

Whether Carotenoids and Quercetin Affect the Proliferation and Expression of PPAR γ Protein in Leukemia K562 Cells

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Abstract

Carotenoids are some of the most abundant pigments in nature, ranging from yellow to red. Carotenoids can divide into two categories: carotenoids composed of hydrocarbon elements and lutein-containing oxygen elements. They are lipid-soluble pigments, and some contain carbonyl or enol groups, which are water-soluble. Carotenoids absorb visible light from 400 nm to 600 nm, while special ones such as octa-hydro lycopene and hexahydrolycopene only absorb ultraviolet light. Quercetin is a flavonoid compound widely found in fruits and vegetables. Its chemical structure phenolic hydroxyl group is the basis of antioxidant action, which can be used for hydrogen to quench oxygen free radicals. It is oxidized to form a highly stable catechol structure. It has a variety of biological activities and pharmacological effects, such as expanding coronary blood vessels and inhibiting tumor cell proliferation. This paper shows that the K562 cell growth rate in carotenoids and quercetin treatment groups was slower than the control group.

Keywords: *carotenoids, PPAR γ protein, K56 cells*

1. Introduction

Carotenoids are important biological antioxidants and natural pigments. It is a 40-carbon isopentane-like polymer, or tetraterpene compound. Typical carotenoids are made from eight isoprene units end to end. The color of carotenoids varies with the number of conjugated double bonds [1]. The yellow, orange-red or red pigments commonly found in animals, higher plants, fungi and algae have been shown by further cell culture and animal experiments to play an important role in the prevention and suppression of cancer, as well as other dietary carotenoids.

Quercetin, a flavonoid compound, is a plant-based flavanol found in plants such as fruits, vegetables and grains. It has a wide range of pharmacological effects such as anti-tumor, anti-oxidation, anti-virus, lowering blood pressure, anti-arrhythmia, anti-platelet aggregation and so on, with low toxicity and side effects. In vitro cell studies in the laboratory have shown that mistletoe can also turn into a carcinogen, but it is not true and this study did not report an increased risk of cancer in animals or humans [2,3,4]. In animal studies, mistletoe has been speculated to reduce the risk of certain cancers [5,6]. An eight-year study found that three flavonoids -- kaempferol, mistletoe and myricetin -- reduced the risk of pancreatic cancer in smokers[7].

PPAR γ , mainly expressed in adipose tissue and immune system, is closely related to adipose cell differentiation, body immunity and insulin resistance, and is the target molecule of insulin sensitizer troglitazone (TZDs). Peroxisome proliferator-activated receptor γ (PPAR γ) is

one of the important factors regulating the development of cancer. Studies have shown that PPAR γ controls cell proliferation in various tissues and organs, including colon and breast, and dysregulation of PPAR γ signaling is associated with tumor development in these organs [7]. What's more, Carotenoids have antagonistic effects on the genes of some carcinogenic agents. Carotenoids have inhibitory effects on the initiation, promotion and invasion stages of cancer

1.1. Hypothesis

In this study, I predict that treatment of K562 CML cells with different concentrations of carotenoid and quercetin (0.5 μ mol/L, 1.0 μ mol/L, 5.0 μ mol/L, 10.0 μ mol/L, 20.0 μ mol/L) for various durations will kill the cells by apoptosis and reduce size of K562 xenograft tumors. Measure killing by MTT and annexin V/PI by FACS and tumor size in the xenografts and PPAR gamma levels. Positive control is taxol, negative control is PBS/DMSO

2. Methods

2.1. Materials

This experiment will use β -carotene; Capsaicin; Carmine orange; Quercetin; RPMI1640 medium; Fetal bovine serum; Cyanobacteria and streptomycin; Tris (Tris) analysis pure.; Dimethyl sulfoxide (DMSO); EDTA, Sodium bicarbonate, sodium dihydrogen phosphate and potassium dihydrogen phosphate were analytical pure grades. β -mercaptoethanol; Bromophenol blue; Acrylamide purchased from; Thiazole blue; N-[2-hydroxyethyl] piperazine N'-[2-ethane-sulfonic acid]

(HEPES); β -actin antibody and PPAR γ antibody; (2,4,6-Tripyridyl-S-Triazine, TPTZ); K562 cells (Chronic myelogenous leukemia cells).

