In vitro Study of Flavonoids, Fatty Acids, and Steroids on Proliferation of Rat Hepatic Stellate Cells

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There is a wealth of evidence that hepatic stellate cells (HSCs) orchestrate most of the important events in liver fibrogenesis. After liver injury, HSCs become activated to a profibrogenic myofibroblastic phenotype and can regulate net deposition of collagens and other matrix proteins in the liver. The proliferation of HSCs is mainly stimulated by the platelet-derived growth factor (PDGF). In this study, some compounds from natural resources have been tested for their activity to inhibit PDGF-driven proliferative activity of rat HSCs. Apigenin, quercetin, genistein, daidzin, and biochanin A exhibited > 75% inhibitory activity against HSC-T6. It was found that, γ-linolenic (γ-Ln), eicosapentanoic (EPA) and α- linolenic (α-Ln) acids showed a high inhibitory effect on proliferation of rat HSCs at 50 nmol/l. Cholest-4-ene-3,6-dione and stigmastone-4-en-3,6-dione are the most active steroids with inhibitory activities > 80% and this is most likely due to the presence of the 4-en-3,6-dione moiety in both compounds. These results revealed that the compounds which effectively blocked HSC proliferation may be beneficial in liver fibrosis. Structure-activity relationships (SAR) may provide a basis for rational structure modification.

Key words: Hepatic Stellate Cells, Steroids, Liver Fibrosis

Introduction

Hepatic stellate cells (HSCs) play a central role in the liver fibrogenesis. During fibrosis, HSCs are activated and undergo a phenotypic change to myofibroblasts, which are highly proliferative and synthesize most extracellular matrix components. The proliferation of HSCs is mainly stimulated by Kupffer/macrophage cell conditioned medium (MCM), especially by the platelet-derived growth factor (PDGF) (Kawada, 2001; Nieto and Friedman, 2002).

Thus, the development of specific inhibitors which can effectively block HSC proliferation are of particular therapeutic interest for hepatic fibrosis. Many reports consider flavonoids as phytoestrogens possessing a free radical scavenging activity, inhibiting apoptosis or tyrosinase (Badria *et al.*, 1996; Badria and El-Gayyar, 2001), antioxidant (Mikhaeil *et al.*, 2004) and hepatoprotective properties (Badria *et al.*, 1994). These remarkable pharmacological actions suggest that some flavonoids may have an unique antifibrotic activity. The role of free fatty acids on proliferation of rat hepatic stellate cells was examined (Lu *et al.*, 1998).

Here, HSC-T6 cells, a myofibroblasts line, were used as target cells and the effects of selected flavonoids (flavones, flavonols, flavanones, anthocyanidins, and isoflavones), fatty acids, and steroids on proliferation of rat HSCs proliferation in response PDGF were tested to provide a theoretical basis for further studies.

Results and Discussion

Activated, but not quiescent, hepatic stellate cells (HSCs) have a high level of collagen and α-smooth muscle actin (α-SMA) expression (Lee *et al.*, 2001). The proliferation of HSC was influenced by many factors, such as cytokines. Manipulation of these cytokines may constitute a significant new approach in the modulation of liver fibrosis by blocking the actions of these cytokines. HSC-T6 cells stimulated with PDGF were used as an experimental model for screening the agents that will effectively block HSC proliferation

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(Friedman, 1997). In this model, the stimulation by PDGF may reflect the effects of the blood bioactive factors, the fibrogenic cytokines released by activated macrophages in local environment and the most mitogenic factor PDGF on HSC proliferation. Colchicine is a well-known anti-fibrotic drug in clinical treatment (Kerchenobich *et al.*, 1988). It was tested in this model as a positive control. The data demonstrated that colchicine could inhibit the proliferation of HSC-T6 cells stimulated by PDGF. It suggests that this model sounds feasible for screening anti-fibrotic agents *in vitro*.

The obtained results (Table I) showed that five flavonoids; apigenin, quercetin, genistein, daidzin, and biochanin A, reduced the increased proliferation of HSC-T6 derived by PDGF. Comparing the structure features of these compounds, we found that the 4′-hydroxy group was closely related to high activity. When the 4′-hydroxy group was missing or when it was changed to 4′-methoxy their activities markedly decreased. Secondly, it did not matter if a 1,4-benzopyranone ring moiety existed or not since similar activity was observed in both groups. These results indicate that the moiety 1,4-benzopyranone was not essential for their biological activities. PDGF is one of the important components in serum and accounts for 50%–70% of the total macrophage-derived mitogenic activity. The results in this report show that the compounds with high activities inhibited proliferation of HSC-T6 cells at the highest concentration with inhibitory rates of about 100%–120% when stimulated by PDGF. All the above results suggest that inhibition of HSC proliferation by 5 flavonoids mainly arose from the blocking of the proliferation action induced by PDGF.

