Puerarin Induces Angiogenesis in Myocardium of Rat with Myocardial Infarction

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Puerarin is a major effective ingredient extracted from the traditional Chinese medicine Ge-gen (Radix Puerariae, RP). Recently, puerarin has been used to treat patients with coronary artery diseases (CAD). However, the mechanisms of puerarin on coronary artery diseases are still not very clear. In this study, we investigated the role of puerarin on angiogenesis in the non-ischemic and ischemic myocardium. We found that puerarin (120, 60 mg/kg, i.p.) could reduce infarct area in the heart of rat with myocardial infarction (MI). Puerarin (120 mg/kg) induced angiogenesis in the non-ischemic and ischemic myocardium, which was one of the mechanisms of curing coronary artery diseases. The gene expression or activation of vascular endothelial growth factor (VEGF), hypoxia-inducible factor 1 (HIF-1α) and endothelial nitric oxide synthase (eNOS) that correlated with angiogenesis were also induced by puerarin. From these results, we suggested that puerarin may induce therapeutic angiogenesis in myocardium of rat with MI. The mechanism may be that puerarin can induce VEGF and eNOS expression.

Key words angiogenesis; puerarin; myocardial infarction; vascular endothelial growth factor (VEGF); endothelial nitric oxide synthase (eNOS); hypoxia-inducible factor 1α (HIF-1α)

Puerarin [4H-1-benzopyran-4-one,8-β-D-glucopyranosyl-7-hydroxy-3-(4-hydroxyphenyl), C31H24O19] is a major active ingredient extracted from the traditional Chinese medicine Ge-gen (Radix Puerariae, RP). The uses of Ge-gen described in pharmacopoeias and in traditional systems of medicine are for the treatment of fever, pain, diabetes, meases, acute dysentery or diarrhea, etc. Puerarin has been shown to be able to block the beta adreno-receptor of isolated organs and the whole animal. In addition, it possesses anti-convulsive activity. It suppresses alcohol intake, attenuates the hyperthermia produced by 2,4-dinitrophenol and improves retinal functions. In particular, it has long been used to treat cardiovascular diseases including coronary artery diseases (CAD), arrhythmia and hypertension. Moreover, some studies of the mechanisms of puerarin on CAD have shown that, for example, puerarin could recover left ventricular function and increase coronary blood flow. It also decreased myocardial lactate production, myocardial oxygen consumption, creatine phosphokinase (CPK) release and the degree of ischemic damage. In addition, it improved the opening and forming of coronary collateral circulation, inhibits the increase of platelet aggregation and the blood viscosity during acute myocardial infarction (AMI). It lowered the plasma levels of free fatty acids, matrix metalloproteinases-9, C-reactive protein, serum interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), inhibited inflammation and stabilized atherosclerotic plaque. It played an important role in regulating the imbalance of endothelin (ET), renin activity (RA), angiotensin I (AT-I) and nitric oxide (NO) of patients with AMI, etc. However, the exact mechanisms of puerarin on CAD are still not very clear.

Angiogenic therapy for the human heart is currently being vigorously pursued. In the past ten years, alternative revascularization/angiogenesis strategies have progressed from bench to bedside. However, most of the strategies involve the delivery of growth factors in which very little success with these strategies has been demonstrated so far for various reasons. Puerarin has been reported to improve the opening and forming of coronary collateral circulation, inhibit the increase of platelet aggregation and the blood viscosity during AMI. Moreover, puerarin showed protective effects in endothelial dysfunction induced by lipid peroxide, chemical hypoxia, and hydroxyl-free radicals. Puerarin could also increase the number of endothelial progenitor cell (EPC) as well as EPC proliferative, migratory, adhesive, and in vitro vasculogenesis capacity in a concentration and time-dependent manner. EPCs have the ability to circulate, proliferate, and differentiate into mature endothelial cells. In additional, puerarin has been shown to promote endothelial cell proliferation. Endothelial cells elongate and align to form a sprout, and the lumen is formed by a curvature inside each endothelial cell. Individual sprouts elongate and eventually join with each other forming loops through which blood begins to flow. Endothelial cells retain the capacity to divide and form new blood vessels in response to specific stimuli in adult tissues.

Since the effect of puerarin on angiogenesis in ischemic myocardium has not been investigated previously, we hypothesized that puerarin could induce angiogenesis in ischemic and non-ischemic myocardium, leading to the curing of ischemic heart disease.

