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Antiatherogenic Effects of *Phyllanthus Emblica* Associated with Corilagin and its Analogue

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(Received December 5, 2004; Accepted April 4, 2005)

Oxidized low-density lipoprotein (ox-LDL) is the main etiologic factor in atherogenesis, and antioxidants are accepted as effective treatment of atherosclerosis. The aim of this study was to clarify whether the mechanism of the antiatherogenic effects of the herb *Phyllanthus Emblica*, which is widely used to treat atherosclerosis-related diseases, is associated with ox-LDL *via* its compounds of soluble tannin, corilagin (beta-1-*O*-galloyl-3,6-(*R*)-hexahydroxydiphenoyl-*d*-glucose), and its analogue Dgg16 (1,6-di-*O*-galloyl-beta-*d*-glucose). Human umbilical vein endothelial cells, ECV-304, were incubated with ox-LDL (50 mg/l), treated with corilagin or Dgg16 at different doses (0.0001—0.1 mmol/l), and then incubated with monocytes. Malondialdehyde (MDA) in the culture media was determined and the number of monocytes adhering to ECV-304 cells was counted with cytometry. In another experiment, the rat vascular smooth muscular cells (VSMC) were incubated in media with or without ox-LDL (50 mg/l), and with corilagin or Dgg16 also at different doses (0.0001—0.1 mol/l), the proliferation of which was assayed with MTT. The results showed that both corilagin and Dgg16 were able to decrease MDA, prevented ECV-304 cells from being adhering to by monocytes, and inhibited VSMC proliferation activated by ox-LDL. The results suggest that the two compounds are effective in inhibiting the progress of atherosclerosis by alleviating oxidation injury or by inhibiting ox-LDL-induced VSMC proliferation, which may be promising mechanisms for treating atherosclerosis.

Key words——corilagin; endothelial cell; smooth muscle cell; oxidation injury; proliferation

INTRODUCTION

Atherosclerosis is a main cause for many cardiovascular diseases,[1] e.g., hypertension, apoplexy, congestive heart failure, aneurysm, and ischemic cardiomyopathy. One well-known mechanism is associated with the injury of endothelial cells by free radicals (usually followed by monocyte adherence) *via* oxidized low-density lipoprotein (ox-LDL), and with the proliferation of vascular smooth muscle cells (VSMC).[2] Scavenging free radicals or inhibiting endothelial proliferation is accepted as an effective method to alleviate atherosclerosis. Many antiatherogenic agents have both properties, such as probucol,[3] vitamin E,[4] and Phenolics,[5] which strongly support the opinion.

Under this hypothesis, many compounds from traditional Chinese medicine which contain promising antiatherogenic components were screened. Recently, two compounds have been extracted from *Phyllanthus embolica* L. (Phy), called Yu-Gan-Zi in Chinese[6]: corilagin [beta-1-*O*-galloyl-3,6-(*R*)-hexahydroxydi-phenoyl-*d*-glucose], and its analogue Dgg16 (1,6-di-*O*-galloyl-beta-*d*-glucose). The whole plant, especially its fruit, is widely used to treat diseases associated with atherosclerosis. Corilagin and Dgg16 are two novel members of the tannin family from the fruit, their structures are similar (Fig. 1), and both show strong antioxidation;[6] corilagin has strong effects against platelet aggregation[7] and inflammation.[8] But there are few reports on their pharmacologic effects related to atherosclerosis. It is necessary to determine the mechanism of the effects of the herbal drug, and to evaluate whether the drug

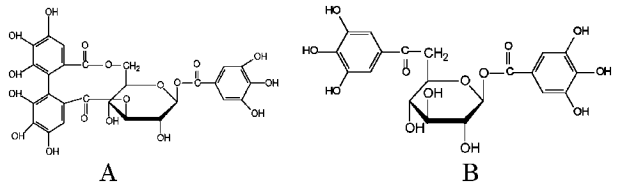

Fig. 1. Structure of Corilagin and Dgg16

A: Corilagin (beta-1-*O*-galloyl-3,6-(*R*)-hexahydroxy- diphenoyl-*d*-glucose), B: Dgg16 (1,6-di-*O*-galloyl-beta-*d*-glucose).

