Research Article


datachip2 and geniposide inhibit the development of atherosclerosis by increasing Wnt1 and inhibiting dickkopf-related protein-1 expression

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Abstract

Background  Our previous study showed that the combined Chinese herbs containing scutellaria baicalensis georgi and gardenia jasminoids ellis inhibited atherosclerosis. In this study, we sought to determine if baicalin and geniposide could inhibit atherosclerosis through Wnt1 and dickkopf-related protein-1 (DKK1).

Methods  The wild-type and ApoE−/− mice were treated with baicalin, geniposide, and baicalin plus geniposide daily by gavage for 12 weeks. Blood lipid levels were measured with an automatic biochemistry analyzer. Aortic atherosclerotic lesion areas were analyzed with Image-ProPlus software. The mRNA and protein expression of DKK1, Wnt1 and nuclear factor-xB (NF-xB) were measured with RT-PCR and Western Blot. Serum levels of interleukin-12 (IL-12) were quantified with ELISA.

Results  The baicalin or geniposide monotherapy as well as combination therapy inhibited the development of atherosclerotic lesions, increased Wnt1 and decreased DKK1 expression and elevated the ratio of Wnt1/DKK1 compared with high-lipid diet group. However, only baicalin or geniposide monotherapy decreased NF-xB expression. Moreover, baicalin and geniposide mono- or combination therapy lowered IL-12 levels. Geniposide reduced both serum total cholesterol and low density lipoprotein levels, while baicalin either alone or in combination with geniposide did not affect serum lipid levels.

Conclusion  Baicalin and geniposide exert inflammation-regulatory effects and may prevent atherosclerotic lesions through enhancing Wnt1 and inhibiting DKK1 expression.


Keywords: Atherosclerosis; Baicalin; DKK1; Geniposide; Wnt1

1 Introduction

Atherosclerosis is the number one cause of death and disability worldwide. Pathologically, this disease is characterized with endothelial dysfunction, vascular inflammation, and buildup of lipids in the vessel intima, leading to the formation of lipid plaque, and so far the “response-to-injury” theory for atherosclerosis is widely accepted. This pathophysiological process is also accompanied by migration and proliferation of vascular smooth muscle cell, as well as endothelial activation triggered by multiple inflammatory pathways.[1]

The Wnt signaling pathway, a highly conserved cellular communication system, is involved in diversified development and physiological processes.[2] Wnt signaling exists with 19 different Wnt ligands that bind and signal through G-protein-coupled receptors of the Frizzled (Fzd) family.[3] Recent evidence shows that Wnt signaling participates in inflammatory regulation.[4] Endogenous inhibitors, in particular dickkopf-1 (DKK-1), control the Wnt pathways. As part of a negative feedback loop, however, DKK1 antagonizes the canonical pathway by inhibiting the interaction of Wnts with low density lipoprotein receptor-related protein 5/6. Meanwhile, DKK1 itself is inhibited by Wnt signaling.[5,6]

Many Wnt proteins are involved in the procedure of atherosclerosis. Wnt1 promotes proliferation of cultured human endothelial progenitor cells and angiogenic function, indicating that Wnt1 plays an anti-atherosclerotic role.[7] Other clinical investigation found decreased Wnt1 levels in patients with premature myocardial infarction.[8] DKK1 has recently been considered as a biomarker for atherosclerosis, the expression of which is increased in atherosclerotic lesions, resulting in an inflammatory response, activation of endothelium, and promotion of coronary atherosclerosis and acute ischemic stroke.[9–11] Whereas it is not different at
baseline compared with controls and significantly increased at one-year follow-up in premature myocardial infarction patients.\textsuperscript{18} Taken together, the Wnt signaling pathway is involved in the process of atherosclerosis.

We have previously found that the micrometer compound rhizoma coptidis containing scutellaria baicalensis georgi and gardenia jasminoids ellis significantly decreased lipid deposition and inhibited the formation of foam cells in the wall of aorta.\textsuperscript{12} Baicalin (C\textsubscript{21}H\textsubscript{16}O\textsubscript{11}), a monomeric flavonoid compound, and geniposide (C\textsubscript{17}H\textsubscript{26}O\textsubscript{10}), a monomeric iridoid glycoside compound, are isolated from scutellaria baicalensis georgi and gardenia jasminoids ellis. Both baicalin and geniposide exert anti-inflammatory effects. Baicalin, for instance, inhibits the nuclear factor-κB (NF-κB) activation.\textsuperscript{13} Baicalin and geniposide also have potential anti-atherosclerotic effects by upregulating foxp3 expression, promoting Treg cells number and function, decreasing dendritic cell number, and inhibiting dendritic cells maturation in bone marrow and subsequent infiltration into lesions.\textsuperscript{14,15}

