Inhibitory effect of quercetin on proliferation of human microvascular endothelial cells *in vitro*[1]

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KEY WORDS quercetin; vascular endothelium; cell division; cytotoxicity; human

ABSTRACT

AIM: To investigate the role of quercetin (Que) in the proliferation of cultured human skin microvascular endothelial cells (MVEC). **METHODS:** Cell count and [*methyl*-^{3}H]thymidine ([^{3}H]TdR) uptake assay were used to measure the effect of Que in the proliferation of cultured MVEC. Cytotoxicity of Que on MVEC was also evaluated by ^{51}Cr release assay. **RESULTS:** When MVEC were treated with Que, the proliferation was significantly inhibited in a time-course and dose-dependent manner. Que 5 μmol/L did not inhibit the proliferation of MVEC. When the concentration of Que increased to 20, 40, 80, and 160 μmol/L, the cell numbers per well were decreased and the inhibition rate was 12.2 %, 23.5 %, 35.3 %, and 54.1 % respectively with IC_{50} of 138 μmol/L. The inhibitory rate of [^{3}H]-TdR uptake was 18.7 %, 34.4 %, 48.9 %, and 62.5 % respectively (IC_{50}=87.5 μmol/L). ^{51}Cr release assay showed that Que 160 μmol/L incubated with MVEC from 1 to 16 h had no clear cytotoxicity compared with control group. **CONCLUSION:** Que greatly inhibited the proliferation of cultured human MVEC *in vitro*. This effect may not be related to the cytotoxicity of Que on MVEC.

INTRODUCTION

The induction of angiogenesis is characterized by the degradation of the vascular basement membrane, endothelial cell migration, proliferation, and tube formation[1,2]. There are growing evidences that anti-angiogenic drugs, especially those inhibiting proliferation of endothelial cells, will improve future therapies of diseases including cardiovascular diseases, chronic inflammation (rheumatoid arthritis), diabetes, psoriasis, adiposity, and other neovascularisation[3,4].

Quercetin (Que, 3,3',5,7-pentahydroxy flavone) is by far the most abundant bioflavonoid. Very wide ranges of biological actions of Que have been reported such as anti-oxidantion[5], anti-aggregation and anti-adhesion of platelets[6,7], vasodilation[8], and antitumorigenic (anti-angiogenesis) effects[9]. Recently, Que has been demonstrated to inhibit proliferation of several types of cell lines including cancer cells[10-12], smooth muscle cells [13], and porcine endothelial cells[14]. However, there is little information about the effect of Que on human endothelial cells, and particularly, the microvascular endothelial cells (MVEC) that plays a crucial role in the angiogenesis. Therefore, in the present study we have evaluated the role of Que in the proliferation and cytotoxicity of MVEC *in vitro*.

MATERIALS AND METHODS

Culture of MVEC MVEC were purchased

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from Clonetics (San Diego, USA). Cells were cultured with MCDB 131 medium on 75-cm^2 tissue culture flasks. The media were supplemented with 5 % fetal calf serum, endothelial cell growth supplement 30 mg/L, epidermal growth factor 10 μg/L, hydrocortisone 10 mg/L, heparin 10 kU/L, and benzylpenicillin/streptomycin as antibiotics. The medium was changed every 2-3 d until subculture. For subculture, the cells were washed twice with phosphate-buffered saline (in g/L: NaCl 8, KCl 0.2, Na_2HPO_4 1.44, KH_2PO_4 0.24) without calcium and magnesium, a digestion buffer (0.25 % trypsin-0.01 % edetic acid dissolved in PBS, pH 7.4) was added and incubated at 37 ºC for 3-5 min until the cells were detached from the flask. The digestion was then stopped by adding the same volume of medium containing 10 % fetal calf serum and centrifuged at 300×*g* for 10 min. The pellets were resuspended with medium and seeded to culture flasks or plates for experiment. Three-seven passages were used for the test.

