行政院國家科學委員會專題研究計畫　成果報告

中藥有效成分鞣花酸和茶多酚對致癌劑誘發老鼠遺傳傷害的拮抗作用機制之研究

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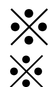

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一、中文摘要

許多研究指出多酚類化合物單離成分鞣花酸(Ellagic acid, EA)和茶多酚((-)-epigallocatechin gallate, EGCG)具有抗癌防癌的效果，然其與抗致癌起始作用間的關係之作用機轉仍值得進一步研究。我們實驗室先前已建立一個化學致癌劑誘發老鼠體內遺傳傷害的動物模式，可同時測量致癌機轉中早期指標基因突變與 DNA 傷害。利用 T-淋巴細胞 cloning 的方法計算老鼠脾臟淋巴細胞 *hprt* 基因突變率，初步的結果顯示預飼老鼠 200 mg/kg 的 EA 或 EGCG 可有效抑制基因致癌物乙基亞硝酸基尿素（N-ethyl N-nitrosourea, ENU）所誘發的 *hprt* 基因突變。為了進一步了解 EA 和 EGCG 的預防化學致癌作用與其可能作用機轉，本研究中使用高敏感度與專屬性的氣相層析儀加質譜儀(gas chromatography/mass spectrometry)的方法定量 ENU 所誘發的主要 DNA adduct 7-ethylguanine (7-EG)的濃度，結果顯示 30 mg ENU /kg 處理組老鼠肝臟組織的 7-EG 為 53 ± 9 pmol/µmol guanine，顯著高於陰性對照組老鼠。若以 200 mg/kg EGCG, 100 mg/kg EGCG 或 200 mg/kg EA 前處理老鼠，分別可降低 ENU (60 or 30mg/kg)誘發的老鼠肝臟組織內的 7-EG ($P<0.05$)。結果顯示 EGCG 與 EA 可減低 ENU 所誘發的 DNA 損傷與基因突變，但 EGCG 的抗 DNA 損傷的效果明顯比 EA 好。DNA 損傷與基因突變可受 DNA 損傷修補酵素功能的影響，鑒於 7-EG 於體內會分別被烷基醣鍵分解酵素(N-methylpurine-DNA glycosylase, MPG)修補，以 RT-PCR 觀察 DNA 修補酵素 MPG 基因的表現情形，結果發現 EA 和 EGCG 可顯著增強老鼠肝臟 MPG 基因的表現。綜合以上的結果，EA 和 EGCG 在體內有抗化學致癌物傷害的效果，可能與其增強體內 DNA 修補酵素有關。

關鍵詞：鞣花酸、茶多酚、DNA 損傷、基因突變、DNA 修補酵素

Abstract

Ellagic acid (EA) and (-)-epigallocatechin gallate (EGCG), natural plant polyphenols, have demonstrated to possess anti-carcinogenic and anti-mutagenic effects in several test systems. However, more studies are needed to understand their underlined mechanisms. Our previous data showed that pretreatment of 200mg/kg of EGCG or 200mg/kg EA by gavage could inhibit N-ethyl nitrosourea (ENU)-induced *hprt* mutations in mouse splenic T lymphocytes. In this study, the anti-genotoxic effects of EA and EGCG were further examined by using ENU-induced DNA adducts 7-methylguanine as biomarkers of genotoxic damage. Mice were orally fed with ellagic acid or (-)-epigallocatechin gallate (EGCG) for consecutive seven days before challenging with ENU. By using our newly developed, highly sensitive and specific gas chromatography/mass spectrometry (GC/MS), pretreatment with 200, 100 mg/kg EGCG and 200mg/kg EA significantly reduced ENU-induced DNA damage (7-methyl guanine) in mice ($P<0.05$). Our data showed that EA and EGCG could reduce ENU-induced DNA damage and mutations. 7-EG is believed to be repaired by N-methylpurine-DNA glycosylase (MPG) *in vivo.* The expression of mRNA of MPG in mouse liver tissue was examined by RT-PCR. EA and EGCG significantly increase the expression of MPG mRNA. These data suggest that the protective effects of EA and EGCG against chemical-induced DNA damage and mutations might at least partly result from its modulating of enzymes of DNA repair.