Using transgenic ApoE\textsuperscript{−/−} mice, the present study sought to determine whether the anti-atherosclerotic effects of baicalin and geniposide acted through regulation of the Wnt signaling pathway. Moreover, we investigated the changes in the blood lipid, aortic expression of the NF-κB, and serum interleukin 12 (IL-12) levels in order to determine the roles of other atherosclerosis-linked factors in the effects of those two compounds.

2 Methods

2.1 Drug administration

Baicalin (CAS Number: 21967-41-9; purity, 98%; molecular formula, C\textsubscript{21}H\textsubscript{16}O\textsubscript{11}; molecular weight, 446.36), and geniposide (CAS Number: 24512-63-8, purity, 98.5%; molecular formula, C\textsubscript{17}H\textsubscript{26}O\textsubscript{10}; molecular weight, 388.37) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in normal saline, then adjusted pH to 7.4 with NaOH.

2.2 Animal models

Eight-week-old male C57BL/6J and ApoE\textsuperscript{−/−} transgenic mice (Bar Harbor, Maine, USA; 23–26 g), were fed at the Animal Center of Huazhong University of Science and Technology, following the Guide for the Care and Use of Laboratory Animals, published by the US NIH. Following adaptation to their environment for one week, the ApoE\textsuperscript{−/−} mice were divided randomly into one of four groups: high-lipid diet group (HLD group) fed with a high-lipid diet containing 1.25% cholesterol and 10% coconut oil; baicalin group (BAI group) fed with a high-lipid diet and baicalin (100 mg/kg per day, 200 μL/kg per day); geniposide group (GEN group) fed with a high-lipid diet and geniposide (100 mg/kg per day, 200 μL/kg per day); or a combined group (BAI+GEN group) fed with a high-lipid diet plus a combination of baicalin (100 mg/kg per day, 200 μL/kg per day) and geniposide (100 mg/kg per day, 200 μL/kg per day). Wild-type (WT) male mice served as the normal control (NC group) and were fed with standard mouse food. The animal in HLD and NC group received normal saline and the other animals received homologous drug by gavage feeding once daily for 12 weeks. The mice were euthanized by chloral hydrate after treatment for 12 weeks. The blood was collected by the angular vein using a capillary siphon and centrifuged at 1000 g for 5 min and harvested the serum for lipid and IL-12 assay. The heart with aortic root and the surplus thoracic aorta were harvested and immersed into liquid nitrogen for atherosclerotic lesions evaluation, western blot and RT-PCR, respectively.

2.3 Blood lipid measure

The automatic biochemistry analyzer (Olympus AU2700, Japan) was used to measure the levels of total cholesterol (TC), triglyceride (TG), and high-density lipoprotein cholesterol (HDL-C). The level of low-density lipoprotein cholesterol (LDL-C) was calculated with the Friedewald formula.\textsuperscript{16}

2.4 The evaluation of atherosclerotic lesions

Aortic lesion areas stained by oil red O were quantified using 5μm cryosections of the aortic root and using Paigen’s method\textsuperscript{17} with Image-ProPlus software. For each section, we chose five visual fields of light microscopy in plaque, respectively. The mean was calculated as lesion area.

2.5 RT-PCR

Following the manufacturer’s instruction, total RNA was isolated with Trizol reagent (Invitrogen, USA). RT-PCR assay was operated following protocol of SYBR PrimeScript RT-PCR kit (Takara, Japan). The expressions of Wnt1, DKK1 and NF-κB mRNA were calculated using the comparative Ct method formula 2\textsuperscript{−ΔΔCT}. Finally, the levels were normalized to β-actin. PCR primers were listed in Table 1.