Cell count MVEC were cultured on 24-well tissue culture plates at a number of 5×10^4 cells per well in 1 mL media. To ensure most of cells entered the DNA synthesis state of cell cycle, the cells were treated with MCDB 131 base media with 0.5 % fetal calf serum and without any stimulators for 2 d. After wash and replacement with normal media, Que (Que was dissolved in 0.05 % NaOH at a concentration of 5 mmol/L as a stocking solution and diluted to desired concentration with media before the experiment) was added and the cells were cultured for another 20 h as desired. At the end of experiment, the cells were digested, centrifuged and re-suspended in count buffer. Cell numbers in each well were counted on a Coulter Counter.

[^{3}H]TdR uptake assay MVEC were cultured on 96-well tissue culture plates at 1×10^4 cells per well in 100 μL media, and treated as described above. [^{3}H]TdR from Amersham (Minneapolis, USA) was added to MVEC at a concentration of 37 kBq per well for 16 h before harvest. At the end of experiment an automated multiwell harvester was used to aspirate, and lyse cells. DNA was transferred onto glass filter paper, while unincorporated [^{3}H]TdR was washed out. Each row of 96-well plate was filled and aspirated 10 times to ensure complete cell transfer and removal of unincorporated thymidine. After wash, each filter strip was washed with 100 % ethanol and transferred to scintillation vials. Scintillation fluid 2 mL was added to each vial. Radioactivity of each well was determined by Beckman liquid scintillation spectroscopy. The data were shown as TBq.

^{51}Cr release assay The cytotoxic effect of Que on MVEC was determined by a method described by Coligan *et al*[15] with modification. Briefly, MVEC were cultured on 96-well plates till 80 % confluence. ^{51}Cr diluted with media was then added at a concentration of 37 kBq per well in 100 μL growth media. The cells were then labeled in a humidified atmosphere at 37 ºC with 5 % CO_2 for 4 h. Unlabeled ^{51}Cr was removed by washing four times with MCDB base media. Que 160 μmol/L was incubated with labeled MVEC for different periods. H_2O_2 (0.01 %) was used as a positive cytotoxic stimulator. At the end of experiment, supernatants from each well were collected and radioactivity of each well was determined by Beckman liquid scintillation spectroscopy. The data were shown as TBq.

Reagents All the reagents were from Sigma (Saint Louis, USA). [^{3}H]Thymidine and ^{51}Cr were purchased from Amersham (Minneapolis, USA).

Statistics Pooled data were analyzed using Microsoft Excel program. All the values were expressed as mean±SD and compared with *t* test.

RESULTS

Effect of Que on the proliferation of MVEC When MVEC were treated with Que, the proliferation of MVEC was significantly inhibited in a time-course manner. Que did not inhibit the proliferation until 1 h. Higher Que (160 μmol/L) had a faster inhibitory effect on the proliferation than that of lower Que (Tab 1). Cell count indicated that Que inhibited the proliferation of human MVEC cultured *in vitro* in a concentration-dependent manner. Que 5 μmol/L did not show any inhibitory effect. When the concentration increased from 20, 40, 80, and 160 μmol./L separately, the inhibitory rate measured by cell number increased to 12.2 %, 23.5 %, 35.3 %, and 54.1 %. The [^{3}H]TdR

Tab 1. [^{3}H]TdR uptake by microvascular endothelial cells (MVEC) in culture treated with Que (80 and 160 μmol/L) at different time points. *n*=4 experiments. Mean±SD.

	Radio activity/TBq				
Que	1 h	4 h	8 h	16 h	32 h
80 μmol/L	728±22	651±15	526±21	440±17	339±7
160 μmol/L	722±21	559±26	421±22	289±35	222±15

uptake by MVEC showed the same results, the inhibitory rate increased to 18.7 %, 34.4 %, 48.9 %, and 62.5 % respectively (Tab 2).

