01 vs control.

Groups	A _{410 nm}	Inhibition rate/%
Control	0.88 ± 0.14	—
Que 5 μmol/L	0.82 ± 0.22 ^a	6.3
10 μmol/L	0.66 ± 0.13 ^b	25.0
20 μmol/L	0.50 ± 0.10 ^b	42.5
40 μmol/L	0.39 ± 0.05 ^c	55.5

PECAM on MVEC. Our data showed that Que inhibited significantly the adhesion of human platelet to MVEC *in vitro*. Pre-treatment of either MVEC or labeled platelets with Que manifest the action, but inhibition is stronger in platelets treated with Que than in MVEC treated with Que. Expression of PECAM on MVEC was also decreased when treated with Que, which might be one of the mechanisms of the inhibitory effect of Que on the platelet adhesion.

It is crucial for MVEC to maintain and carry out normal functions. Prevention of the platelet adhesion to damaged MVEC was beneficial for pathogenesis and development of various diseases such as cardiovascular disorders, inflammatory process, and connective tissue diseases, *etc.*^[7-9]. Our previous study showed that Que could inhibit aggregation of platelets and protect arrhythmia from ischemic reperfusion. Present study confirmed that Que could also decrease the adhesion of platelets to human MVEC *in vitro*. The block of the adhesion could further prevent the aggregation of platelets and the following biological reaction with endothelial cells and matrix of sub-endothelium. So our study would be very helpful to understand the cardiovascular pharmacology of Que and its potential clinical use for the treatment of diseases such as atherosclerosis and other related diseases.

To evaluate the possible mechanism of the action of Que, we chose PECAM as a marker, since PECAM is very important for the platelet adhesion to MVEC. Up-regulation or down-regulation of PECAM expression on both MVEC and platelet will significantly influence the degree of adhesion of platelets to MVEC. Our experiment indicates that Que can inhibit the expression of PECAM on MVEC in a manner of concentration-independence. In conclusion, Que can inhibit the platelet adhesion to MVEC and this inhibitory effect may be related to the decreased expression of PECAM on MVEC.

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槲皮素对体外血小板与微血管内皮细胞粘附的影响¹

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关键词 血管内皮; 槲皮素; 粘附; 酶联免疫吸附法; 血小板

目的: 研究槲皮素(Que)对血小板与人皮肤分离的微血管内皮细胞(MVEC)粘附的影响. **方法:** 用³H-腺嘌呤标记的血小板与 MVEC 共同孵育, 研究 Que 对血小板与 MVEC 粘附所起的作用. Que 对 MVEC 上血小板内皮细胞粘附分子(PECAM)表达的影响用酶联免疫法评价. **结果:** 血小板加入 MVEC 中后立

即发生粘附并于 30 min 达到最大值. Que 能浓度依赖地抑制血小板粘附, Que 5 μmol/L 无显著的抑制作用, 但 Que 浓度为 10、20 和 40 μmol/L 时, 抑制率分别为 10.5 %、20.0 % 和 42.2 %. Que 与标记的血小板预先孵育 30 min, 也能浓度依赖地抑制其粘附, Que 10、20 和 40 μmol/L 的抑制率分别为 18.2 %、29.8 % 和 65.3 %, 而 Que 5 μmol/L 在两种处理中均无效. 当用 Que 10 - 40 μmol/L 处理 MVEC 时, Que 能浓度依赖地减少 MVEC 上 PECAM 的表达, 但 Que 5 μmol/L 无显著效果. **结论:** Que 能抑制血小板与 MVEC 的粘附, 此作用可能与减少 MVEC 上 PECAM 的表达有关.

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