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acts against atherosclerosis through its chemicals of tannin like corilagin and Dgg16.

According the hypothesis, the experiment was designed to determine whether the two compounds can 1) alleviate endothelial oxidation injury by testing against malondialdehyde (MDA), 2) prevent endothelial cells from being adhered to by monocytes, or 3) inhibit VSMC proliferation. Vitamin E (alfa-tocofecol), a well-known bioreducer, was used as a positive control in the study.

MATERIALS AND METHODS

Materials Corilagin and Dgg16 were provided by Kunming Institute of Botany, Chinese Academy of Sciences, China. DMEM and RPMI 1640 culture media were purchased from Gibco BRL, USA. Fetal bovine serum was purchased from Hangzhou Sijiqing Bio-material Co., Ltd., China. ox-LDL, collagenase, elastase, and vitamin E (alfa-tocofecol) were purchased from Sigma, USA. MDA kits were purchased from Nanjing Jiancheng Bioengineering Institute, China. Human umbilical vein endothelial (ECV-304) cells, was purchased from Shanghai Institute of Materia Medica, Chinese Academy of Sciences, China. The CO_2 incubator was manufactured by Hereaus, German. The cytometer (model Ridmodei-3550) was manufactured by Biotech, Italy. The clinical electrophotometer (model CL-770) was manufactured by Shimadzu, Japan. Other reagents used in this study were of analytic purity from China.

Preparation of Human Monocyte and Rat Aortic VSMC Monocytes were separated from healthy human venous blood (from Nanking Blood Bank, China) by density gradient centrifugation, and were cultured using the method of Rajesh et al.[9]

Aortic VSMC were prepared from healthy Sprague-Dawley rats (from Kunming Medical College, China, certification number: 2001034) according to the method of Watanabe et al.[10] with some modifications. Briefly, rat thoracic aorta was separated, cleaned, and washed. After the tunica adventitia and tunica intima were removed, the vessel was scissored in DMEM solution. Small pieces were incubated in DMED solution with 10% fetal bovine serum at 37°C in the CO_2 incubator. Another washing was carried out 24 h later with the culture medium containing benzylpenicillin (200 IU/ml) and streptomycin (200 IU/ml). Then, it was digested with a compound enzyme solution containing collagenase 2 g/l and elastase 0.25 g/l. When disrupted cells appeared, the digestive reaction was ceased with 20% fetal bovine serum. VSMC were separated by centrifugation, and their primary culture and subculture were carried on to harvest enough VSMC for study.

MDA Assay in Culture Media and Adhesion of Monocytes ECV-304 cells were incubated with RPMI 1640 containing 15% fetal bovine serum in the CO_2 incubator at 37°C. When the cells covered about 80% of the full area of the flask, they were digested to disrupted cells with 0.5% trypsin solution. The disrupted cells (1.0×10^5/ml, 1.0 ml per well) were transplanted to 96-well plates and incubated in the same conditions. When the cells adhered, corilagin, Dgg16, or vitamin E at a dose of 0.0001, 0.001, 0.01, or 0.1 mmol/l was added to the culture media with ox-LDL (50 mg/l), and incubated for an additional 4 h. The culture media were sampled for the MDA test with MDA kit.

When the above media were removed, 3.0×10^5 monocytes (20 μl) were placed into every well of 96-well plates (containing ECV-304 cells 5.0×10^5 per well) and incubated in the same conditions mentioned above for another 1 h. Then the total number and the number of the monocytes viable but not adhering to ECV-304 cells were counted with cytometry.[11] The adhesion was calculated using formula (1).

$$A(\%) = \frac{N_{\text{total Mc}} - N_{\text{non-adhering Mc}}}{N_{\text{total Mc}}} \times 100\% \quad (1)$$

A: adhesion;

$N_{\text{total Mc}}$: Number of total monocytes;

$N_{\text{not adhering Mc}}$: Number of nonadhering monocytes.