Tab 2. Effect of Que on the proliferation of human MVEC assayed by cell count and [^{3}H]TdR uptake. *n*=4 experiments. Mean±SD. ^{a}*P*>0.05, ^{b}*P*<0.05, ^{c}*P*<0.01 *vs* control.

Group	Cell number per well	Inhibitory rate/%	[^{3}H]TdR uptake/TBq	Inhibitory rate/%
Control	106 192±6 787		900±32	
Que 5 μmol/L	102 451±3 427^a	3.5	861±57^a	4.3
20 μmol/L	93 167±1 718^b	12.2	732±17^b	18.7
40 μmol/L	81 237±7 840^b	23.5	590±17^c	34.4
80 μmol/L	68 673±2 567^c	35.3	459±23^c	48.9
160 μmol/L	48 769±2 607^c	54.1	337±17^c	62.5

Cytotoxic effect of Que on MVEC Confluence MVEC were labeled with ^{51}Cr at 37 ºC for 4 h. Que 160 μmol/L or 0.01 % of H_2O_2 in MCDB 131 base medium were added and incubated as designed. The cytotoxicity was mainly measured by the release of labeled ^{51}Cr from MVEC into the medium. Que did not have significant cytotoxicity to MVEC even 16 h after incubation but 0.01 % of H_2O_2 indicated a clear cytotoxicity to MVEC in a time-dependent manner compared with control group (P<0.05, Tab 3).

Tab 3. Chromium-51 released from MVEC in culture treated with Que (160 μmol/L) at different time points. The value indicates the TBq per group. *n*=4 experiments. Mean±SD. ^{a}*P*>0.05, ^{b}*P*<0.05, ^{c}*P*<0.01 *vs* control.

Time/h	Radio activity/TBq		
	Control	Que	H_2O_2 (0.01%)
1	102±5	112±6^a	200±11^b
4	186±7	201±10^a	356±32^b
8	232±10	254±13^a	479±35^c
16	379±16	391±27^a	633±38^c

DISCUSSION

The previous study showed that Que inhibited the platelet adhesion to human MVEC cultured *in vitro*, and this role was related to the down-regulation of PECAM expression on the endothelial cells[6].

Several *in vitro* studies indicate that Que has multiple effects on cancer cells, including the inhibition of cell proliferation[10-12], little is known about the effect of Que in non-malignant cells. Que showed a significant inhibitory effect on the proliferation of cultured MVEC *in vitro* monitored by cell count and [^{3}H]TdR. MVEC proliferation or re-generation play a key role in the process of angiogenesis which is associated with lots of diseases such as cardiovascular disease, chronic inflammation, diabetes, and cancers. The anti-proliferate role of Que on MVEC will help us to understand the regulation of angiogenesis and may introduce new therapy for the treatment of diseases associated with angiogenesis. Further study need to be done *in vivo* to confirm the anti-angiogenesis of Que.

The mechanisms of Que in inhibiting proliferation of cultured MVEC are not clear. ^{51}Cr release assay showed that there was no clear cytotoxicity of MVEC treated with Que. Lipopolysaccharide (LPS)-induced endothelial cytotoxicity can also be inhibited by Que *in vitro*[16]. These results might indicate that the inhibitory effect of Que on the proliferation of MVEC is not due to its cytotoxicity.

Several other mechanisms, including inhibition of lactate transport, suppression of glycolysis and ATP production, inhibition of various tyrosine protein kinases, interference with ion pump systems, and various signal transduction pathways and perturbule network, have been cited for the anti-proliferative activity of Que[10,-11, 17-19]. Que suppressed proliferation of Ishikawa cells through down-regulation of EGF and cyclin D1[20]. MVEC can enter the stage of proliferation because of unbalance between pro-angiogenic and antiangiogenic factors. In our model, EGF and endothelial cell growth supplement were used as stimulators of proliferation. Further study is underway in our lab to see whether the anti-proliferative activity of Que on the MVEC is related to regulation of the expression of VEGF and other anti-angiogenic factors.

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