The results revealed that flavonoids, which are considered as natural phytoestrogens, fatty acids, a precursor for lipid peroxidation, and steroids can effectively inhibit the proliferation of PDGF-stimulated HSCs. Accordingly, we can conclude that these compounds may serve as potential natural anti-fibrotic agents. Their activity will be evaluated *in vivo*.

In conclusion, the obtained results showed that these compounds effectively blocked HSC prolif-

Table I. Effect of some natural flavonoids on proliferation of PDGF-stimulated HSCs.

Compound	OH substitution	Glycosylation	A_{595}*	Inhibition of proliferation (%)
Negative control	– –	– –	0.59 ± 0.02	– –
Positive control	– –	– –	0.90 ± 0.05	– –
Flavones				
Luteolin	5,7,3′,4′		0.83 ± 0.08	22.58
Luteolin-4′-glucoside	5,7,3′	4′-glucose	0.89 ± 0.07	3.23
Apigenin	5,7,4′		0.64 ± 0.02	83.87
Chrysin	5,7		0.89 ± 0.05	3.23
Luteolin-5,7-diglucoside	5,4′	3′,7-diglucose	0.89 ± 0.05	3.23
Flavonols				
Quercetin	3,5,7,3′,4′		0.63 ± 0.06	87.10
Myricetin	3,5,7,3′,4′,5′		0.81 ± 0.04	29.03
Morin	3,5,7,2′A′		0.86 ± 0.07	12.9
Rutin	5,7,3′,4′	3-rutinose	0.81 ± 0.03	29.03
Kaempferol	3.5,7,4′		0.81 ± 0.02	29.03
Flavanone				
Naringenin	5,7,4′	4′-OCH_3	0.81 ± 0.04	29.03
Hesperetin	3,7,3′	4′-OCH_3	0.72 ± 0.03	58.06
Hesperedin	5,3′	7-rutinose	0.82 ± 0.07	25.81
Anthocyanidin				
Delphinidin	3,5,7,3′,4′,5′		0.82 ± 0.03	25.81
Cyanidin	3,5,7,3′,4′	3′,5′-di-OCH_3	0.82 ± 0.02	25.81
Malvidin	3,5.7,4′	3′ 5′-di-OCH_3	0.82 ± 0.05	25.81
Isoflavones				
Genistein	5,7,4′	7-glucose	0.64 ± 0.02	83.87
Genistin	5,4′	7-glucose	0.74 ± 0.04	51.61
Daidzin	4′	4′-OCH3	0.67 ± 0.03	74.19
Biochanin A	5,7	–	0.67 ± 0.07	74.19

* A_{595}, absorbance at 595 nm.

Table II. Effect of free polyunsaturated fatty acids on proliferation of HSCs-T6 (50 nmol/l).

Compound	A_{595}*	Inhibition (%)
Arachidonic acid (AA)	0.89 ± 0.05	3.7
Linoleic acid	0.86 ± 0.03	14.8
γ-Linolenic acid (γ-Ln)	0.72 ± 0.04	66
Eicosapentanoic acid (EPA)	0.76 ± 0.03	51.9
α-Linolenic acid (α-Ln)	0.74 ± 0.04	89.3

* A_{595}, absorbance at 595 nm.

Table III. Effect of steroid compounds on proliferation of HSCs-T6 (50 nmol/l).

Compound	A_{595}[a]	LC_{50} [μg/ml]
1	0.89 ± 0.05	11.7
2	0.86 ± 0.03	13.8
4	0.74 ± 0.04	90.5
5	0.72 ± 0.04	56
7	0.76 ± 0.03	51.9
6, **8**	0.82 ± 0.02	19.6
9	0.74 ± 0.04	87.4
C	0.90 ± 0.04	–
M	0.60 ± 0.07	–

[a] A_{595}, absorbance at 595 nm.

eration and they may by beneficial in liver fibrosis. The relationship of structure-bioactivity may provide a basis for rational structure modification.