<table>
<thead>
<tr>
<th>Table 1. Primers used in RT-PCR.</th>
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<tbody>
<tr>
<td>Wnt1 237 bp</td>
</tr>
<tr>
<td>Forward: 5′-CATCTTGGCAATCCTTCGG-3′</td>
</tr>
<tr>
<td>Reverse: 5′-GCCCTGTTGTGGTGAAGTT-3′</td>
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<tr>
<td>DKK1 205 bp</td>
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<tr>
<td>Forward: 5′-TACCTTGGCTGAGATGAA-3′</td>
</tr>
<tr>
<td>Reverse: 5′-CTCGAGAAAATGGCTGTGG-3′</td>
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<tr>
<td>NF-κB 196 bp</td>
</tr>
<tr>
<td>Forward: 5′-ACCTGGCTACTGTTGCAAC-3′</td>
</tr>
<tr>
<td>Reverse: 5′-TCTCCTGAGAGACCTTGGGA-3′</td>
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<tr>
<td>β-actin 240 bp</td>
</tr>
<tr>
<td>Forward: 5′-CAGCATGAGGGGGCCGAGGAT-3′</td>
</tr>
<tr>
<td>Reverse: 5′-TAAGACCTCTATGCAACAGAT-3′</td>
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2.6 Western blot for Wnt1, DKK1 and NF-κB

Protein expression in the aorta was detected by Western blot, using the antibodies for rabbit anti-Wnt1, rabbit anti-DKK1 and rabbit anti-NF-κB (Santa Cruz, USA). Total protein (30 μg) per sample was loaded on 10% SDS-PAGE and transferred onto a PVDF membrane (Millipore, Billerica, MA, USA). The membrane was blocked for 1 h with 5% skimmed milk in Tris-Buffered Saline Tween-20 [50 mmol/L Tris, 0.15 mol/L NaCl (pH 7.5)] containing 0.1% Tween 20, and then incubated with the 1: 2000 primary antibody (Wnt1, DKK1 and NF-κB) at 4°C overnight, followed by secondary HRP-conjugated antibodies (1: 5000). Protein expression was probed using enhanced chemiluminescence (Amersham Pharmacia Biotech) per manufacturer’s instructions. Equal protein loading was normalized to β-actin.

2.7 Immunofluorescence

The culture of human umbilical vein endothelial cells (HUVECs), the extraction of oxidation of LDL-C were executed according to our previous methods.[18] HUVECs were plated into 24 wells plate in DMEM medium with 10% FBS for 24 h. The cells were divided into five groups: control group, oxidized low density lipoprotein (ox-LDL) group (50 mg/L), ox-LDL+baicalin (5 mg/mL) group, ox-LDL+geniposide (5 mg/mL) group and ox-LDL+baicalin (5 mg/mL)+geniposide (5 mg/mL) group. After 48 h incubation, the cells were fixed with 4% paraformaldehyde for 20 min, incubated in 0.3% Triton X-100-PBS for 10 min at room temperature, followed by blocking with 5% goat serum at 37°C for 30 min. The cells were then incubated with rabbit anti-Wnt1 and rabbit anti-DKK1 antibody (1: 200) at 4°C overnight. The samples were soaked in 20% paraformaldehyde for 20 h with 4% paraformaldehyde, and then incubated with the 1: 2000 primary antibody (Wnt1, DKK1 and NF-κB) at 4°C overnight, followed by secondary HRP-conjugated antibodies (1: 5000). Protein expression was probed using enhanced chemiluminescence (Amersham Pharmacia Biotech) per manufacturer’s instructions. Equal protein loading was normalized to β-actin.

3 Results

3.1 Effects of baicalin and geniposide on atherosclerotic lesion area

Atherosclerotic lesions were stained with oil red O after administration of normal diet or high lipid diet plus baicalin or/and geniposide fed to both WT and ApoE−/− mice (Figure 1). ApoE−/− mice showed significantly increased atherosclerotic lesions, whereas NC group mice had little atherosclerotic lesions (HLD vs. NC, P < 0.01). The atherosclerotic lesions in BAI group, GEN group and BAI+GEN group also increased as compared with the NC group (P < 0.01), but decreased as compared with HLD group mice (P < 0.01). However, there were no significant changes in the atherosclerotic lesion area among BAI group, GEN group and BAI+GEN group (Figure 1).

3.2 Effects of baicalin and geniposide on blood lipids

Serum TC and LDL-C were increased in the BAI group, GEN group, BAI+GEN group and HLD group compared to the NC group after treatment for 12 weeks (Figure 2). TC and LDL-C levels were significantly lowered in GEN group compared to HLD group (P < 0.05). There were no differences between BAI group and BAI plus GEN group. The ApoE−/− mice had lower levels of HDL-C and higher TG due to disturbance of lipid metabolism compared to those of the NC group (Figure 2).