Keywords：Ellagic acid, (-)-epigallocatechin gallate, DNA adduct, *hprt* mutant frequency, DNA repair enzyme

二、緣由與目的

Recently cancer has climbed up to become the number one cause of death in Taiwan. The preventive approach has become one of the main foci in cancer research. Cancer is a disease caused by pathological alterations in the genes. The pathological alterations of the genes can be affected by environmental factors, individual life styles and endogenous sources in the body. These harmful agents attack the human genes to produce lesions or even mutations. Hence, decreasing genetic damages and mutations might be closely related to cancer prevention or anti-aging.

Recently the theory of carcinogenesis supports the notion that the products of the reactions between the genotoxicants and the DNA molecules, termed DNA adducts, are thought to be the earliest damage markers in carcinogenesis. Therefore, analysis on the level of DNA adducts caused by the genotoxin in the live organisms can reflect

the extent of damages to the genes.

Ellagic acid (EA) is a common ingredient in many Chinese medicinal herbs. Some common fruits found in Taiwan such as strawberry, mango, and guava contains high amounts of EA (8). Catechin is the major polyphenol compound found in the green tea leaves. It includes (+)-catechin, (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechingallate (ECG), and epigallocatechin gallate (EGCG). People who drink green tea often tend to have a lower chance of developing certain types of cancer, as reported by recent epidemiological reports. EA and EGCG have demonstrated to possess anti-carcinogenic and anti-mutagenic effects in several test systems. However, more studies are needed to understand their underlined mechanisms.

We have established an animal model to not only systematically test the chemopreventive potential of Chinese medicine, but also study the mechanisms involved in achieving the effect. Our previous data showed that pretreatment of 200mg/kg of EGCG or 200mg/kg EA by gavage could inhibit N-ethyl nitrosourea (ENU)-induced *hprt* mutations in mouse splenic T lymphocytes. In this study, the anti-genotoxic effects of EA and EGCG were further examined by using ENU-induced DNA adducts 7-methylguanine as biomarkers of genotoxic damage. Furthermore, the expression of mRNA of N-methylpurine-DNA glycosylase (MPG), responsible for 7-EG repair, was examined by RT-PCR to evaluate the modulating effect of MPG in 7-MG formation in mice.

三、研究方法

Animals and treatment

C57BL6 male mice aged 4-5 weeks were obtained from the National Laboratory Animal Breeding and Research Center. The animals were randomly assigned to 6 groups: control, ENU, EGCG 200 mg/kg (EGH), EGCG 100 mg/kg (EGL), EA 200 mg/kg (EAH), and EA 100 mg/kg (EGL). The chemicals were given orally, once per day, consecutively for 7 days. Two hours after the dosing on the 7th day, ENU (30mg/kg) was injected via i.p. route in all but the control group mice. Then the animals were sacrificed 2 hours after the ENU injection. The brain, liver, spleen, and kidneys were quickly frozen in liquid nitrogen and stored in –80°C fridge for future use.

Isolation of DNA is followed the standard phenol/chloroform extraction protocol.

Fifty micrograms of DNA were spiked with $^{15}N_5$-labeled 7-MG and heated at 100^oC for 15 min before immediately putting in ice. Then 150 μl of 1 N HCl was added and centrifudged at 3200 rpm. The supernatant was dried in vacuum and added 50 μl of tert butyl nitrite and 20 μl of 6 N HCl at 4^oC for 4 hr. After being dried in vacuum, samples were extracted with 50 μl of dd H_2O and 150 μl of ethyl acetate. The aqueous phase was recovered and dried *in* vacuum. Each residue was reacted with 150 μl of 10% PFBBr in acetonitrile and 5 mg K2CO3 (degydrated at 60o at least 1 hr) at room temperature for 20 hr, followed by evaporation under nitrogen at 45oC and extracted three times with ethyl acetate. The combined supernatants were dried under nitrogen at 85^oC. The residue was dissolved in 50% ethyl acetate/hexane solution and subjected to solid-phase extraction using silica gel. The eluent was dried under nitrogen at 85^oC and raised in dry toluene.

