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Effect of genistein and quercetin on proliferation, collagen synthesis, and type Ⅰ procollagen mRNA levels of rat hepatic stellate cells[1]

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KEY WORDS genistein; quercetin; liver cirrhosis; cell division; collagen; reverse transcriptase polymerase chain reaction; messenger RNA

ABSTRACT

AIM: To study the effects of genistein (GE) and quercetin (QU) on proliferation, collagen synthesis, and procollagen messenger RNA (mRNA) expression of rat hepatic stellate cell line HSC-T6 cells. **METHODS**: Cell proliferation was measured by crystal violet staining assay. Collagen synthesis was determined by [^{3}H]proline incorporation assay. Type Ⅰ mRNA level was determined by reverse transcription polymerase chain reaction (RT-PCR). **RESULTS**: GE (25 - 70 μmol/L) and QU (6.25 - 50 μmol/L) concentration-dependently reduced serum-driven HSC-T6 cell proliferation and collagen synthesis associated with a suppression of type Ⅰ procollagen mRNA level. **CONCLUSION**: GE and QU inhibited hepatic stellate cell proliferation and collagen synthesis that might have a protective role against liver fibrosis.

INTRODUCTION

Hepatic stellate cells (HSC) and the derived myofibroblasts have been documented to play a central role in liver fibrogenesis, in experimental models of liver fibrosis, as well as in human chronic liver disease[1]. In the course of fibrogenesis, HSC undergo activation to a myofibroblast-like phenotype, proliferate and synthesize excess extracellular matrix components, particularly collagen. Therefore, it is important to find out some agents that might limit the proliferation and/or matrix component synthesis of HSC. We have developed an *in vitro* assay to screen anti-proliferation agents using an immortalized rat stellate cell line, HSC-T6[2,3]. Several kinds of Chinese herbal components are assessed, including alkaloids, flavonoids, and other compounds isolated from Chinese medicines used in the treatment of liver diseases. Genistein and quercetin have strong antioxidative activity and have therapeutic potential in some diseases, such as arteriosclerosis and cancer[4,5]. However, their effect on liver disease remains unclear. The aim of this study was to assess the antifibrotic effects of genistein (GE) and quercetin (QU) on proliferative activity and collagen synthetic capacity as well as type Ⅰ procollagen mRNA expression of HSC-T6 cells.

MATERIALS AND METHODS

Reagents GE and QU (Sigma, St Louis, USA) were dissolved in methyl sulfoxide (Me_2SO) as a stock solution and diluted with Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Karlsruhe, Germany) when test. [^{3}H]Proline was obtained from China Institute of Atomic Energy, Chinese Academy of Sciences. Taq polymerase was from Shanghai Bioasia Co. Deoxynucleotides, oligo (dT) 15 primer, and random primers were from Promega (Wisconsin, USA). Supertranscript Ⅱ RT, first-strand synthesis buffer, DTT, and TRIZOL reagent were purchased from Gibco BRL.

Cell line and cell culture HSC-T6 cell, an immortalized rat myofibroblast line, which had the stable phenotype and biochemical characters, was kindly provided by Dr S L FRIEDMAN (liver Center Laboratory, San Francisco General Hospital, USA). The cells were cultured in DMEM with 10 % calf serum at 37 ℃

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in a humidified atmosphere of 5 % CO_2 + 95 % air.

Measurement of HSC proliferation Proliferation of HSC-T6 cells was determined by crystal violet assay[2]. Briefly, HSC-T6 cells (1×10^4 per well) were incubated in DMEM medium containing 10 % serum for 24 h. Thereafter, the medium was removed and cells were incubated in the same medium containing different concentrations of GE or QU. For the untreated control, an equal amount of drug solvent (DMEM containing 0.1 % Me_2SO) was added. After a 48-h incubation, cell density was measured and expressed as A_{595}.

Collagen assay Intracellular collagen synthesis was measured by [^{3}H] proline incorporation assay[2]. HSC-T6 cells (3.5×10^4 per well) were incubated in DMEM with 10 % calf serum for 24 h. Then the medium was removed and cells were incubated in the same medium containing ascorbic acid 50 mg/L together with drug or drug solvent (0.1 % Me_2SO) for 24 h. To assess intracellular collagen synthesis, [^{3}H] proline 7.4 kBq per well was added and the microplate was returned to the incubator for a further 12 h. The cells were then harvested onto glass fibers and the radioactivity was measured using a scintillation counter. Intracellular collagen synthesis was expressed as Bq.