3.3 Effects of baicalin and geniposide on mRNA and protein expression

The mRNA and protein of Wnt1, DKK1 and NF-κB were all increased in the HLD group compared with the normal diet group after twelve weeks. BAI or GEN mono-therapy, and BAI+GEN treatment enhanced Wnt1 mRNA and protein expression. BAI or GEN mono-therapy, and BAI plus GEN treatment, however, lowered expression of DKK1 mRNA and protein compared to HLD group (all P < 0.05). The NF-κB mRNA and protein were lowered significantly in BAI and GEN mono-therapy group (all P < 0.05) as well. The ratio of Wnt1/DKK1 was elevated significantly in BAI, GEN, and BAI plus GEN group compared to HLD group (all P < 0.05). However, there was no difference in the ratio between HLD group and the NC group (Figures 3 & 4).

3.4 Effects of baicalin and geniposide on Wnt1 and Dkk1 in HUVECs

Ox-LDL decreased Wnt1 expression and increased DKK1 protein expression in HUVECs. After treatment with baicalin, geniposide+baicalin+geniposide, the effects of ox-LDL on Wnt1 and Dkk1 were reversed (Figure 5).
Figure 1. Staining imaging of atherosclerotic lesion areas in aortic root stained with oil red O (× 100 times). (A): NC group; (B): HLD group; (C): BAI group; (D): GEN group; (E): BAI+GEN group; and (F): the area of atherosclerotic lesion. *P < 0.01 vs. NC group; †P < 0.01 vs. BAI, GEN and BAI+GEN group; N = 5 per group. BAI: baicalin; GEN: geniposide; HLD: high-lipid diet; NC: normal control; WT: wild-type mice.

Figure 2. The effects of baicalin and geniposide on levels of blood lipids. *P < 0.01 vs. NC group; †P < 0.05 vs. HLD group; n = 5 per group. BAI: baicalin; GEN: geniposide; HDL-C: high-density lipoprotein cholesterol; HLD: high-lipid diet; LDL-C: low-density lipoprotein cholesterol; NC: normal control; TC: total cholesterol; TG: triglyceride; WT: wild-type mice.
3.5 The effect of baicalin and geniposide on IL-12 levels

There was significantly lowered IL-12 expression in BAI, GEN, and BAI+GEN groups compared to the HLD group (Figure 6).

4 Discussion

Our results showed that baicalin, geniposide and baicalin plus geniposide all attenuated the formation of atherosclerotic plaque in high-lipid diet group, compared to that in untreated control group. This piece of data is consistent with that we have previously observed. The anti-atherosclerotic mechanism of baicalin and geniposide may involve increasing Wnt1 and the ratio of Wnt1/DKK1, inhibiting inflammation or adjusting blood lipid according to our results. To our knowledge, this is the first study to find that geniposide and baicalin can inhibit atherosclerosis through regulating the Wnt1 and DKK1 expression.

Baicalin and geniposide are known to exert anti-inflammatory and immune-regulatory actions. They are widely used to treat the inflammatory diseases. Many studies have confirmed that baicalin and geniposide possess the anti-atherosclerotic roles by inhibiting inflammation, reducing oxidative stress, and regulating blood lipid. Geniposide dramatically lowered the serum TC and LDL-C. Neither baicalin alone nor combined with geniposide lowered the TC and LDL-C levels compared with the high-lipid diet group mice. It seems that baicalin damaged the regulating lipid potential of geniposide because the TC and LDL-C levels
levels had an increasing tendency in baicalin plus geniposide group compared with geniposide group.

Atherosclerosis is a chronic progressive disease derived from vascular inflammation due to vascular endothelial damage. Recent reports have confirmed that the Wnt signaling play a role in regulating inflammation. The canonical

**Figure 4.** The effects of baicalin and geniposide on mRNA expression of Wnt1, DKK1, NF-κB and relative expression of Wnt1/DKK1. *P < 0.05 compared with NC group; †P < 0.05 compared with HLD group; n = 4 per group. BAI: baicalin; DKK1: dickkopf-related protein-1; GEN: geniposide; HLD: high-lipid diet; NC: normal control; NF-κB: nuclear factor-κB; WT: wild-type mice.

