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Effects of Ursolic Acid Isolated from *Eriobotrya japonica* on c-myc and c-Ha-ras Oncogene Expression at Sarcoma 180 Cell

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Abstract

The sarcoma 180 cells were treated with ursolic acid which was previously extracted from leaves of *Eriobotrya japonica* Lindy (Rosaceae) and identified as a potent anticarcinogenic agent. Suppressing effects of the compound with testing changes in selected oncogenes' expression were examined by using the northern hybridization method. Ursolic acid significantly suppressed c-myc oncogene expression. However, c-Ha-ras oncogene expression was lowered slightly with the ursolic acid treatment. Therefore, it was concluded that preproven anticarcinogenic effects of ursolic acid should be partly ascribed to the modified oncogenic expression.

Key words : sarcoma 180, c-myc, c-Ha-ras, oncogene, northern hybridization, ursolic acid

INTRODUCTION

Many reports available stated that a major fraction (50%~60%) of human cancer is caused by largely exogeneous factors and therefore, potentially preventable[1]. Many of the exogeneous compounds known as "tumor promoters" are not mutagen but act instead in an epigenetic manner, perhaps in increasing the risk of cumulative mutations by sustaining the proliferation of cells or in evoking infrinting events that could inactivate alleles of tumor suppressor genes[2,3].

Moreover, the external factors are probably the most operable variables in the majority of human cancer. This knowledge led to the investigation of anti-carcinogenic components in diets and edible plants. A recent paper stated that a search of the literature from the National Library of Medicine database and from individual journal articles yielded more than 500 compounds to have anti-carcinogenic activities. This vast array of agents includes synthetic compounds and compounds naturally present in foods[4].

Leaves of *Eriobotrya japonica* Lindy (Rosaceae) have been used as traditional medicines for lung and stomach diseases and have been found to be effective against influenza virus, inflamation, and tumors. Furthermore, there were several reports concerning the active ingredient in plants which is triterpenoids (ursolate)[5]. Recently, Tokuda et al.[6] also reported that ursolic acid (UA) had an antipromotion effect in the carcinogenic process. Besides, Park[5] and Fang[7] reported its cytotoxic effects in some tumor cell lines.

Based on this information, an implementation was carried out to marry this folk medicine with modern science and to produce the basis for an effective new treatment. In order to study the possible involvement of ursolic acid in oncogene expression, a conventional approach in RNA level was adopted by exposing sarcoma 180 cells to the compound, and by looking for changes in the levels of mRNA

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expression of c-myc and c-Ha-ras oncogenes.

MATERIALS AND METHODS

Cell culture, total RNA isolation and northern blot

Sarcoma 180 cells were grown with 10% heat-inactivated fetal calf serum (FCS) in Eagle's minimal essential medium (EMEM) as the reference group. For the treated group, ursolic acid was added to the medium by 7.5×10^{-5} mole. Total RNA's were isolated from the cells of two groups in 24hours by using the Acid Guanidium Thyocyanate-Phenol-Chloroform Extraction method[8].

The concentration of RNA's isolated were measured with UV absorbance at 260nm, and 50ug aliquots of the total RNA's were subjected to an electrophoresis on formaldehyde-denaturated agarose gel (1%). The RNA was transfered to a nitrocellulose membrane[9]. The filter were hybridized to each of the selected probes, respectively[10] : A freshly denatured herring sperm DNA was added to the 9.8ul of a prehybridization solution (50% formamide ; 5x SSC ; 5x Denhardts' solution ; 25mM sodium phosphate, pH 6.5), mixed and placed in a heat-sealable bag along with the filter. The filter was incubated at 420° C in the sealed bag for 3hours. For hybridization, the probe and the herring sperm DNA were denatured for 10 minutes in a boiling water bath followed by chilling on ice and added to hybridization solution (40% formamide ; 5x SSC ; 1x Denhardts' solution ; 20mM sodium phosphate, pH6.5; and 5% dextran sulfate). The filter was hybridized at 42° C overnight. The filter was washed in 250ml of 2x SSC/0.1% SDS, followed by another wash in buffer for 1hour in buffer #1 (0.1M Tris-HCl, pH7.5 and 0.15M NaCl) and soaked for 1hour in buffer #2 (3% bovine serum albumin, Fraction V in buffer #1). The filter was soaked in Streptavidin-Alkaline Phosphatase (SA-AP) conjugate (0.1ug/ml) for 10minutes and washed in buffer #1 followed by wash in buffer #3 (0.1M Tris-HCl, pH9.5 ; 0.1 M NaCl, and 50 mM $MgCl_2$). The filter was soaked in a dye solution. The dye solution was prepared by mixing 33ul nitroblue tetrazolium (NBT) solution to 7.5ml buffer #3 and 25ul bromo-chloro-iodolyl Phosphate (BCIP) solution[10].

Probe preparation

Plasmids bearing c-myc and c-Ha-ras, respectively, were amplified by using *E. coli*, HB101 cells as host, purified; digested; and separated by methods previously described. The information, sources, sizes, cloning sites, and cloning vectors of c-myc and c-Ha-ras oncogenes were listed in Table 1. The inserted fragments were separated with electrophoresis on a low-gelling temperature agarose gel. The bands of fragmented DNA were excised from the gels, labeled with biotin-labeled dUTP by random priming and used for northern hybridization.

RESULTS

The effect of UA on c-myc and c-Ha-ras expressions were measured on the mRNA levels. The total RNA was extracted after treatment of sarcoma 180 cells with UA. The quantity and stability of the RNAs extracted were shown in Figs. 1 and 2. The presence of 28S and 18S rRNA bands were clearly shown, and the quantity of both rRNA's were observed to be approximately identical (internal control). The results of northern blot clearly dictated that the extent of reduction in c-myc expression by UA was prominent (Fig. 1). It was suggested that antipromotional effect of UA on tumor could be related with the inhibition of c-myc expression. However, no significant difference was observed in c-Ha-ras oncogene expression of the UA group when compared with that of the reference group (Fig. 2).