MATERIALS AND METHODS

Rat Myocardial Infarction Model All the experiment were performed on Sprague–Dawley male rats weighing 250—350 g and kept under standardized housing condition. The temperature in the chamber was maintained at 22—24 °C. All the animals were maintained on a 12 h light/dark cycle.
Rats were anesthetized with urethane (1 g/kg i.p.). The surgical procedure was performed according to the method of Grzegorz Heba et al.\(^{25}\) In brief, the trachea was incised longitudinally and cannulated. The chest was opened under ventilation with room air (Rodent Ventilator Tkr-200C) by left thoracotomy. After opening the pericardium, the left anterior descending (LAD) coronary artery was ligated near its origin by a 6.0 prolene suture. Thereafter, the chest and the skin were closed.

**Treatment** After surgery, rats were randomly divided into five groups. The model group consisted of rats without any treatment. The three treatment groups consisted of rats treated with puerarin (Beijing Union Pharmaceutical Factor, Pure degree % = 99.80) at the dosage of 120, 60 and 30 mg/kg i.p. Puerarin was administered for consecutive 30 d after surgery. The rats in the sham group underwent the same surgical procedure except the LAD ligation. All rats were killed 30 d after surgery and hearts were removed and treated as described below.

**Measurement of Infarct Size**\(^{26}\) Infarct size was measured in eight horizontal sections between the point of ligation and the apex. The non-infarcted and infarction areas were demarcated after incubation with 1% triphenyltetrazolium chloride (TTC) phosphate buffered solution (pH 7.4) at 37°C for 15 min and fixed in 10% formalin to increase the contrast of the TTC staining. The infarct area was determined by outlining the myocardial sections on paper. With the use of Scion Image, the volumes of infarct myocardium were calculated. Infarct size was reported as a percentage of the total area.

**Immunohistochemical Staining** The hearts were fixed in 10% neutral-buffered formaldehyde for 12 h and embedded in paraffin, cut into section (5 μm thick) and deparaffinized with a graded series of xylene and ethanol solutions.

Immunohistochemical staining was performed using UltraSensitive TM S-P kit (MAIXIN-Bio, China) according to the manufacturer’s instruction. In brief, sections were deparaffinized and microwave-treated for 10 min twice in 10 mm sodium citrate (pH 6.0). Endogenous peroxidase in the section was blocked by incubating them in endogenous peroxidase blocking solution for 10 min at room temperature. A rabbit polyclonal antibody against Von Willebrand Factor (vWF) protein (maixin) was used as primary antibody in a 1:70 dilution at 4°C for 18 h. After washing three times with PBS, sections were incubated with biotin-conjugated anti-rabbit second antibody for 10 min. They were then washed 3 times with PBS, treated with streptavidin-peroxidase for 10 min and then washed again with PBS 3 times. Finally, specimens were incubated in diaminobenzidine (DAB) for 5 min, followed by haematoxylin counterstaining. Images from the entire sections were acquired using digital camera system (Leica DM IL, DC 300). The slides were processed by a computerized image analyzer (Leica IM50) for quantitative assessment of vascular density in the myocardium. The number of blood vessels was counted in 10 random fields (magnification 200×). The average of the 10 high power fields (hpf) was calculated and the vascular density was defined as blood vessels/hpf.

**RT-PCR** The non-ischemic and ischemic cardiac tissue was obtained from rats of model group and treated with puerarin at the dosage of 120 mg/kg. Ischemic zones consisted of normal appearing myocardium surrounding the infarct zone. Non-ischemic zones are normal zones distant from the infarct and peri-infarct zones. The infarct zone could be readily distinguished from the normal myocardium surrounding it by its distinctive pale coloration. In the sham operated rats, the entire ventricle was quick-frozen.

RNA was isolated from cardiac tissues by the TRIZOL Reagent (Invitrogen). Transmural samples (ca. 100 mg) of ventricle after 30 d of surgery were excised and cleaned of adherent fat in ice-cold 0.9% NaCl solution. RNA was extracted, precipitated, washed according to the manufacturer’s instruction, and stored in DEPC-treated H₂O at −80°C until analysis.