One μl of sample solution was injected into a Fision 8060 gas chromatograph coupled with Platform mass spectrometer (Micromass, Weesp, Netherlands) operated in the selective ion mode. The gas chromatography was performed using a J&W Scientific (Folsom, USA) ~ 30 m, 0.32 mm, and 0.1 μm film thickness DB-5 MS capillary column. Helium was used as a carrier gas, and head pressure was set at 15 psi. The source temperature was set at 180℃, and methane (1×10^{-4} mbar). The injector port and initial column temperature was set at 290℃ and 100℃, respectively. After 1 min, the column temperature was ramped at 10℃/min to 250℃, and held for 2 min, at 10℃/min to 290℃, and held for 1 min.

Isolation of RNA

Following the manufacturer's protocol, the isolation was done with TRIzol Reagent and additional Chloroform. Briefly, homogenize 100 mg of tissue in 1 ml TRIzol Reagent. Add 200µl of Chloroform and shake vigorously by hand. Centrifuge at 4 ^oC, 14000xg for 10 minutes. Transfer the supernatant to a new Eppendorff and precipitate the total RNA with 500µl of (-20 ^oC) Isopropanol. Wash the pellet with 1ml (-20 ^oC) of the mixture of 75%Ethanol, and 25%DEPC. Discard the wash solution and dry briefly. Dissolve the pellet in DEPC treated water and quantitate with a spectrophotometer.

Primer Sequences

The prime sequences are as follows.

MPG 3': TCCGGCGGAGTATCTACTTCTCCAGC.

MPG 5': CTTGACTAGAGACATTCAAGCAGAAG.

α-actin 3': GGAGCAATGATCTTGATCTTC.

α-actin 5': CCTTCCTGGGCATGGAGTCCT.

First Strand cDNA Synthesis

Six μg of total RNA was mixed with 1.0 μl of 0.1M DTT, 24 U of RNAse Inhibitor and 3.2 μl of 10 μM of reverse primer of interest in a final volume of 14 μl. Then the reaction mixture was incubated at 65 °C for 5 minutes and 37 °C for 10 minutes to anneal. After the annealing stage, 4.0 μl of 5X RT reaction buffer (250 mM Tris-HCl pH 8.3 at 25 °C, 375 mM KCl, 15mM $MgCl_2$ and 50mM DTT), 1.0 μl of 25 mM dNTP, and 200 units of MMLV reverse-transcriptase (Promega) were added to the reaction mixture to give a final volume of 20 μl. The reaction mix was incubated at 37 °C for 10 minutes and 42 °C for 120 minutes. The termination step was done at 95 °C for 5 minutes and the cDNA synthesized was stored at –20 °C.

Polymerase Chain Reaction

The reaction mix contains 5.0 μl of cDNA, 0.5 μl of 25 mM dNTP, 5.0 μl of 10X PCR reaction Buffer (500 mM KCl, 100 mM Tris-HCl pH= 9.0 at 25 °C, 15 mM $MgCl_2$ and 1% Triton X-100), 4.0 μl of 25 mM MgCl2, 1.0 μl of each 10μM primer of interest, and 1.0 unit of Taq polymerase in a total volume of 50 μl. The mix was incubated in a thermocycler (Hybrid PCR Express). The program consists of 3 minutes at 94℃, various cycles of 45 seconds at 94 °C, 30 seconds at 59 °C, and 1 minute at 72 °C. The final extension was carried out at 72 °C for 7 minutes and the product kept in –20 °C fridge. The MPG PCR reactions went thru 27 and 30 cycles while the α-actin 22 and 25 cycles.

Analysis of Amplified Products

The PCR products were electophoresized on 1.8% agarose gel. The gel photo was taken by using a photo-image system (Stratagene Eagle Eye II) and band intensities adjusted to the α- actin ones from the same RNA. Then the ratios were analyzed and compared among different conditions within the same RNA group.

四. 結果與討論

Pretreatment with 200, 100 mg/kg EGCG and 200mg/kg EA significantly reduced ENU-induced 7-methyl guanine in mouse liver DNA (P<0.05)(Table 1).

