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Role of quercetin on inhibiting collagen in human embryonic lung fibroblast by MAPKS signaling pathway

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ABSTRACT

Objective To explore the effect of Quercetin on inhibiting collagen synthesis in Human Embryonic Lung Fibroblast (HELF) by regulating MAPKs signaling pathway. Methods The silicotic alveolar macrophages(AM) were collected by bronchoalveolar lavage and incubated in vitro with DMEM medium containing SiO_2(50 mg/L) for 18 hours. Then the AM supernatant incubated for 18 hours was collected with quercetins as stimulus supernatant. HELF was isolated by organize paste block method. four generations of pulmonary fibroblasts were divided into control group,AM group,SiO_2+AM group and quercetin in low, middle, high (10,20,40μmol/L) concentration group. The proliferation in the HELF was detected with MTT method. The expressions of MAPKs in the HELF are detected by western blot. Collagens were measured by Hydroxyproline Kit. Results Compared with control group,group SiO_2 + AM and group SiO_2 increased collagen and proliferation of HELF and MAPKs（$P<0.05$）. Quercetin inhibited collagen and proliferation of HELF and MAPKs,the higher the concentration inhibition stronger. Conclusions Quercetin has potent protective effects against silicosis through inhibiting collagen synthesis by suppressing the MAPKs activation.

KEYWORDS

Quercetion; Human embryonic lung fibroblasts; Alveolar macrophage; MAPKs; Collagen.

INTRODUCTION

Silicosis is a sort of destructive lung disease caused inhalation of crystalline silica, and is characterized by interstitial pulmonary fibrosis. Although many different cell types and cytokines have been implicated to have a role in fibrotic diseases, there is increasing evidence that Alveolar macrophages and fibroblasts play important roles in the progress of pulmonary fibrosis.

Quercetin (QE), one of the main flavone in human diets, has a variety of biological actions against many diseases, including ischemic heart disease, atherosclerosis, liver fibrosis, renal injury, and biliary obstruction[1-3]. In vitro, quercetin inhibits keloid fibroblast proliferation, collagen production and keloid contraction by suppressing transforming growth factor (TGF)‑β /Smad signaling[4]. In vivo, quercetin has been shown to improve liver histology and reduce collagen content in rats with carbon tetrachloride-induced cirrhosis[5]. However, the regulatory mechanism of signal transduction for quercetin inhibiting collagen synthesis in silicotic model has not been well determined. Thus, the current study was to address these unanswered questions.

MATERIALS AND METHODOLOGY

Materials

SiO_2 dust was provided by the National Institute of Occupational Health and Poison Control, Chinese Center for Disease Control and Prevention (Beijing, China). SiO_2 processed was suspended in Dulbecco's modified Eagle's medium (DMEM) without fetal calf serum (FCS) by 50 μg/mL and stored at 4°C. DMEM and FCS were bought from Tianjin Blood Institute(Tianjin, China). Quercetin (Sigma, USA) was suspended in dimethylsulfoxide (DMSO) and stored at -20°C. DMSO, p-ERK1/2,p-P38MAPK and p-JNK were purchased from Sigma. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Tianjin Hao Yang biological Co. Ltd.(Tianjin, China). Hydroxyproline Kit and Horseradish peroxidase conjugated secondary antibody were provided by Wuhan Boshide Biological Engineering Co. Ltd, China.

Methodology

Collection supematant of alveolar macrophages

The information of lung lavage was obtained from the medical record of Beidaihe Sanatorium for China's Coal Miners. All subjects signed informed consent before lung lavage. All subjects were 20 years duration of silica exposure,and without other complications. Silicosis was diagnosed stage Ⅲ by local pnumoconiosis diagnosis groups under China's"diagnostic criteria of pneumoconiosis"(GBZ70-2009). Patients were under general anesthesia and physiological salaine was perfused to the lung. The lavage fluid was collected. The mucus was removed using sterilized double gauze. After filtration, the fluid was centrifuged,washed three times with PBS,the total cell number was counted. $5x10^6$AM was added to DMEM medium in six-well cultural plate, adherent culture 2 hours at 37°C for purifying. After replacement of medium,the AMs were cultured for another 24 hours. Meanwhile, the morphological characteristics of AMs was observed under optical microscope(HE staining and Wright staining and acid-fast stain),which was excluded lung-related diseases such as pneumonia,lung cancer,active tuberculosis,etc. Then, AMs were divided into 2 groups and cultured for 18 hours: 1) supematant Ⅰ: only treated with DMEM; 2) supematant Ⅱ: instilled of 50 μg/mL SiO_2 and treated with DMEM. After that, supematant was collected and stored at -20°C and -70°C,AMs were harvested and lysed.

