Naringenin Exerts Its Anti-Tumor Effect Via Antiangiogenesis

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Summary

Naringenin inhibited the proliferation and induced apoptosis in human non-small cell lung carcinoma (A549) cells and human umbilical vein endothelial (ECV304) cells. The development of microvessel structures in ECV304 cells and VEGF mRNA expression were inhibited by naringenin in a dose-dependent manner. Naringenin was also able to prevent A549 cell migration and invasion in vitro and to inhibit Lewis Lung Carcinoma (LLC) growth in the subcutaneous mouse model.

Introduction

Naringenin (4,5,7-trihydroxyflavanone), a natural predominant flavanone, has a wide range of pharmacology properties, such as anticancer [1], antimutagenic [2], and anti-inflammatory [3]. However, its mechanisms by which inhibits tumor growth remain unclear. Here, we report for the first time that naringenin exerts its anti-tumor effect by the inhibition of angiogenesis.

Methods

Cell Viability Assays The effects of naringenin on ECV304 and A549 cell viability were assessed by standard MTT assays. After 24, 48 or 72 hours treatment of the cells with naringenin, cell viability was determined by measuring the converted formazan at 590 nm using a plate reader (Bio-Rad 3550).

Annexin V/PI staining Cells were plated in 6-well plates and exposed to naringenin for 48 hours. Propidium iodide (PI) and FITC-conjugated Annexin V double-stained kit (Jingmei Biotech Co., China) was used to stain cells

according to the manufacture's instructions and analyzed by flow cytometry.

RT-PCR Total RNA was isolated from cells treated with naringenin for 24h using TRIzol (Invitrogen, USA) and reversely transcribed to cDNA. Primers for human VEGF were 5'-TCGGGCCTCCGAAACCATGA-3' and antisense primer 5'-CCTGGTGAGAGATCTGGTTC-3'. Primers for â-actin, which as the control, were sense primer 5'-CATGTACGTTGCTATCCAGGC-3' and antisense primer 5'-CTCCTTAATGTCACGCACGAT-3'.

In vitro angiogenesis assay The effect of naringenin on angiogenesis was assessed through the development of microvessel structures in ECV304 using an angiogenesis assay kit (Chemicon) according to the manufacturer's instructions.

In vitro migration and invasion assays A549 cell migration was analyzed using the cell culture inserts (pore size 8 ìm, Millipore). The invasive properties were analyzed by using the inserts covered with ECMatrix. For both assays, 100 µl of cell suspension (1×10^5 cells) was added to the upper wells and indicated concentrations of naringenin were added to the lower wells. After incubated the chambers at 37°C for 24 h, cells on the lower side of the filter were stained with 0.1% crystal violet and photographed. Then the cell-bound crystal violet was quantitated by spectrophotometric analysis at 570 nm.

In vivo suppression of tumor by naringenin Mice were injected subcutaneously in the right flank of C57Bl/6 female mice with 0.2 ml of cell suspension containing 5×10^5 LLC cells. Mice (eight in each group) received naringenin (100 mg/kg body weight/day), or vehicle (2%) by oral gavage once per day for 15 days, starting on the day after tumor implantation. Tumors were measured using vernier caliper twice a week and calculated as $(L\times W^2)/2$, where L is the length in millimeters and W is the width in millimeters.

Results

Inhibition of viability by naringenin in endothelial and tumor cells As shown in Fig. 1, the proliferation of ECV304 and A549 cell lines was inhibited by naringenin in a dose and time dependent manner. The viability of both ECV304 and A549 cells was slightly influenced by naringenin at 200 µM for 24h. Therefore, the concentration of naringenin was lower than 200 µM that was used in all subsequent experiments only except for the analysis of apoptosis.

Induction of apoptosis by naringenin Compared to control, the apoptotic cells including the early and late apoptosis were significantly increased after treatment with 500 µM naringenin for 48h in both ECV304 and A549 cell lines (Table 1).

