行政院國家科學委員會專題研究計畫成果報告
豬隻冠狀動脈再阻塞之基因療法

The Molecular Mechanisms Of Ginsenoside G115 On Balloon Injury-Induced Neointimal Formation

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摘要

在美國每年大約有百分之 30 **至** 50(**約二十五萬)的病人，在接受冠狀動脈氣球擴張術的治療之後，六個月之內有血管再阻塞的現象。其病因可能是由於機械性的破壞，再加上自由基之釋放，血液中生長因子的刺激，進而促使平滑肌細胞的增生。由於人蔘在心血管方面有良好的保護作用，再加上它的抗氧化作用，使得我們認為人蔘在避免氣球擴張所引起之血管再阻塞方面應扮演一重要角色。本篇實驗分成體內及體外兩種研究方式。在體外冷光實驗中，大鼠平滑肌細胞的細胞分裂速率能被人蔘抽取物** G115 **降低約** 24%。**另外在離體的實驗中, 利用** Norepinephreine **所誘導的血管收縮分別受到** 1.44mg/ml **及** 2.88mg/ml **的** G115 **抑制達** 21%**及** 44%。**而體內的動物實驗，藥物在手術前一個星期和後兩個星期給予，被氣球破壞的血管作橫相切片後並予以染色，從結果得知** G115 **能有效避免血管內層細胞再增生的比例為** 77.3%。**這些結果顯示人蔘在心血管方面能提供保護作用，對於必需接受氣球擴張手術的病人而言，提供了一種預防性的治療選擇。**

Abstract

After percutaneous transluminal coronary angioplasty (PTCA), 30-50% of the patients may present with restenosis within 6 months. The aim of this study was to search for a preventive remedy against the balloon injury-induced neointima formation. Ginseng, with its wide indications on immune and cardiovascular functions, has prompted us to explore its role in neointima formation. In the present study, we aimed to explore if a standardized *Panax Ginseng* extract G115 was able to inhibit neointimal formation. With BrdU luminencence assay, maximal proliferation of rat smooth muscle cells was reduced to 24% of control values by G115. Norepinephrine-induced vasocontraction was antagonized in 21% and 44% by 1.44mg/ml and 2.88mg/ml of G115, respectively. Neointima-to-lumen area ratio of balloon-injured rat carotid arteries was reduced 77.3% by G115 as compared to the sham control. These results demonstrate the preventive effects of ginsenosides on angioplasty-mediated neointima formation.

Keywords: Restenosis, Balloon Injury, Ginseng, G115

II、Background and Specific Aims

Restenosis is the angiographic narrowing at the angioplasty site in 30-50% of patients within 6 months of PTCA without stenting of the arteries, frequently accompanied by recurrence of angina symptom [7]. Therefore, even though PTCA has been proven to be an effective therapy for saving people from myocardial infarction, restenosis has limited the benefit of angioplasty in clinical application. In many animal studies, it was found that gene therapy in dilating vessels or regulating NO synthesis was effective in preventing restenosis [8,9]. However, gene therapy is still away from perfection due to unsatisfied gene transferring efficiency and cytotoxicity. We therefore aimed to search for a pharmacological agent in preventing abnormal cell proliferation as well as inducing vascular relaxation to suppress balloon injury-induced restenosis.

Panax Ginseng has long been used in Chinese since Han Dynasty about 2000 years ago. Its main indications are to enhance stamina and

capacity to cope with fatigue and physical stress [10]. The major active principles of *Panax Ginsen* are ginsenosides, the derivatives of the triterpene dammarane structure [11,12]. Minor components are amino acids, peptides, and minerals. There have been about 20 different ginsenosides extracted from roots, leaves, and flower buds of ginseng [11]. Aglycones of the common ginsenosides are 20(S)-protopanaxadiol (Rb1, Rb2, Rc, and Rd) or 20(S)-protopanaxtriol (Re, Rf, Rg1, and Rg2) structures, whose nomenclatures derive from the mobility of the ginsenosides in a one-dimensional thin-layer chromatographic system [13-15]. Various sugar moieties are found present in *Panax Ginseng*, including glucose, maltose, fructose, and saccharose. Conventional HPLC, as well as electrospray HPLC and mass spectrometry are now available to quantitate and purify ginsenosides [14-16].