Preparation and cuture of Fibroblasts

Human embryonic lung fibroblasts were collected according to a previously described procedure[6]. Cells were maintained in a complete culture medium DMEM supplemented with 10%-FBS,100 U/ml penicillin/streptomycin in a 37°C incubator with 50 mL/L CO_2. At confluence, cells were split with 0.25% trypsin at the ratio 1:3 every passage. Cells from 4 to 9 passages were used in this study. When cells reached sub-confluence, they were cultured in DMEM containing 0.5% FBS for 24 hours and most cells were in quiescent state. Then, they were divided into 6 groups: 1) control (10%-FBS DMEM 400 μL); 2) AM (supematant Ⅰ 200 μL+20%-FBS DMEM 200 μL); 3) SiO_2+AM (supematant Ⅱ 200 μL+20%-FBS DMEM 200 μL); 4) Que (supematant Ⅱ+20%-FBS DMEM +quercetin): final concentration quercetin was 10,20,40 μmol/L, 10%-FBS DMEM. Cells were cultured for 30min and harvested. After 48 hours supematant was collected and stored at -70°C.

Cell viability and proliferation

To study the effects of quercetin on cell proliferation and viability, according to the arrangement groups, fibroblasts ($5x10^3$/well) were plated in 96-well plates and cultured for 18 hours. After that, cells were washed once with medium, supplemented with 10% FCS containing 20μl MTT (5 mg/ml), for 4 h. The culture solution was swilled, 150μl DMSO was added to each well and the solution was subsequently shaken to completely dissolve the blue-purple precipitate obtained from MTT. A microplate reader (BIO-RAD company) was used to test the absorbance (A) of each well at 540 nm and average values were obtained. Experiments were repeated ≥3 times and data are presented as the mean±SD.

Western blot analysis

The cells were cultured for 30min and rinsed with 0.01 M PBS three times, then were lysed by lysis solution (200 μL) with freshly prepared for 30 min. The homogenates were centrifuged for 15 mins at 4°C 12000r/min and supematant was collected. The total protein content was quantified by a protein assay (PC0020, Solarbio; China). The proteins (80 mg/lane)

were separated in 10% gel by SDS-PAGE and electro-transferred to a nitrocellulose membrane(180mA,1h). Membranes were blocked with 5% non-fat milk and incubated overnight at 4°C with the primary antibody [anti-phosphorylation-ERK1/2, anti-phosphorylation-P38MAPK,anti-phosphorylation-JNK] followed by alkaline phosphatase-conjugated secondary antibodies. Target bands were visualized by addition of DAB. Results were normalized with total protein.

Assessment of collagen by hydroxyproline kit

Human embryonic lung fibroblasts in 6 groups were cultured for 48 hours and supematant was collected for 0.5 ml in every group. The concentration of Hydroxyproline in supematant was determined by Hydroxyproline Kit following the suggested manufacturer's protocol. The collagen content (ug /ml) were calculated for 13% with the mass percent concentration of hydroxyproline.

Statistical analysis

Values were expressed as mean±SEM. All statistical analyses were performed using the SPSS software, version 16.0. Comparisons between multiple independent groups were conducted using one-way ANOVA followed by post hoc test. Group differences resulting in p-values of less than 0.05 were considered to be statistically significant

RESULTS

Effect of quercetin on the proliferation of HELF

TABLE 1 : Effect of Quercetin on the proliferation of HELF Activated by the Silicotic Alveolar Macrophages($\bar{x} \pm s$,n=5)

Groups	OD
control group	0.414±0.062*
AM group	0.490±0.064$^{\blacktriangledown *\triangle}$
SiO_2+AM group	0.620±0.096$^{\blacktriangledown \triangle}$
Que10 group	0.448±0.075$^{*\triangle}$
Que20 group	0.406±0.070*
Que40 group	0.334±0.050*

$^{\blacktriangledown}P<0.05$ vs control group; * $P<0.01$ *vs* SiO_2+AM group; $^{\triangle}P<0.01$ *vs* Que40 group

As shown in TABLE 1, compared with the control group, AM group and SiO_2+AM group promoted HELFs proliferation significantly, especially SiO_2+AM group from the overall trend. While this effect was significantly reversed by quercetin, and the effect was obvious with more quercetin. The result were 72.3%,65.5%,53.9% of that in SiO_2+AM group, respectively. The proliferation of HELFs in Que20 group and Que40 group was decreased significantly compared with AM group. But the results were no significant in quercetin groups and the control group. We can see that quercetin can inhibit the proliferation of HELF and the higher the concentration inhibition stronger.

Collagen content in supematant of HELF

We next examined expression of Hydroxyproline in supematant of HELF. As we all know that fibrosis is characterized by collagen deposition, and the HP content reflects the proportion of collagen fibers. The supematant of HELF in the control group contained 15.553±0.995μg collagen/ml (TABLE 2). The collagen content began to increase significantly in AM group and AM+SiO_2 group. The collagen content in AM+SiO_2 group was increased by 1.33 fold compared with AM group, by 2.48 fold compared with control group, respectively. Quercetin noticeably reduced the collagen, especially in Que20 group and Que40 group, which was 72.15%, 47.76% of that in SiO_2+AM group, respectively.