**Figure 5.** The effects of baicalin and geniposide on Wnt1, DKK1 expression in HUVECs stimulated by ox-LDL. The Wnt1 and DKK1 located in cytoplasm. BAI: baicalin group; DKK1: dickkopf-related protein-1; GEN: geniposide group; HUVECs: human umbilical vein endothelial cells; ox-LDL: oxidized low density lipoprotein.
Wnt/β-catenin pathway activation could accelerate adhesion and the trans-endothelial migration of monocytes, regulate monocyte and macrophage inflammatory response, and induce the survival of endothelial cells. All these findings indicate complicated roles of Wnt pathway in atherosclerosis. The study reveals the changes in Wnt1 and DKK1 signals in atherosclerotic mice induced by a high-lipid diet and the effects of baicalin and geniposide on atherosclerotic plaque and Wnt1, DKK1 expression.

High DKK1 levels are found in atherosclerotic plaques and it could activate the platelet-mediated inflammation in atherogenesis and destabilize atherosclerotic plaque. We found that a high-lipid diet enhanced the DKK1 mRNA and protein expression accompanied with Wnt1 mRNA and protein expression increase compared with the normal diet group. But the increased degree of DKK1 was higher than that of Wnt1. So the ratio of Wnt1/DKK1 mRNA and protein had no difference between normal and high-lipid diet group, which indicated that high-lipid diet exacerbated the atherosclerosis through increasing DKK1 expression. The reasons for Wnt1 increase maybe the feedback mechanism to withstand the deleterious role of DKK1. After administering baicalin and geniposide alone or combined, the Wnt1 mRNA and protein expression was further elevated and DKK1 mRNA and protein expression decreased. The ratio of Wnt1/DKK1 was further enhanced compared with the high-lipid diet group, which was the first discovery that baicalin and geniposide could regulate the Wnt signal pathway to inhibit atherosclerosis progression. Unexpectedly, mRNA and protein of Wnt1 and DKK1 in baicalin plus geniposide group were not further increased or decreased compared to baicalin or geniposide monotherapy group. This suggested that the baicalin combined geniposide treatment had contradictory effects on Wnt1 and DKK1 expression similar to the effects on blood lipid. Ox-LDL accelerated atherosclerosis development through activating NF-κB. We observed Wnt1 and DKK1 expression in HUVECs induced by ox-LDL to further confirm the roles of Wnt1 and DKK1 in this procedure. In HUVECs, we found that ox-LDL decreased Wnt1 and increased DKK1 protein expression. After treatment with baicalin, geniposide and baicalin plus geniposide, the effects of ox-LDL on Wnt1 and DKK1 were reversed, which indicated that Wnt1 and DKK1 involved in the development of atherosclerosis.

The function of Wnt proteins is quite different, based upon its subtypes. For example, Wnt4 is able to inhibit the NF-κB activation via noncanonical Wnt signaling in the mouse model, but Wnt5a induces inflammation by activating the NF-κB transcriptional pathway in vascular endothelial cells. These studies indicated that the Wnt signal also regulate the NF-κB activity. Our results show that the high-lipid diet remarkably increased NF-κB mRNA and protein expression, which was reversed by baicalin or geniposide. This was accompanied by enhanced Wnt1, attenuated DKK1 and parallel increase in Wnt1/DKK1 ratio. We therefore speculate that Wnt1 could be an inhibitor of the NF-κB activation. Although the mechanism of baicalin or geniposide in inhibiting the NF-κB expression remains unclear, it might be linked with the increased Wnt1 signaling. This should be further confirmed in the future investigation.

As long as from the results of blood lipid changes, the combination of baicalin and geniposide cannot further inhibit the NF-κB expression, which indicates that baicalin and geniposide had overlapping effects on Wnt1 and DKK1. The down-regulation of NF-κB contributes to anti-inflammatory effects of baicalin and geniposide, since the activated NF-κB modulates numerous signals that mediates inflammation and the development of atherosclerosis. We found that baicalin and geniposide also inhibited the IL-12 level after lowering the NF-κB expression. Studies found that IL-12 contributed to the atherosclerotic plaque form and accelerated the progression of diabetic macrovascular complications. So both baicalin and geniposide possess the anti-atherosclerotic potential via inhibiting the inflammation.

In conclusion, our results demonstrate that while baicalin and geniposide enhance Wnt1 signaling, they attenuate DKK1 expression, which is associated with the inhibition of transcription factor NF-κB and maybe further inhibit downstream cytokine expression. Taken together, all these ac-
tions by baicalin and geniposide may contribute to the mechanisms underlying their anti-atherosclerotic actions in vivo observed. Moreover, geniposide also significantly lower the total serum levels of cholesterol and LDL-C, but baicalin seems to counteract this action. Thus, baicalin or geniposide might be useful for clinical treatment of atherosclerotic diseases.

Acknowledgements

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