2.2. Reagent preparation

RPMI medium: weigh and take RPMI-1640 6.7g, NaHCO₃ 1.0g, Penicillin 0.0305g, Streptomycin 0.076g, HEPES 1.19g, add 500mL fresh double-distilled water, adjust pH to 7.2-7.4, 0.22 μ m microfiltration membrane for sterilization, and CVD at -20 °C.

PBS buffer: weigh and NaCl 0.1g, NaCl 4.0 g, Na₂HPO₄ · 12 h₂O 0.71 g, KH₂PO₄ 0.135 g, and deionized water, 400 ml dissolved, adjustable pH 7.3 ~ 7.4 after constant volume to 500 ml, high temperature and high pressure sterilization and preservation at room temperature.

4% Trypan blue mother liquor: weigh 4g trypan blue, add a little distilled water to grind and dissolve, then add double distilled water to 100mL, filter it with filter paper, and store it at 4°C. When used, dilute to 0.4% with PBS buffer.

MTT solution: weigh 250mg MTT, add 50mL PBS solution, stir and dissolve for 30min, filter with 0.22 μ m microporous membrane for sterilization, and store at 4°C after packaging. The final concentration should not be less than 0.5mg/mL.

10% ammonium persulfate solution: take 0.05g ammonium persulfate, add water to 0.5mL.

Membrane transfer buffer: Weigh Glycine 2.9g; Tris 5.8 g; SDS 0.37g is fully dissolved by adding 800mL deionized water, and fully mixed by adding 200mL methanol.

1 \times Tris-Glycine electrophoresis buffer: Tris 1.51g; Glycine 9.4 g; SDS 0.5 g, keep the volume of deionized water to 500mL at room temperature.

2 \times SDS buffer solution: 100mmol/L Tris-HCl, containing 20% glycerol, 0.2% bromophenol blue, 4% SDS, 20 mmol/L DTT.

TBST buffer solution (Western hybrid film cleaning solution): Weigh 8.8g measure 1 mol/L Tris-HCl (pH 8.0) 20mL; 0.1% Tween 20 0.5mL, add deionized water constant volume to 1L.

PBST: Take 0.01 mol/L (pH 7.5) PBS 1000mL, add 0.5 mL Tween 20.

Protein inhibitor : 0.02% sodium azide; 100 μ g/mL PMSF; 1 μ g/mL Aprotinin; 1 μ g/mL Pepstatin A; 2 μ g/mL Leupeptin.

Single detergent cracking buffer : 50 mmol/L TRIS-HCl (pH 8.0); 150 mmol/L NaCl; 1% Triton X-100 or NP-40, plus protein inhibitor when used.

Lichun Hong S storage solution: Take Lichun Hong S 2G; Trichloroacetic acid 30g; Sulfosalicylic acid 30g; Add water to 100mL to dissolve and store, dilute the storage solution 10 times when using.

5% skim milk powder (sealer): Weigh 1g skim milk powder and add 20mL TBST to dissolve thoroughly.

SDS-PAGE separation glue: deionized water 6.9ml; 30% Acrylamide 4.0 mL; 1.5 mol/L Tris HCl (pH 8.8) 3.8 mL; 10% SDS 0.15 mL; 10% ammonium persulfate 0.15mL, TEMED 0.009mL; 10% ammonium persulfate and ① TEMED should be added last, and used now.

SDS - PAGE Concentrated glue: 4.1ml deionized water; 30% Acrylamide 1.0 mL; 1.0 mol/L Tris - HCl (pH 6.8) 0.75 mL; 10% SDS 0.06 mL; 10% ammonium persulfate 0.06mL, TEMED 0.006mL; 10% ammonium persulfate and TEMED are added last. Available now.

Cell resuscitation. Take out a tube of clocking K562 cells from liquid nitrogen, quickly put it into 37°C warm water, melt and centrifuge at 1000rpm, suck out supernatant, add it to 1mL medium blow and transfer it to culture bottle and add it to 10mL medium for cultivation.