VSMC Proliferation Assay with MTT[12] Rat aortic VSMC 80 μl (1×10^5/ml) subcultured to the third passage were placed in every well of 96-well plates incubated in the CO_2 incubator at 37°C. When the cells adhered, corilagin, Dgg16, or vitamin E at the dose of 0.0001, 0.001, 0.01, or 0.1 mmol/l was added to every well. ox-LDL (50 mg/l) was added 8 h later. Once they were incubated for another 12 h, 20 μl of MTT (5%) was added and incubated for an additional 4 h. The viable cells were stained with MTT scanned with the clinical electrophotometer at 570 nm, and the number of VSMC was counted based on their absorption.

Statistical Analyses Values are expressed as mean±S.E. One-way ANOVA was performed to compare the means with the control group. Statistically significant differences were accepted at $p<0.05$

or <0.01. The concentration of the three agents was transformed to logarithmic (Lg) value except for zero in statistical figures.

RESULTS

Corilagin and Dgg16 decreased MDA in ECV-304 Cells Culture Media Without treatment or at 0.0001 mmol/l in every group, MDA due to ox-LDL was high, and when corilagin or Dgg16 was added, MDA decreased significantly. Corilagin or Dgg16 was more potent than the control (vitamin E) at the same dose, and showed dose dependence (Fig. 2).

Corilagin or Dgg16 Prevented Endothelial ECV-304 Cells Adhesion ox-LDL was able to activate monocytes adhering to endothelial cells, and when corilagin or Dgg16 was added, adhesion decreased quickly at the dose of 0.001 mmol/l or higher. Vitamin E had less effect, although it had a similar maximal effect of about 55% (Fig. 3).

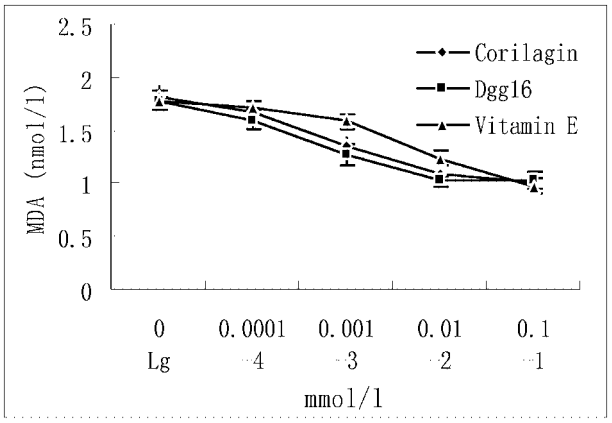

Fig. 2. Corilagin and Dgg16 Decreased MDA in ECV-304 Cells Culture Media (Mean±S.E., $n=6$)

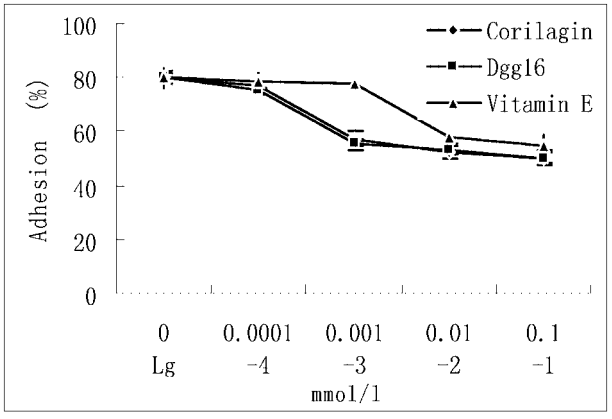

Fig. 3. Corilagin and Dgg16 Decreased Adhesion of ECV-304 Cells and Monocytes (Mean±S.E., n=6)

Corilagin or Dgg16 Inhibited VSMC Proliferation In culture media containing ox-LDL 50 mg/l, either corilagin or Dgg16 powerfully inhibited VSMC proliferation. Their positive control, vitamin E, also inhibited VSMC proliferation, but relatively weakly (Fig. 4(A)). To verify whether corilagin or Dgg16 was able to inhibit VSMC proliferation alone, another experiment was carried out. VSMC with corilagin, Dgg16, or vitamin E were added to the medium free of ox-LDL, and VSMC proliferation showed almost no change (Fig. 4(B)).