Inhibition of angiogenesis by naringenin Naringenin markedly decreased

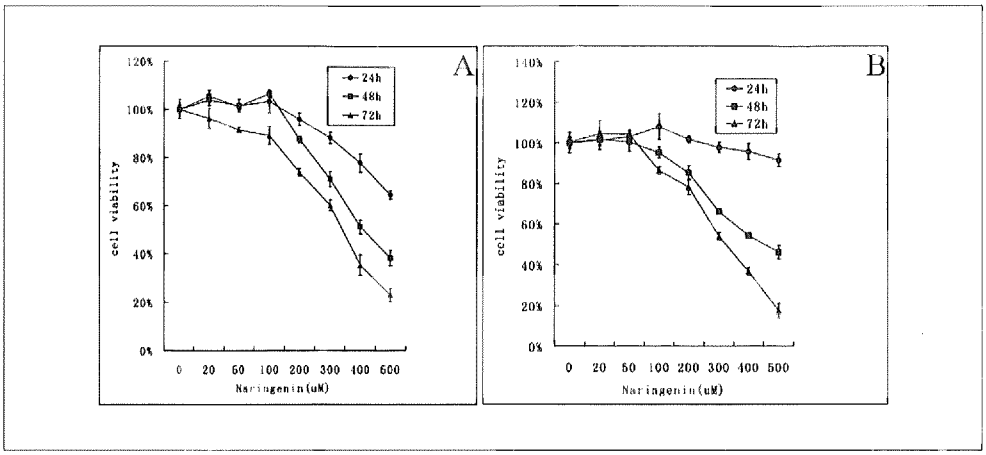

*Fig.1 Effect of naringenin on the viability of A549 (**A**) and ECV304 cells (**B**). Bars: ± SD.*

*Table.1 Naringenin induces apoptosis in different cell lines. Data are expressed as mean ±SD (n = 3). ** P < 0. 01, ***P < 0.001 compared with the group of control.*

Cell line	Apoptotic cells (%)		
	Control (2.5‰DMSO)	Naringenin (200μM)	Naringenin (500μM)
ECV304	5.12±0.37	7.46±1.02	21.25±1.98**
A549	4.39±0.73	6.03±0.81	30.34±3.56***

VEGF mRNA transcription in a dose-dependent manner in A549 and ECV304 cells (Fig. 2A & B). Compared to the well-formed neovascularization of

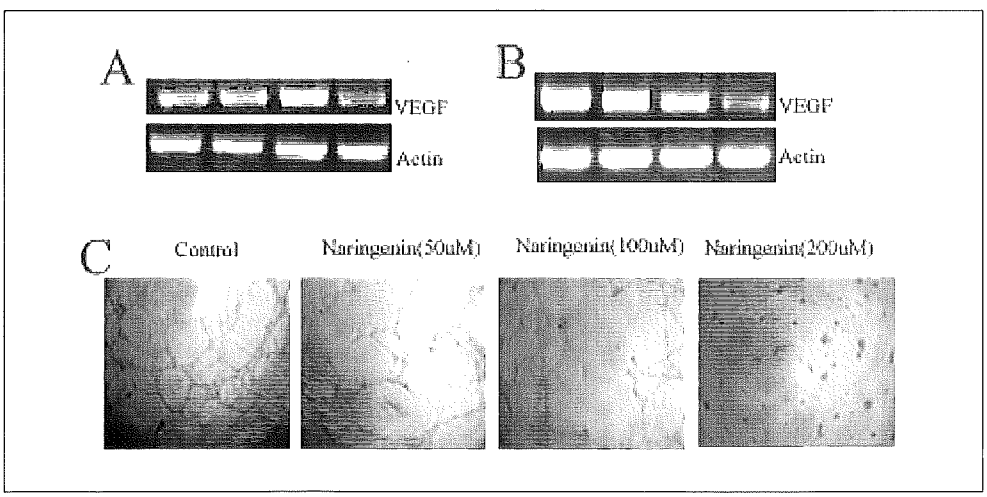

*Fig.2 Naringenin inhibits VEGF mRNA expression in A549s (**A**) and ECV304s (**B**), and the tube formation by ECV304s. Photographs of tube formation (×40) were taken (**C**).*