Cell culture. After resuscitated, the cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum, 100U/mL penicillin and 100U/mL streptomycin, and grew in a 5%CO₂ incubator at 37°C.

Cell passage. K562 cells were subculture according to the conventional suspension cell culture method. The culture medium containing cells was transferred to a 10mL centrifuge tube, centrifuged at 1000 RPM for 5min, the supernatant was removed, and 10mL new culture medium was added to make cell suspension, which was inoculated into a new culture flask for culture.

Cryopreservation of cells. The CVD K562 cells overgrown are centrifuged, mixed with cryotherapy and then blown, transferred to the CVD tube (marked on the CVD tube), immediately put it in the freezer 4°C for half an hour and then -20°C for 2-3 hours, take it out and hang it over the liquid nitrogen overnight, the next day, put the CVD tube into the VAT in the liquid nitrogen.

2.3. Cell proliferation was detected by trypan blue staining

K562 cells were subculture according to the above method, and cells at logarithmic growth stage were collected. The cell density was adjusted to 5 \times 10⁵-10⁶ /mL and the cells were inoculated into 24-well plates. The cells were treated with carotenoid and quercetin at concentrations of 0.5 μ mol/L, 1.0 μ mol/L, 5.0 μ mol/L, 10.0 μ mol/L and 20.0 μ mol/L, respectively. All the operations were carried out in the dark. No drugs were added in the control group, but the corresponding content of THF (anhydrous ethanol for orange grade of Carmine tree and DMSO for quercetin group) was added, and 3 parallel holes were set for each concentration. The cells were cultured for 24h, 48h, 72h, 96h and 120h, respectively. After mixing 0.4% Trypan blue with cell

suspension at a ratio of 1:1, the number of living cells was counted under a microscope with a cell counting plate. Each group had three multiple Wells, and the average was taken to evaluate the effect of carotenoid and quercetin on cell proliferation.

2.4. The cell inhibition rate was detected by MTT(thiazolyl blue) method

The logarithmic growth phase cells were collected and the cell density was adjusted to $5 \times 10^5 - 10 \times 10^5$ /mL. The cells were then inoculated into 96-well plates and treated with carotenoid and quercetin at concentrations of 0.5 μ mol/L, 1.0 μ mol/L, 5.0 μ mol/L, 10.0 μ mol/L and 20.0 μ mol/L, respectively. The 96-well plates were placed in an incubator for 3 to 5 days, then 200 μ L of diluted MTT solution was added to each well, so that the final concentration of MTT in each well was 0.5mg/mL. The 96-well plates were placed in an incubator for further cultivation for 4 hours. The supernatant was carefully discarded by centrifugation, 150 μ LDMSO was added to each well, and the MTT reduction product was fully dissolved by horizontal vibration for 10 min. Then the absorbance value (OD value) was measured by BIO-RAD 3350 Microplate Reader at 570 nm. Results Cell inhibition rate =(CONTROL group OD value - treatment group OD value)/ control group OD value \times 100%.

2.5. Detection of PPAR γ protein expression in cells by Western-blotting

CVD K562 cells were collected and CVD (4-CVD C), washed with PBS for 3 times, and CVD was CVD with CVD single detergent lysis liquor (including albumin inhibitor) for 30 min, CVD was CVD at CVD 12000 \times g for 10 min, and CVD supernatant was collected and stored in the -20 $^{\circ}$ C refrigerator. Total Protein concentration was determined by PIERCE Biological BCA Protein Assay Kit(PIERCE, CA, USA). 10% polyacrylamide gel was prepared, and 30 μ g protein sample was taken from each well. After electrophoresis, the protein in the gel was transferred to the cellulose membrane for antigen and antibody reaction. The primary antibody was rabbit anti-human PPAR γ polyclonal antibody diluted at 1:1000. The secondary antibody was horseradish peroxidase labeled sheep anti-rabbit polyclonal antibody, which was 1: Finally, the substrate color was detected by chemiluminescence. The strip strength on the film was analyzed by NH Image [8].