DISCUSSION

The pathogenesis of atherosclerosis is not completely clear, however, two possible mechanisms have

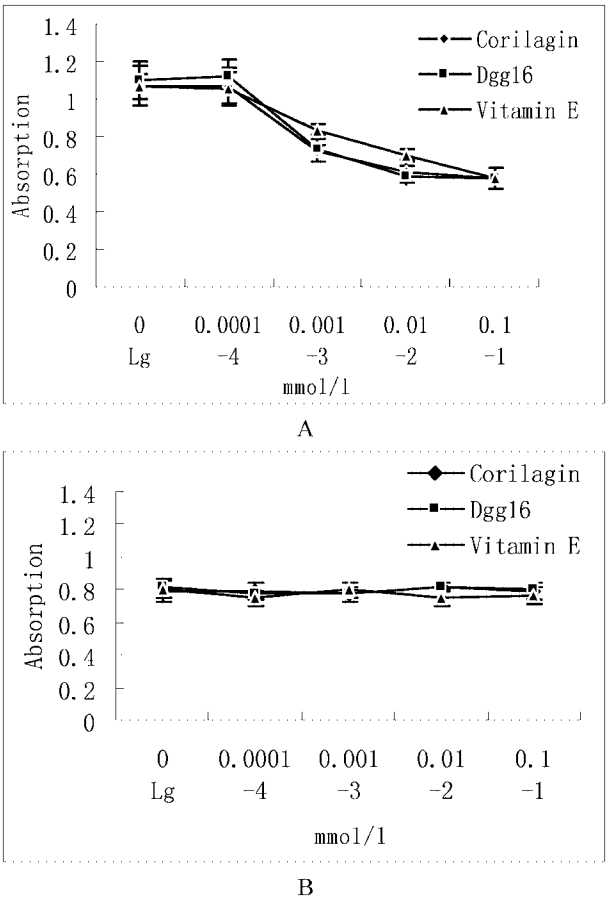

Fig. 4. Corilagin or Dgg16 Effects on Aortic VSMC Proliferation (Mean±S.E., $n=6$)

VSMC proliferation was measured as their absorbance of MTT. A: ox-LDL stimulated VSMC proliferation at 50 mg/l. At 0 or 0.0001 mmol/l, there were no significantly different effects between corilagin, Dgg16, and vitamin E. But at the dose of 0.001 mmol/l or higher, all inhibited VSMC proliferation ($p<0.01$) and showed a logarithmic dose dependence. Corilagin and Dgg16 were more effective than the control. B: Without ox-LDL, corilagin or Dgg16 showed almost no effect on VSMC proliferation ($p>0.05$), and neither did control vitamin E.

been proposed.[2] One is endothelial injury caused by free radicals. The injured cells are assumed to trigger monocytes or other inflammatory cells like macrophages adhering to them, and then inflammatory cells infiltrate inside the vessel wall and enclose the lipid or lipoid. When they devour adequate lipid or lipoid, they transform themselves into foam cells, and atherosclerosis begins. The other mechanism is associated with proliferation of the smooth muscle cells beneath the endothelium, which are usually activated by inflammatory cells or other factors. This mechanism is credible because if VSMC could not proliferate, atherosclerosis would end in the state of preatherosclerosis. In general, the two mechanisms were regarded as two consecutive states of the pathology, namely, two vital links in the atherosclerosis chain, either one or both of which if broken will halt the progress of atherosclerosis. Oxidation injury begins, monocyte adhesion follows, and VSMC proliferation is inevitable.