Table 1. Gene probes

Gene probe	c-myc	c-Ha-ras
Source	human	human
Insert size	9 kb	350bp
Cloning site	EcoRI/Hind Ⅲ	EcoRI/Hind Ⅲ
Vector plasmid	pBR322	pUC18

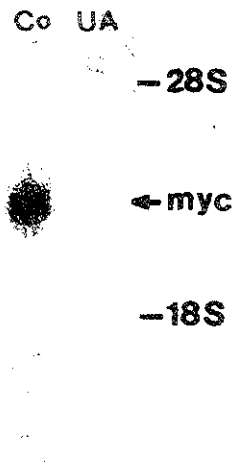

Fig. 1. Northern blot. Total RNA (50ug per lane) was fractionated on a agarose formaldehyde gel (1%) and transfered to a nitrocellulose membrane. The blot was hybridized with a 9 kilobase pair c-myc cDNA probe which was labeled with biotin-labeled dUTP.

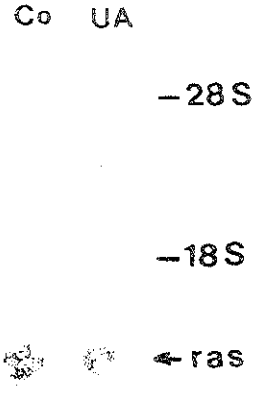

Fig. 2. Northern blot. Total RNA 50ug per lane was fractinated on a agarose formaldehyde gel (1%) and transfered to nitrocellulose membrane. The blot was hybridized with a 350 base pair c-Ha-ras cDNA probe which was labeled with biotin-labeled dUTP.

DISCUSSION

In this study it was shown that UA reduced c-myc oncogenes' expression levels significantly. The data therefore, suggested that the antipromotional effects of UA on tumors previously reported could be ascribed to the reduction in the oncogene expression. The c-myc is the "immediate early" gene (jun and rel) and a member of a class of genes which are rapidly induced when resting cells are treated with mitogen, suggesting that they may be involved in a cascade initiating cycling. Thus, their activities are likely to be associated with initiating or promoting the growth of tissue. Expression of the protooncogene, myc is particulary required for the entry into G_1 from G_0 and additionally required for continuous cell proliferation. It can be therefore, expected that an increase in their activities could result in carcinogenesis[20]. The independency of c-Ha-ras mRNA expression level on UA was shown in Fig. 2. While the exact function of ras proteins remained unclear, it has been established that the p21s encoded by ras genes have GTPase activity and interact with a GTPase-activating protein. The abnormality in cell function caused by ras family can occur by biochemical malfunction with point mutation as well as overexpression of the wild type alleles.

As additional information, the activity of hepatic glutathione-S-transferase, and the contents of non-protein-SH and protein-SH were not significantly changed by ursolic acid and linolic acid. Therefore, it can be said that the activities of these particular compounds are not associated with enzymes which mediate reactions that enhance the solubilization and elimination of carcinogens, or that increase the concentration of glutathione(unpublished data).

The effects of ursolic acid are thought to suppress tumor promotion, which leads to advanced stages in the tumorigenesis process required to expand the population of oncogene-expression cells.

In cell proliferation, the elements in growth control pathways may be either the carriers of intracellular signals or their receptors, or the proteins that are responsible for transducing signals from the receptors to critical targets. The second elements in a growth-regulating pathway are the receptors. An al-

tered growth factor receptor that behaves as if it is constitutively binding its ligand, could act as an oncogenic protein. The subsequent elements in the pathway are the intracellular transducers of signals. Examples of this, such as ras-encoded proteins, seem to act by receiving growth-stimulatory signals and passing them on to downstream targets.

Oncogene products may act in the conjugation of some of the following mechanisms. The oncogene products may themselves be the enzymatic agent of phosphorylation or they may be a factor that elicits phsphorylation. They can regulate adenylate cyclase (products of Ras 1 and Ras 2 in yeast stimulate adenylatecyclase). They may regulate transcription. For example, the product of myc may regulate transcription from other genes. They may also regulate DNA replication.

Some of oncogene products are associated with polypeptide growth factor (PGF) or their receptors and the majority of those are involved in the transfer of stimulus from the cell surface to the intracellular machinery. The products of ras play roles in connecting occupied receptors with appropriate effector molecules. The products of myc have proven to be effective markers in growth factor stimulation.

In this version, it can be concluded that the well known anticarcinogenic effects of UA could be due partly to the reduction in oncogene expression level, since the reduced oncogene products may be correlated with any stage of the biochemical pathway mentioned above.

In further study, to elucidate the mechanism of anticarcinogenic effects associated with the reduction in oncogenic expression, the effects of altered oncogene expression to the signal transduction machinery will be studied.

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Sarcoma 180 세포에서 비파엽에서 분리한 울솔레산이 c-myc 과 c-Ha-ras 암유전자 발현에 미치는 영향

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요 약

비파엽에서 분리동정한 활성성분인 울솔레산을 sarcoma 180 cells에 처리하여 c-myc 과 c-Ha-ras 암유전자 발현에 있어서 변화를 조사하였다. 그 결과 c-myc유전자의 발현에서는 뚜렸한 감소가 관찰되었으나 c-Ha-ras 유전자 발현은 대조군과 거의 차이가 없었다. Cell proliferation에 중요한 역할을 하는 것으로 추측되고 있는 c-myc 유전자 발현의 감소는 지금까지 보고된 그 물질의 antipromotional effect와 관계있는 것으로 보여진다.