2.6. Reagent Preparation

40 mmol/L hydrochloric acid solution, ferric chloride solution and 0.3 mol/L sodium acetate buffer solution (pH=3.6) were prepared, and placed in a dark place for reserve.

10 mmol/LTPTZ solution: 0.0312g TPTZ sample was weighed, and the volume was reduced to 10mL with 40mmol/L hydrochloric acid, and the TPTZ sample was stored in the refrigerator for later use.

FRAP working solution: Vo.3mol/L sodium acetate buffer solution: concentrated conical side V10mmal/L1FYZ concentration: V-cone side =10:1:1. Use it now.

2.7. Drawing of standard curves

Draw 2.5mL series FeSO₄ solution in concentrations of 100, 200, 300, 400, 500, 600, 800, 1000 μ mol/L, with 2.5ml10 Mmol /LTPTZ solution (prepared with 40mmol/LHCI) and 25mL300mmol/L acetate buffer (pH3.6), heated to 37 $^{\circ}$ C, the absorbance value was read at 593 nm. Draw a standard curve using the concentration of FeSO₄ solution as the horizontal coordinate and the light absorption value as the vertical coordinate.

2.8. Antioxidant capacity determination of carotenoids and quercetin

β -carotene (THF dissolved), capsaicin (THF dissolved), carmine orange (anhydrous ethanol dissolved), quercetin (DMSO dissolved) were prepared into 1.0 mmol/L solution, 0.2mL sample solution was absorbed, and 6mlTPTZ working solution was added, after mixing37 $^{\circ}$ C 10 min, 593 nm Absorbance \times Was measured. After the reaction, the blank was directly applied to TPTZ working solution (TPTZ colored has an absorbance value of A reaction curve) [9]. The oxidation activity of the sample was expressed as the number of millimoles FeSO₄ needed to achieve the same absorbance. Conical side by side AE side -A conical side. The difference between the obtained carotenoids on the standard curve and quercetin corresponding FeSO₄ the concentration of the.

2.9. Determination of antioxidant capacity of carotenoids and quercetin combined with them

β -carotene, capsaicin, carmine orange, quercetin 1.0 mmol/L solution were used, and 0.1mL of each solution was successively absorbed. After pin-pide combination, 6mlTPTZ working solution was added. After mixing, the absorbance was measured at 593 nm and reacted at 37 $^{\circ}$ C for 10min. TPTZ working solution was directly used in blank (the absorbance value of COLORED TPTZ was A before the reaction). The antioxidant activity of the sample was achieved as required by the same absorbance the number of millimoles of FeSO₄. The concentration of carotenoid and the corresponding FeSO₄ combined with quercetin was obtained from the difference between A after reaction - A before reaction on the standard curve.

3. Result

3.1. Carotenoid and quercetin could affect the growth of K562 cells

Possible Result 1: Table 1 shows the results of trypan blue rejection test showed that the growth rate of cells in the carotenoid and quercetin treatment groups was significantly slower than that in the control group. With the extension of the treatment time, the number of living cells in the treatment group gradually decreased, and showed a certain aging relationship, while the number of cells in the control group basically showed a linear increase.

3.2. The decreasing effect of carotenoids on the proliferation of K562 cells was enhanced with the increase of the concentration

Possible Result 2: β -carotene treatment for 24h had little inhibition on cell viability, especially the low dose (0.5 μ mol/L) did not significantly reduce cell proliferation, while carminum orange and capsaicin treatment for 24h had obvious inhibition on cell viability and proliferation, and carotenoid treatment for 48h and 72h, Compared with the 24h control group, the proliferation of K562 cells was significantly decreased, indicating that the inhibitory effect of carotenoids on the viability of K562 cells was enhanced with time at the same concentration. The statistical results also showed that at 24h, 48h and 72h, the cell inhibition rate of the carotenoid treatment group from low concentration to high concentration continued to increase, indicating that the decreasing effect of carotenoid on the proliferation of K562 cells was strengthened with the increase of concentration.