ox-LDL is a powerful oxidant to biomembranes. ox-LDL can produce, or help to produce, free radicals. MDA, regarded as an indicator of membrane injury, is a relatively stable intermediate product yielded during such reactions. The present data indicate that corilagin and Dgg16, also scavengers for free radicals, were able to inhibit lipid oxidation, prevent endothelial injury, and prevent endothelia from being adhered to by monocytes in a dose dependant manner. The results support the fact that ox-LDL is not only a powerful activator in endothelial injury but also an effective trigger of VSMC proliferation. The present study also verified that VSMC proliferation and monocyte adhesion are associated with oxidation injury. The potency of corilagin, Dgg16, and vitamin E in preventing proliferation and adhesion are in proportion to their antioxidant activity to a certain extent. Therefore, the results suggest that the two natural chemicals and vitamin E can break both vital links of the atherosclerosis chain as well as the bioreducer probucol.[3] The results agreed with the report that corilagin can scavenge hydroxide superoxide anion radicals and hydrogen peroxide.[13] But the antioxidation of Dgg16, never reported before, has similar antiatherogenic effects to those of corilagin.

It has been reported that the expression of some chemotaxis cytokines, *e.g.*, vascular cell adhesion molecules,[14,15] intercellular adhesion molecules,[14] and monocyte chemoattractant protein,[14] are upregulated in injured endothelial cells caused by ox-LDL, which attract monocytes or other inflammatory cells, and immediately damage more endothelial cells, and then a vicious circle begins. Corilagin and Dgg16, as well as vitamin E, are able to alleviate adhesion by preventing endothelial cells from oxidation injury, which support the hypothesis.

Another experiment[16] showed that the ox-LDL molecules can infiltrate inside the vessel wall easily; they not only stimulate VSMC secretion, but also activate their proliferation, migration into the tunica media of the artery, and swallowing of abundant lipid or lipoid, eventually becoming foam cells. How ox-LDL triggers such a chain of reactions is still not well understood. It has been reported that ox-LDL can upregulate the expression of proliferating cell nuclear antigen (PCNA) that facilitates control proteins related to the cell cycle, like P_{27}, and P_{57}, binding to PCNA,[17] which activates c-myc or other gene expression, and allows VSMC allometric proliferation and anomalistic differentiation.[18] As far as promoting smooth muscle proliferation is concerned, these results suggest that bioreducers like vitamin E, corilagin, and Dgg16 inhibit VSMC proliferation mainly by their intrinsic reduction rather than by other effects, although corilagin has other mechanisms against atherosclerosis,[7,8] since corilagin and Dgg16 were not able to affect routine VSMC proliferation without ox-LDL stimulation.

The fruit of Phy is effective in treating diseases associated with atherosclerosis in traditional Chinese medicine. Modern science agrees with the theory that the effect of an herb may be caused by a chemical or a group of chemicals. Corilagin and Dgg16, easily binding to lipid or lipoid, are the main soluble components in the fruit of Phy and have many reductive phenol hydroxide groups.[6] The hydroxides in them are useful free radical scavengers like vitamin E or probucol.[3] As has been reported, corilagin can inhibit platelet aggregation[7] and local inflammation,[8] which may also participate in its antiatherogenic effects.

In summary, the two compounds from Phy are effective in inhibiting the progress of atherosclerosis by alleviating oxidation injury and by inhibiting ox-LDL-induced VSMC proliferation, which may be promising mechanisms for Phy.

Acknowledgments We thank Prof. I. Kouno, T. Tanaka (Faculty of Pharmaceutical Sciences, Nagasaki University, Japan) and Y.J. Zhang, C.R. Yang (Laboratory of Phytochemistry, Kunming Institute of Botany, Chinese Academy of Science, China) for providing the standard compounds of corilagin and its analogue.

The study is supported by Mega-project of Science Research for the 10th Five-year Plan (No. 2002AA2E3785) of Ministry of Science and Technology of PRC.

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