Total RNA (5 μg) was reverse-transcribed in 20 μl volumes using SuperScript™ III Reverse Transcriptase (Invitrogen) with 0.5 μg oligo dT15 (Promega). For each RT product, aliquots (1—2 μl) of the final reaction mixture were amplified in four parallel PCR reactions using VEGF, eNOS, HIF-1α, or GAPDH specific primers and Taq DNA polymerase (Fermentas). The sequences of the primers used were as follows:

- **VEGF forward**: 5′-CAGAAGCCCATGAAGTGGT-3′,
  reverse: 5′-CTATGGTGCCTTTGTTGTA-3′;
- **HIF-1α forward**: 5′-ATTCTCAAGCCCTCCGA-3′,
  reverse: 5′-TCATCGCATGACTGCCCC-3′;
- **eNOS forward**: 5′-ATGGCAGACCGTGTGAAG-3′,
  reverse: 5′-ATTTGGGTCCTGGGGTGTAG-3′;
- **GAPDH forward**: 5′-CAACTCCCTCAAGATTGTGAGC-3′,
  reverse: 5′-CCCTGTTGCTGTAGCCCATATCC-3′.

Reactions were run on an ABI Thermoblock thermocycler. Following an initial denaturation at 94°C for 3 min, the reaction mixtures were subjected to 30 cycles of denaturation at 94°C for 45 s, annealing at 55°C (58°C for GAPDH) for 45 s, extension at 72°C for 1 min and a final extension at 72°C for 7 min.

PCR products were analyzed by electrophoresis in TBE buffer with ethidium bromide at 120 V in 0.8% agarose gel. The product sizes of VEGF, HIF-1α, eNOS and GAPDH were 250, 601, 797 and 548 bp respectively.

The level of gene expression was assessed by densitometric measurement of the amount of PCR products on scanned agarose gels. The units of expression were calculated as the ratio of the amount of PCR product of studied mRNA (VEGF, HIF-1α, eNOS) to the amount of PCR product of the constitutively expressed housekeeping gene GAPDH, the amount of which was assumed to be expressed constantly in the cell. The measurements were made with a BIO-RAD analysis system.

**Statistical Analysis** All values are given as the mean ± S.D. Differences between the groups were calculated by analysis of variance (ANOVA) followed by Duncan’s multiple range test or Student’s t-test whichever appropriate. A value of p < 0.05 was considered as significant.

**RESULT**

Among 88 rats that underwent coronary ligation, 36 died on the day of the surgery; the survival rate was 59.09%. The 52 rats that survived were randomly divided into four groups.
In the course of treatment, except for high dose group, one died in each group. The mortality rates were not significantly different in the treatment groups. The mortality in sham-operated rats was zero.

**Measurement of Infarct Size** Infarct size expressed as a percentage of total myocardium size was shown in Fig. 1. Infarct area was reduced in the hearts of the rats treated with high-dose and middle-dose puerarin as compared to that of the model group ($p<0.01$ or $p<0.05$). No significant difference was observed in the infarct area between low dose group and model group.

**Immunohistochemistry** Myocardial vessel density was significantly increased in ischemic zones (the border area of infarct) and non-ischemic zones in myocardial sections of rats that underwent LAD coronary artery occlusion as compared to that in sham-operated rats ($p<0.01$ or $p<0.05$). In ischemic zones, myocardial vessel density was significantly increased in high-dose puerarin-treated rats compared to that of the model group ($p<0.05$). A slight increase but no significant difference was observed in the middle and low dose puerarin-treated rats compared to the model group ($p>0.05$) (Figs. 2, 4). In non-ischemic zones, myocardial vessel density was significantly increased in high and middle dose puerarin-treated rats compared to that of the model group ($p<0.05$). A slight increase but no significant difference was observed in low dose puerarin-treated rats as compared to that of model group ($p>0.05$) (Figs. 3, 4).

**RT-PCR** HIF-1α, VEGF, eNOS mRNA expressions were investigated in the non-ischemic and ischemic zones of sham-operated group, model group, and treatment group. In ischemic zones, HIF-1α, VEGF, eNOS mRNA expressions were...
were significantly increased in the model group and treatment groups as compared to that of sham-operated rats ($p<0.05$ or $p<0.01$). The expression of these genes was significantly higher in the treatment group than in model group ($p<0.05$ or $p<0.01$). In non-ischemic zones, there was no difference in the expression of HIF-1α mRNA among the sham-operated, model and treatment groups, VEGF mRNA expressions were only observed in the treatment groups. The expression of VEGF mRNA was too weak to be quantified in sham-operated and model groups. eNOS mRNA expression was significantly increased in model and treatment groups as compared to that of sham-operated group ($p<0.05$ or $p<0.01$). The expressions of eNOS were slightly increased but no significant difference was observed in treatment group as compared to the model group ($p>0.05$) (Fig. 5).