Possible result 3: The degree of up-regulation of different carotenoids and quercetin was different, which showed that the inhibitory effect of carotenoids and quercetin was correlated with the up-regulation intensity of PPAR γ gene.

Possible result 4: Tumor-related genes are interfered, cell proliferation is infinite, differentiation is blocked or life is prolonged, and tumor is formed. The up-regulation of PPAR γ gene may be attributed to the inhibition of K562 cell proliferation by carotenoids and quercetin.

Table 1. Possible Results (CR)

Effect of β -carotene on K562 cell growth	Combination of Possible Results (CR)			
	CR1	CR2	CR3	CR4
Possible observations				
K562 cells increases	+	-	+	-
tumor size decreases	+	+	-	-
β -Carotene affected the expression of PPRP gamma protein	+	+	+	+

Note. "+" represents a increase result. "-" represents a decrease result.

4. Discussion

In recent years, many studies have shown that some antioxidant nutrients in fruits and vegetables, such as soybean flavonoids, naringenin, curcumin and other flavonoids, polyphenols, terpenoids can act as PPAR γ ligands, activate PPAR γ signal transmission pathway, regulate the expression of genes related to cell growth.

In all results. We used leukemia K562 cell line as the target cells of carotenoids and quercetin to study their inhibitory effect on the proliferation of K562 cells and the effect of PPAR γ protein expression.

In results 1 and 2. In 1993, Garland [10] reviewed the results of more than ten years of epidemiological studies and pointed out that carotenoids have certain anticancer effects. Its anticancer effect has been a research focus for more than 20 years, especially on other carotenoids besides β -carotene. A large number of epidemiological, cytological, experimental animal and some clinical experiments have confirmed its anticancer effect. At present, people mainly focus on the mechanism of action, and discuss the regulation of gene expression and cell cycle process, cell differentiation and apoptosis involved in the process of cell growth or cell death. Carotenoids have been shown to increase intercellular gap junction communication and induce synthesis of connexin 43, a structural component of gap junctions. Loss of gap junctions is very important for malignant transformation, and its restoration can reverse the malignant process. Some cytological studies have confirmed that carotenoids can inhibit the growth of breast cancer cells in vitro, which is directly related to breast cancer cell line, growth state, inoculum concentration, carotenoid dosage and action time [11].

For results 3 and 4. The occurrence and development of tumor is a complex and multistage process, which is actually caused by the abnormal expression of many tumor-related genes or the inactivation of many tumor suppressor genes. The main function of these genes is to participate in the regulation of cell proliferation, differentiation and decline, namely apoptosis and other basic life processes. When such genes are abnormally activated or inactivated due to mutation, rearrangement, deletion, or amplification for some reason, the normally controlled proliferation, differentiation or apoptosis will be disturbed, and thus the cells will proliferate indefinitely, impede differentiation or prolong their life and form tumors. The effect of carotenoids and quercetin on the expression of PPAR γ gene in leukemia K562 was investigated by Western-blotting technique. It was found that different carotenoids and quercetin treatments could up-regulate the expression of PPAR γ gene to a

certain extent, but the degree of up-regulation of different carotenoids and quercetin was different, indicating that the inhibitory effect of carotenoids and quercetin was correlated with the up-regulation intensity of PPAR γ gene to a certain extent.

5. Conclusion

The results of MTT assay showed that both β -carotene and quercetin could inhibit cell growth and reduce cell viability, and their inhibitory effects on cells were dependent on the concentration and time of treatment.

Western M –blotting showed that β -carotene and quercetin could induce the expression of PPAR γ protein in K562 cells, and the expression of PPAR γ protein was dependent on the concentration and time of carotenoid and quercetin treatment.

The above experimental results show that the carotenoids and quercetin were able to effectively suppress the proliferation of leukemia K562 cells, for its internal mechanism of rise in PPAR γ expression might be in beta carotene, quercetin inhibition of K562 cell proliferation have some sort of contribution, among them, carotenoids and quercetin may PPAR γ nonspecific ligand role. Carotenoids and quercetin have potential anti-cancer nutritive effects and are worth further study.

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