A preliminary screening was conducted to disclose the toxicity of the tested polyunsaturated fatty acids (PUFAs) and natural oils on HSCs-T6. Eicosapentanoic acid exhibited the highest cytotoxicity (LC_{50} 6.25 μg/ml) among all other tested PUFAs. It was found that, arachidonic acid (AA) and linoleic acid had an effect on proliferation of HSCs, 25 nmol/l of AA promoted HSCs proliferation, but 50 and 100 nmol/l had an inhibitory effect and showed cytotoxicity on HSCs (Table II).

The lipid composition plays an important role in the structural and metabolic functions of cell membrane in particular the production of inflammatory mediators such as prostaglandins and leukotrienes (Fennekohi *et al.*, 2000; Grimble and Tappia, 1998).

The results indicated that compounds **4** and **9** showed the highest inhibitory activity on the proliferating HSCs (Table III). The main features of these compounds are the presence of a 4-en-3,6-dione.

Experimental

Materials

HSC-T6, a myofibroblast line, which had the stable phenotype and biochemical characters, was kindly provided by Dr. S. Friedman as a gift (Mountain of Sinai Hospital, NY, USA). Flavonoids (some isolated in our lab and others) and fatty acids (arachidonic, linoleic, α- and γ-linolenic and eicosapentanoic acids) were purchased from Sigma (St. Louis, MO, USA), and the tested steroids were prepared by hydroxylation of both cholesterol and stigmasterol with *n*-bromosuccinimide to the corresponding 5α,6β-diols followed by oxidation and dehydration reactions. Their structures were established by IR, UV, and mass spectroscopy (Table IV) (Dawidar *et al.*, 1980).

Table IV. List of steroid derivatives tested for blocking HSCs-T6 proliferation.

Compound	Composition	M.p. [°C]	M^+	IR absorption bands [cm^{-1}]
5α-Cholestone-3β,5-diol-6-one-3-acetate (**1**)	$C_{29}H_{48}O_4$	233	460	3400 (OH), 1730, 1270 (acetate), 1710 (> C=O)
5α-Cholestone-3β,5-triol-3,6-diacetate (**2**)	$C_{31}H_{52}O_5$	160	– –	3395 (OH), 1730, 1250 (acetate)
5α-Cholstane-5-ol-3,6-dione (**3**)	$C_{27}H_{44}O_3$	243	– –	3300 (OH), 1710 (> C=O)
Cholest-4-en-3,6-dione (**4**)	$C_{27}H_{42}O_2$	118	398	1690, 1600 (-C=C-CO)
5α-Stigmastone-3β,5-diol-6β-triol (**5**)	$C_{29}H_{52}O_3$	250	– –	4420 (OH)
5α-Stigmastone-3β,5-,6β-triol-3,6-diacetate (**6**)	$C_{33}H_{56}O_5$	157	– –	3430 (OH), 1730, 1250 (acetate)
5α-Stigmastone-5-diol-6-one (**7**)	$C_{29}H_{50}O_3$	251	446	3400 (OH), 1710 (> C = O)
5α-Stigmastone-3β,5-diol-6-one-3-acetate (**8**)	$C_{31}H_{52}O_4$	248	– –	3400 (OH), 1730, 1270 (acetate), 1710 (> C=O)
Stigmastone-4-en-3,6-dione (**9**)	$C_{29}H_{46}O_2$	160	426	1690, 1605 (-C=C-CO)

Methods

The cells were cultured in Dulbeco Medium Eagle (DME) with 10% calf serum at 37 °C in a humidified atmosphere of 5% CO_2 + 95% air (v/v).

HSC-T6 cells (1×10^4 cells/well) were plated in a 96-well microplate for 24 h. The cells were then incubated in DME with 10% calf serum in the presence of serial concentrations of the tested compounds. For the untreated controls, an equal amount of compound-solvent (DME containing 0.1% dimethylsulfoxide) was added.

After a 48 h incubation, the cell density was measured by the crystal violet assay and expressed as A_{595}. In PDGF-stimulated proliferation, the medium was removed after a 24 h incubation.

Finally DME with 0.4% calf serum was added for another 48 h. Compounds and DME (10 μg/l) were added into medium. After 24 h, the cell density was measured. The inhibitory rate was calculated according the equation: Inhibition % (PDGF) = $(A_{\text{control}} - A_{\text{compound}})/(A_{\text{control}} - A_{\text{medium}})$, where A_{control} is the absorbance of equal amount of compound-solvent (DME containing 0.1% dimethylsulfoxide), A_{compound} is the absorbance of dissolved compound in DME and 0.1% dimetylsulfoxide and A_{medium} is the absorbance of DME.

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