RNA extraction Approximately 24 h after culture, HSC-T6 cells were treated with or without drug for 24 h as described above. Total RNA was extracted from control and experimental cell cultures by the Trizol-phenol-chloroform method.

Reverse transcription polymerase chain reaction Levels of mRNA for α1 (Ⅰ) procollagen and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were analyzed by the reverse transcription polymerase chain reaction method. Total RNA 1 μg prepared from HSC-T6 cells was reverse transcribed using supertranscript Ⅱ RT (200 U) at 42 ℃ for 1 h. One microlitre of 20 μL total RT reaction was used as template DNA for polymerase chain reaction (PCR). PCR was performed by 30 s denaturation at 94 ℃, 30 s hybridization at 58 ℃, and 1 min extension at 72 ℃ with specific primers for 35 cycles. Primers were based on published nucleotide sequences for α1 (Ⅰ) procollagen (5′-CCA ATC TGG TTC CCT CCC-3′; 5′-AGG TTG AAT GCA CTT TTG G-3′)[6], GAPDH (5′-ACC ACA GTC CAT GCC ATC AC-3′; 5′-TCC ACC ACC CTG TTG CTG TA-3′)[7]. Polymerase chain reaction products were electrophoresed in 1.8 % agarose gel containing ethidium bromide 0.5 mg/L and photographed under UV light. The polymerase chain reaction experiments were performed at least three times with similar results.

Statistics Data were analyzed by ANOVA and Student t-test, and data were expressed as $\bar{x} \pm s$.

RESULTS

Effect of genistein and quercetin on HSC-T6 cell proliferation GE (25 – 70 μmol/L) and QU (6.25 – 50 μmol/L) concentration-dependently suppressed HSC-T6 cell proliferation (Tab 1). The antiproliferative effects were not related to nonspecific toxicity of both compounds because cells showed normal morphology upon phase-contrast microscopy and trypan blue staining.

Tab 1. Effect of genistein and quercetin on rat hepatic stellate HSC-T6 cell proliferation. The cells were incubated in DMEM containing 10 % serum with drug or drug solvent (0.1 % Me_2SO). Proliferation was assessed by crystal violet assay and expressed as A_{595}. n = 6. $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* control.

Treatment/μmol·L^{-1}		A_{595}	Inhibition/%
Medium		1.30 ± 0.01	
Control		1.30 ± 0.01	
Genistein	25	1.23 ± 0.07^a	5.5
	35	1.14 ± 0.02^c	12.5
	50	0.85 ± 0.05^c	34.4
	70	0.70 ± 0.06^c	46.1
Medium		0.96 ± 0.04	
Control		0.98 ± 0.06	
Quercetin	6.25	0.95 ± 0.06^a	3.1
	12.5	0.87 ± 0.07^b	11.2
	25	0.66 ± 0.05^c	32.7
	50	0.48 ± 0.04^c	51.0

Effect of genistein and quercetin on collagen synthesis Exposure of HSC-T6 cells to GE for 48 h markedly reduced intracellular collagen synthesis by 38.1 %, 55.5 %, and 78.7 % at concentrations of 35, 50, and 70 μmol/L, respectively. Collagen synthesis was markedly reduced by 10.6 %, 18.5 %, and 30.3 % at QU 12.5, 25, and 50 μmol/L, respectively (Tab 2).

Effect of genistein and quercetin on mRNA expression of type Ⅰ procollagen By RT-PCR analysis, HSC-T6 cells expressed high basal level of α1 (Ⅰ) procollagen mRNA when grown in the presence of 10 % calf serum. Under these experimental conditions,

Tab 2. Effect of genistein and quercetin on intracellular collagen synthesis of HSC-T6 cells. The cells were incubated in DMEM containing 10 % serum and ascorbic acid 50 mg/L with drug or drug solvent (0.1 % Me_2SO). Intracellular collagen synthesis was assessed as [^{3}H]proline uptake by cells. $n = 3$. $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* control.