Activity analysis of ginseng revealed that the ginsenoside content depends on the species of ginseng, the manner of sample preparation, and the age and part of the plant extracted. The current available ginseng preparations may therefore differ greatly in pharmacological aspects. To provide the public with reliable information on ginseng preparations, American Botanical Council has analyzed several hundred of ginseng products using standard HPLC techniques. These results have encouraged the publics and research groups to use standardized ginseng to gain consistent effects. G115, a specific ginseng preparation used in many studies, is standardized to contain 4% ginsenosides [13,17].

It is generally believed that the injury-induced restenosis is partly resulted from the uncontrolled smooth muscle cell proliferation and vascular contraction due to endothelial dysfunction [18]. Souza et al. [19] indicate that excessive free radical generation early after arterial balloon injury may also account for restenosis. This is supported by the finding of Libby and Ganz [20] that reactive oxygen species enhance transcription of nuclear factor (kappa) B in balloon-injured cells resulting in smooth-muscle-cell migration, replication, and accumulation and remodeling of extracellular matrix. Ginsenoisdes has been reported to prevent proliferation of vascular smooth muscle cells from rats [21]. A similar proliferation-inhibitory activity was also found in mesangial cell cultures by ginsenosides [22]. In addition to the anti-proliferative effects, ginsenosides are also found to enhance acetylcholine-induced vascular relaxation [23]. Due to the extensive effects of ginsenoside on cell proliferation and vascular relaxation, it is then feasible to propose that G115 may play a critical role in preventing balloon injury-induced restenosis. In order to assess the preventive effects of standardized ginseng G115 against restenosis, in vitro smooth muscle cell proliferation, ex vivo vascular relaxation, and in vivo neointimal formation affected by G115 were evaluated in the present study.

III、Results and Discussion

Quantitative analysis of the ginsenoside content in G115

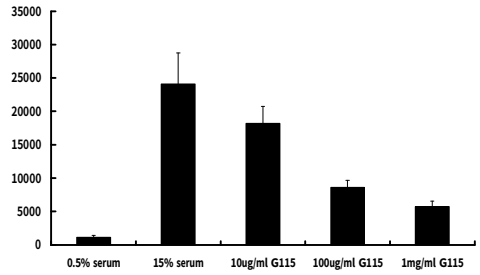
G115 was known as a standardized ginsenoside mixture. Five concentrations of Rb2 (25, 50, 62.5, 125, 250 ppm) subjected to HPLC analysis resulted in peak height of 4325, 9685, 12157, 23347, 41815 RU (relative units), respectively. A standard curve was then constructed accordingly.HPLC chromatograms demonstrated the content of Rb2 in standardized G115 to be 86.46 $\pm$ 1.30 ppm or 84.46 $\pm$ 1.30 μg/ml (n=3).

Chemiluminescence analysis of ginsenosides on smooth muscle cell proliferation

G115 was first tested *in vitro* for its antiproliferative effects on culture smooth muscle cells prior to its *in vivo* analysis. BrdU incorporation using chemiluminescence immunoassay was a sensitive and alternative approach to the [^{3}H]-thymidine incorporation assay in assaying DNA synthesis. Cells were stimulated with 15% serum in the presence of 10μg/ml, 100μg/ml, and 1mg/ml of G115 for 20 hours at 37^{o}C followed by 4 hours of BrdU pulse incubation. Cells with more BrdU incorporation into DNA emitted stronger luminencesce upon reacting with luminol and

4-iodophenol substrates. While 10μg/ml of G115 did not demonstrate a significant inhibition on cell proliferation, the maximal intensity of luminescence caused by 15% serum induction was significantly reduced to 64% and 76% of control by 100μg/ml and 1mg/ml of G115, respectively (Figure 1, n=5, $p< 0.05$).