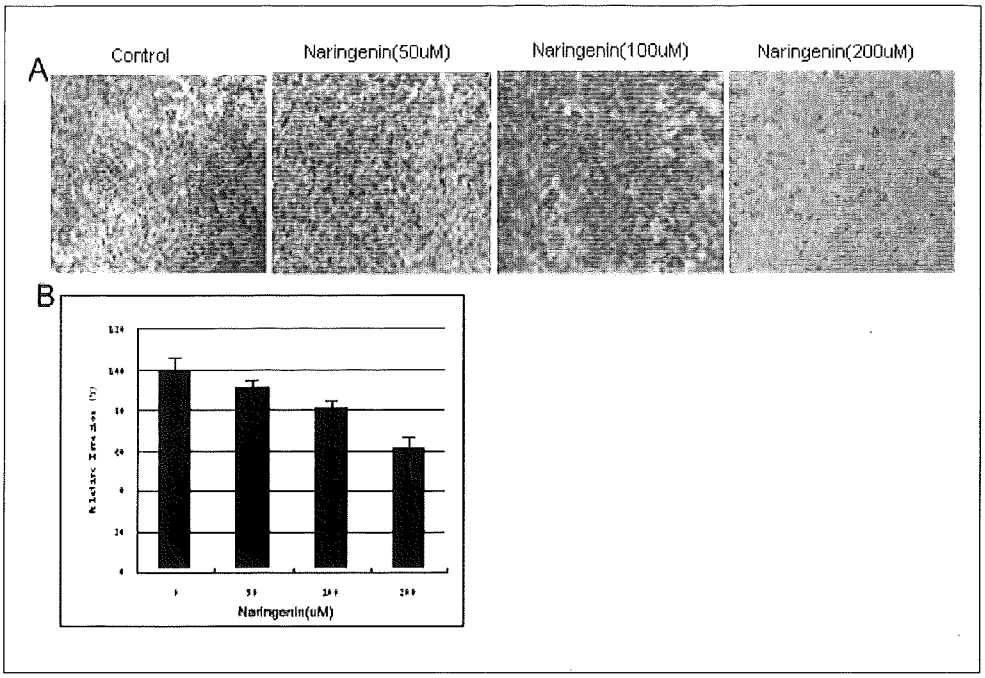

*Fig.3 Naringenin inhibits the migration and invasion by A549 cells. Photographs of migrated A549 cells (× 100) were taken (**A**). Relative invasion rate was compared to the control group (**B**). Bars: ± SD.*

control, the net-work structures appeared incomplete and broken, and the tube formation could hardly been observed when the ECV304 cells were treated by 200 μM naringenin (Fig. 2C).

Inhibition of migration and invasiveness by naringenin in vitro As shown in Fig. 3A, contrasted with the control, 200 μM naringenin significantly inhibited A549 cell migration. Using ECMatrix as extracellular matrix components, we observed cell invasion was inhibited by naringenin at 50, 100 and 200 μM by 91%, 80%, and 61%, respectively (Fig. 3B).

Suppression of tumor growth in vivo In mouse model of subcutaneous inoculation of LLC cells, the average tumor volume of naringenin treatment group was much smaller than that of the control group ($p<0.050$, Fig. 4).

Conclusions

We report here that naringenin exerts its anti-tumor effect by the inhibitory activity against VEGF expression. The results provide a potential application

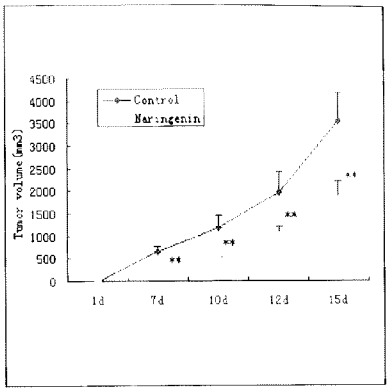

*Fig.4 Effect of naringenin on tumor growth in mice bearing lewis lung carcinoma. ** P < 0. 01, Bars: ± SD.*

for naringenin to the anti-cancer effects through antiangiogenesis in cancer therapy.

References

[1]. Kanno S, Tomizawa A, Hiura T, Osanai Y, Shouji A, Ujibe M, Ohtake T, Kimura K, Ishikawa M. Inhibitory effects of naringenin on tumor growth in human cancer cell lines and sarcoma S-180-implanted mice. Biol. Pharm. Bull. 28: 527-530. (2005)

[2]. Bear WL and Teel RW. Effects of citrus flavonoids on the mutagenicity of heterocyclic amines and on cytochrome P450 1A2 activity. Anticancer Res. 20:3609-3614. (2000)

[3]. Lin HY, Shen SC, Chen YC. Anti-inflammatory effect of heme oxygenase 1: glycosylation and nitric oxide inhibition in macrophages. J. Cell Physiol. 202:579-590. (2005)