Treatment/μmol·L⁻¹		Radio activity/Bq	Inhibition/%
Medium		107 ± 15	
Control		106 ± 15	
Genistein	25	104 ± 14[a]	2.1
	35	65 ± 16[c]	38.1
	50	47 ± 16[c]	55.5
	70	23 ± 8[c]	78.7
Medium		123 ± 10	
Control		123 ± 9	
Quercetin	6.25	113 ± 10[a]	7.8
	12.5	110 ± 5[b]	10.6
	25	100 ± 10[b]	18.5
	50	86 ± 18[c]	30.3

exposure of HSC-T6 cells to GE (50 and 70 μmol/L) decreased α1(I) procollagen mRNA expression to 50 % and 36 % of control. QU (25 and 50 μmol/L) decreased α1(I) procollagen mRNA expression to 72 % and 64 % of control, respectively (Fig 1).

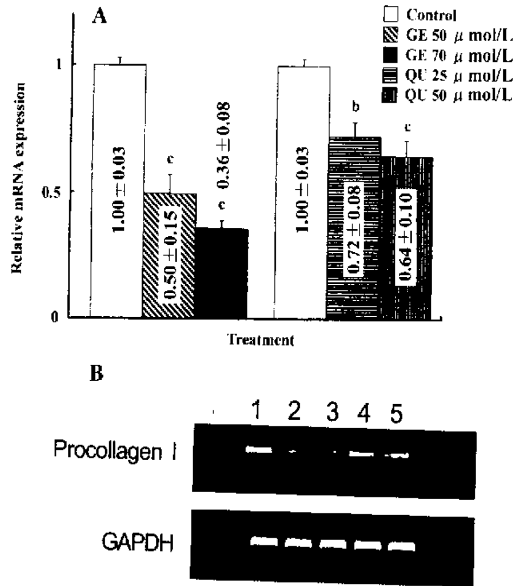

Fig 1. Effect of genistein and quercetin on the expression of α1(I) procollagen. Expression of GAPDH was used as an internal standard. (A) Quantitative determination results. $n = 3$. $^bP < 0.05$, $^cP < 0.01$ *vs* control. (B) Corresponds to a typical PCR result. Lane 1, 2, 3, 4, and 5 (from left to right) represented control, GE 50, 70 μmol/L, and QU 25, 50 μmol/L, respectively.

DISCUSSION

The present report describes that the effects of genistein and quercetin on the proliferation, collagen synthesis, and type I procollagen mRNA expression of rat HSC-T6 cells. We showed that genistein and quercetin inhibited serum-stimulated HSC-T6 cell proliferation. Our results are in keeping with the inhibitory effects of genistein and quercetin observed in mouse NIH/3T3 fibroblasts and effect of quercetin on rat primary hepatic stellate cells[8,9]. Isoflavone genistein and flavonol quercetin have been shown to be potent inhibitors of several kinases involved in signal transduction of cell proliferation, mainly protein kinase C, tyrosine kinase, and phosphatidylinositol 3 kinase[10]. Thus, the anti-proliferative activities of genistein and quercetin might be related to their inhibitory actions on these kinases. In addition, genistein and quercetin concentration-dependently reduced serum-driven intracellular collagen synthesis. This was associated with a decrease in α1(I) procollagen mRNA. These results indicate that the inhibition of collagen synthesis might occur at pretranslational level.

In summary, genistein and quercetin inhibited HSC proliferation and collagen synthesis. Because HSC play a central role in liver fibrosis, we suggest that inhibitory effects of genistein and quercetin on HSC might reduce the pool of HSC and deposit of extracellular matrix such as collagen within the liver. Their therapeutic potential should be studied further.

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金雀异黄素和槲皮素对大鼠肝星状细胞增殖、胶原合成及Ⅰ型原胶原 mRNA 水平的影响[1]

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关键词 金雀异黄素；槲皮素；肝硬化；细胞分裂；胶原；逆转录聚合酶链反应；信使 RNA

目的：研究金雀异黄素和槲皮素对 HSC-T6 大鼠肝星状细胞增殖和胶原合成及Ⅰ型原胶原 mRNA 表达的影响．**方法**：细胞增殖和胶原合成分别采用结晶紫染色法和[^{3}H]-脯氨酸掺入法．原胶原 mRNA 水平用 RT-PCR 法测定．**结果**：金雀异黄素(25-70 μmol/L)和槲皮素(6.25-50 μmol/L)浓度依赖抑制 HSC-T6 细胞增殖和胶原合成，对Ⅰ型原胶原 mRNA 表达也有抑制作用．**结论**：金雀异黄素和槲皮素抑制肝星状细胞增殖和胶原合成可能对肝纤维化有保护作用．

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