Figrue 1.



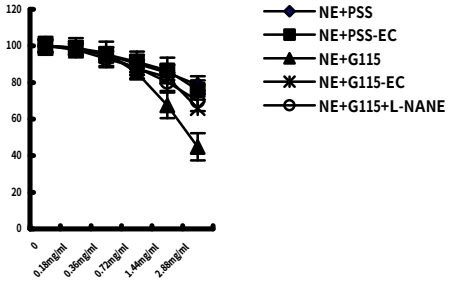
Antagonism of G115 against the inotropic effects of norepinephrine on aortic strip

To determine if the preventive effects of G115 on balloon injury-induced stenosis are mediated by vasodilation, spirally cut strips of rat thoracic aorta were subjected to co-incubation of norepinephrine and G115. G115 reduced the norepinephrine-induced vasocontraction in a dose-dependent manner. At concentrations lower than 0.72 mg/ml, G115 showed no inhibition on the inotropic effects of norepinephrine (10^{-7} M). While the doses of G115 increased to 1.44 and 2.88 mg/ml, the percentage of maximal contraction in reference to the PSS control was reduced by 21% and 44%, respectively (Figure 2, n=8, $p<0.05$). Such vascular relaxing effects of G115 were reversed when vessels were denuded on endothelium or pretreated with NO synthase inhibitor, L-NAME (Figure 2).

In vivo assay of ginsenosides on balloon injury-induced restenosis

Rats pretreated with low dose (2mg/kg) and vhigh dose (200mg/kg) of G115 were killed

Figure 2



two weeks after balloon injury. The carotid arteries were isolated and sectioned for hematoxylin and eosin staining. As shown in figure 4, panel A demonstrated the normal carotid artery without neointima formation. There was a significant neointima formation over the medial layer of the balloon-injured vessel (Figure 3B). The thickness of the neointimal layer was significantly reduced by either low or high dose of G115 (Figure 3C and 3D). The intact contralateral artery

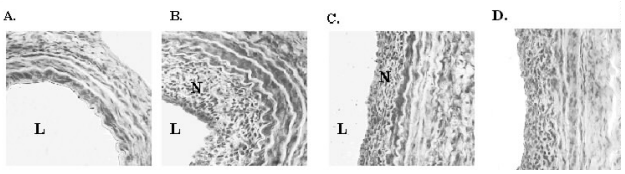


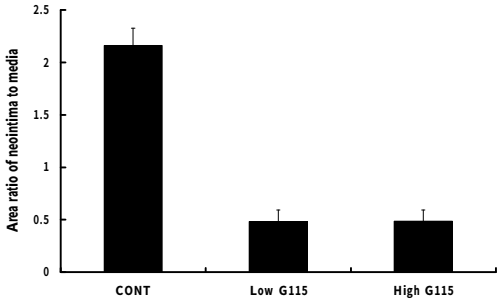
Figure 3

without balloon injury in the G115 treatment groups had no significant morphologic changes as compared to that of the sham controls. Using computer-assisted image analysis, the area ratio of neointima to media was compared between different groups. Figure 4 showed an area ratio of 2.16 for the balloon-injured vessels. This high ratio was significantly reduced to 0.49 and 0.46 in low dose and high dose of G115-treated vessels, respectively.

IV. Self Evaluation

The aim of this study is to elucidate the pharmacological mechanism of gensonsides in preventing balloon injury-induced stenosis. The in vivo assay of G115 on balloon injury-

Figure 4



induced neointimal formation in the present study revealed an effective remedy in preventing vascular restenosis in animal models. Pretreatment of G115 may provide a preventive therapy on vascular smooth muscle cell proliferation caused by balloon injury. Oral administration made this pharmacological therapy more convenient for those patients who are indicated for PTCA. To the best of our knowledge, the present study is the first report showing the preventive effects of ginseng extracts on balloon injury-induced stenosis. Although the precise doses of G115 to reach its optimal effects remained to be determined for clinical uses, this study provides a potential pharmacological therapy in preventing PTCA-induced restenosis.

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