**DISCUSSION**

Therapeutic angiogenesis that may be beneficial in the treatment of ischemia has recently been substantiated by a large amount of experimental data. Bioactive compounds from natural sources may be used as regulatory agents. An increasing number of bioactive compounds from natural sources with elucidated chemical structures are reported as potent inducers of angiogenesis.\(^\text{27-30}\)

Drug development from natural products is a fast emerging field to provide people with more readily available and affordable healthcare. Ischemic heart diseases develop as a consequence of coronary atherosclerotic lesion formation. Coronary collateral vessels and microvascular angiogenesis develop as an adaptive response to myocardial ischemia, which ameliorate the function of the damaged heart. Angiogenesis is outgrowth of new vessels from existing vessels, it is a complex process. During angiogenesis, endothelial cells detach from the pre-existing destabilised vessel, migrate into the perivascular space and proliferate to finally mature and form new vascular structures. A number of growth factors, proteases, adhesion molecules and other angiogenic mediators which enable endothelial cell migration or proliferation regulate this process. VEGF is considered one of the most important growth factors in angiogenesis.\(^\text{31}\)

VEGF activates eNOS by the induction of calcium flux, the recruitment of heat-shock protein 90 (Hsp90) and the phosphorylation of NOS via the phosphatidylinositol-3-OH-kinase [PtdIns(3)K]-Akt pathway.\(^\text{32}\) Upon activation, eNOS catalyzes l-arginine to l-citrulline and NO. NO contributes to a variety of endothelial cellular events including proliferation, migration and anti-apoptosis and so on, the events are essential early steps required for neovascularization.\(^\text{32}\) In addition, NO induces the mobilization and expansion of EPCs in the bone marrow, EPC can differentiate into mature endothelial cell.\(^\text{33}\)

Previous studies have shown the effect of puerarin on cultured endothelial cells. Puerarin has been shown to promote endothelial cell proliferation\(^\text{22}\) and prevent endothelial dysfunction, apoptosis and viability loss induced by lipid peroxide, chemical hypoxia, TNF-α, hydroxyl-free radicals.\(^\text{18-20,24}\) Puerarin could also increase the number of EPCs as well as EPC proliferative, migratory, adhesive, and in vitro vasculogenesis capacity in a concentration and time-dependent manner.\(^\text{21}\)

Angiogenesis can be quantified by different methods based on the microscopic evaluation of tissue vascularization using antibodies with affinity for specific epitopes on the endothel-
The angiogenic effect of VEGF is predominantly mediated by eNOS. eNOS is essential for angiogenesis in ischemic tissues in vivo. The production of NO by eNOS has been shown to play an important role in angiogenesis in many different vascular beds, e.g., fetal myocardium, ischemic hind limb, wound healing, and coronary collateral development. The activity and expression of eNOS have been shown to be increased by hypoxia in endothelial cells. In ischemia myocardium, ischemia acts as a stimulus for eNOS activation resulting in increased eNOS activity and increased NO release. We also checked the eNOS expression and found that eNOS expression was induced by puerarin in ischemic myocardium and non-ischemic myocardium of the rats with or without an increase of HIF-1α expression. We hypothesized that angiogenic effect of puerarin may attribute to inducing VEGF and/or eNOS expression. The detailed mechanism will need to be further examined. Taken together, these results indicated that puerarin did not only successfully promote angiogenesis or decrease infarct size of the ischemic heart, but it also stimulated angiogenesis of non-ischemic region to compensate blood supply to the heart. The underlying mechanism may be that puerarin can increase VEGF and eNOS expression.

The effect of puerarin on angiogenesis in vivo has not been investigated previously. The finding that puerarin bears the angiogenic properties in myocardium of rat with MI is novel and preliminary. The explicit mechanisms of how puerarin induces in vivo angiogenesis will be further investigated.

REFERENCES