Treatment	**7-EG (pmol/ μ mol guanine)***
Control (water)	ND (< 1) **
ENU (30 mg/kg)	53 ± 9
ENU + EGCG (100 mg/kg)	21± 19 #
ENU + EGCG (200 mg/kg)	19 ± 4 ##
ENU + EA (100 mg/kg)	54 ± 9
ENU + EA (200 mg/kg)	31 ± 10 #

*7-EG (pmol/ μ mol) = [m/z =359 area / (m/z =363) area] x (1 pmol of internal std.)/DNA content (μ mol of guanine)

Table 1: Inhibitory effects of EGCG and EA on ENU-induced 7-MG in mouse liverDNA

Mean and standard deviation of three mice.Control：water only; ENU：ENU 30 mg/kg; EGCG 100：EGCG 100 mg/Kg; EGCG 200：EGCG 200 mg/Kg; EA 100：EA 100 mg/Kg; EA 200：EA 200 mg/Kg; # $P<0.05$, ##$P<0.01$ compared with the values of ENU

In summary, both EGCG and EA have the ability to protect the animals from DNA damages. In EA groups, the effect is more prominent in the high dosage group only, while in the EGCG groups, the effect is more marked than the EA groups, suggesting EGCG is more effective in the prevention of DNA damages, or better, in the prevention of cancer.

The expression of MPG gene in mouse livers from different treatments was analyzed using RT-PCR. The electrophoresis gel photo can be seen in Figure 1.

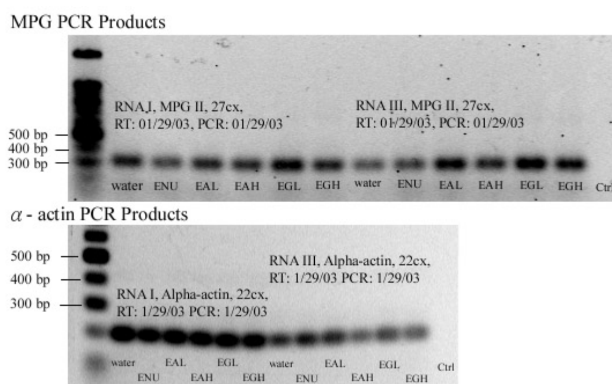

Figure 1: The Electrophoresis gel photos of the MPG and α-actin PCR Products.

The MPG expression level of each sample was adjusted to its corresponding α-actin sample. Then the comparison was made in reference to the positive control group, ENU. The ratios of each condition from all 3 sets of RNA were pooled and

averaged. The result was listed in Table 2.

	Water	**ENU**	**EAL**	**EAH**	**EGL**	**EGH**
AVG	1.27	1.00	1.33	1.58	1.85	1.65
STDEV	0.41	0.00	0.21	0.34	0.42	0.48

Table 2: The MPG Expressions (adjusted to α-actin expressions) among various groups.

As the ratios suggest, EA seems to have an effect on recovering the lowered MPG expression, seen obviously in both the low and high dosage groups. The EAL group and the control group are very close in the level of MPG expressions, suggesting that the MPG expression might have been restored due to EA. The EAH did show a significant increase in the MPG level as well. In terms of EG expressions, the level was increased obviously to 1.85 ±0.42 and 1.65 ±0.48. Yet, there was no obvious dose effect observed in the EG groups.

By comparison to the MPG expression level in the ENU group, the control expression level was at 1.27 ± 0.41, suggesting that the MPG expression level in the ENU group might have gone down due to the effect of the ENU injection. Although the EAL group has similar intensity ratio as the control group, it actually suggests that the lowered expression induced by ENU (seen in ENU group) is recovered by the low dosage EA. In addition, the results from other groups, further suggests that both EA and EGCG can effectively protect the organisms from DNA damages. Moreover, judging from the results of the experiment, the MPG expression of the EGCG groups is higher than that of the EA groups, inferring EGCG has higher preventive effects than EA.

五、計畫成果自評

As mentioned earlier in the introduction, the possible mechanisms for the preventive capabilities of Chinese medicine might involve the inhibition of the metabolism that activates the toxins, increasing the level of detoxification or strengthening the DNA repair capability. Being a direct genotoxin, ethylnitrosourea (ENU) does not need to be metabolized to dissociate to give rise to the ethyl- group which attacks the nuclear bases to form DNA adducts. Thus, the preventive function might come from increasing the level of detoxification or strengthening the DNA-Repair capability, or maybe both. In this experiment, the outcome does support

the idea that the preventive effects of EGCG and EA result from stimulating the DNA-repair system, for MPG is one of the key enzymes involved in the Base-Excision- Repair of DNA. Nonetheless, the role and involvement of the detoxification in EGCG and EA's preventive capability still needs further investigation.

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