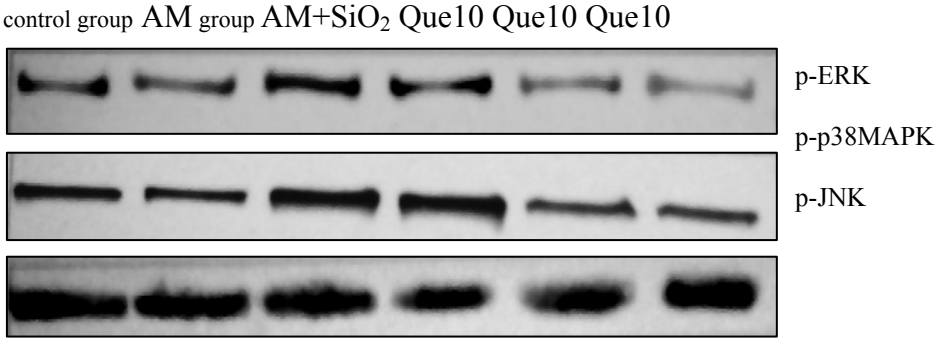
TABLE 2 : Collagen content in supematant of HELF($\bar{x} \pm s$) （n=5）

Groups	Collagen content
control group	15.553±0.995
AM group	28.984±0.599*
AM+SiO_2 group	38.615±0.710$^{*\triangle}$
Que10 group	35.107±0.576*
Que20 group	27.861±0.551$^{*\#}$
Que40 group	18.442±0.460$^{*\#}$

P<0.01 vs control group;$^{\triangle}$P<0.01 vs AM group; $^{\#}$ P<0.01 vs AM+SiO_2 group

Effect of Quercetin on MAPKs Signaling in HELF

Western Blot results revealed that ERKs/JNKs/p38MAPK levels in HELF were significantly elevated in AM group and AM+SiO_2 group, especially in AM+SiO_2 group, and inhibited by quercetin (Figure 1). As shown in Figure 1, the expressions of ERKs, JNKs, and p38MAPK in AM+SiO_2 group increased by 2.69, 3.46 and 2.97 fold, respectively, compared with the control group; and increased by 2.02, 2.07 and 2.03 fold, as compared with the AM group. The up-regulation of ERKs, JNKs, and p38MAPK observed was significantly reversed by 20μmol/L quercetin by 62.26%, 58.09% and 62.62% of AM+SiO_2 group, respectively; by 68.24%, 65.09% and 72.42% of AM group, respectively. Quercetin 40μmol/L decreased the expression of ERKs, JNKs, and p38MAPK by 38.68%, 61.83% and 53.85% of AM+SiO_2 group, respectively; by 42.64%, 71.87% and 62.84% of AM group, respectively.



DISCUSSION AND CONCLUSIONS

Although the underlying basis of fibrosis is unclear, there is increasing evidence that Alveolar macrophages and fibroblasts play important roles in the progress of silicosis. After deposition, SiO_2 is engulfed by the macrophages. However, silica is toxic to the macrophages, leading to cell damage, death and liberation of free silica which is subsequently taken up by other macrophages. This recurring cycle of macrophage phagocytosis perpetuates the silicosis process[7]. Furthermore, those macrophages are being activated to many inflammatory mediators which will intensify the chronic inflammation[8]. Activated macrophages also secrete cytokines to promote pulmonary fibroblasts to proliferate and synthesize excess collagen, which directly contributes to formation of silicotic nodules and interstitial fibrosis[9] We have previously shown that transforming growth factor-β (TGF-β)/platelet-derived growth factor(PDGF)/tumor necrosis factor-α (TNF-α)/ interleukin-1(IL-1) were important cytokines released by Macrophages after cultured in vitro[10]. The training process of macrophages in vitro may be accumulation of growth factors. The culture supernatant of Macrophages at different time points can promote significantly fibroblast proliferation, compared with the control group, especially at 18 hour. Therefore, we collected the supernatant of Macrophages after cultured 18 hour as stimulating factors.

Quercetin is a dietary flavonoid ubiquitous in nature. A significant number of chemical properties and pharmacological effects have been attributed to this agent[11,12]. Quercetin has been shown to have anti-inflammatory properties in various in vitro and in vivo systems, in hepatic cirrhosis, and in pulmonary influenza virus infection[13-15]. Quercetin has also been reported to have an antiproliferative effect on cultures of fibroblast cells obtained from mice and from human keloids[16,17]. The results of the present study show that quercetin inhibited proliferation of HELF and collagen synthesis in silicotic model in vitro.

Quercetin also has inhibitory effects on several proteins, such as tyrosine and serine/threonine kinases, including MAPKs[18]. The MAPK super-family is composed of three major sets of kinases: the extracellular-receptor kinases (ERK), the c-Jun N-terminal kinases/stress-activated protein kinases (JNK/SAPK) and the p38 MAPKinases. p44/42 (ERK1/2), p38, and JNK/SAPK play a critical role in the regulation of cell growth and differentiation and in the control of cellular responses to cytokines and stress. MAPK pathways play a role in the progression of fibrosis[19]. In this study, the results showed that QE markedly decreased the levels of p44/42 (ERK1/2), p38, and JNK,which may indicate the machine that quercetin inhibited collagen synthesis and proliferation of HELF. QE seems to be a potent nephroprotective drug and its use in maintaining a healthy lung and preventing silicosis fibrosis deserves consideration and further examination.

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