行政院國家科學委員會補助專題研究計畫

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以薑黃素為例-研發作用於血管先驅細胞的抗動脈粥狀硬化中草藥

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以薑黃素為例—研發作用於血管先驅細胞的抗動脈粥狀硬化中草藥

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摘要

動脈粥狀硬化可引起冠狀動脈、腦血管和周邊血管障礙的慢性發炎。由骨髓延伸的血管先驅細胞，包括內皮先驅細胞(EPCs)和平滑肌先驅細胞(SMPCs)對生理與病理狀況下的血管癒合與重組扮演重要角色。研究認為血管先驅細胞在肺高壓、血管癒合以及動脈硬化發展具治療潛力。

具動脈粥狀硬化解護作用的中藥，例如薑黃素、銀杏、丹參酚酸B、厚朴酚與白藜蘆醇，可降低心血管疾病發生。中藥裡的抗氧化成分似乎可抑制動脈粥狀硬化的發展。有幾種抗氧化中草藥(如銀杏、葛根素和白藜蘆醇)被發現可提升周邊血中EPCs的數目和功能，但主要機制仍不清楚。

薑黃素是鬱金的黃色染料，同時也被作為美容和在一些醫學製劑。在我們正在執行中的研究發現，薑黃素可能透過抗氧化和抗發炎特性調節EPCs的功能和活性。本計畫將以薑黃素為例，繼續深入探討中草藥對骨髓細胞分化出之血管內皮與平滑肌先驅細胞的作用與角色。

本已經成功建立體外EPC與SMPC細胞培養模型，進行薑黃素之體外調節研究。每年度研究計畫中將進一步分成三部分：第一部份為SMPC體外薑黃素培養模型，單核球細胞將利用梯度離心來分離純化，細胞培養於血清纖維結合蛋白塗佈上的培養皿。經過4天的培養後，黏附的細胞以薑黃素處理。細胞將利用免疫螢光染色觀察鑑定。細胞老化、增生、遷移和試管內血管生成活性將利用β-galactosidase染色，MTT方法，Boyden chamber和試管內血管生成套組分別進行分析，一氧化氮合成酶和細胞內的信號傳遞路徑也將被深入研究。第二部分為篩選數種具抗動脈硬化作用的中草藥(包括銀杏、厚朴酚、丹參酚酸B以及白藜蘆醇)，觀察這些抗動脈硬化中草藥對於EPCs與SMPCs的影響，另外其調節機制也將進一步被比較。第三部分為觀察高血糖或發炎環境下，數種具抗動脈硬化作用的中草藥對於血管先驅細胞的保護作用與機制。

本研究計畫希望能有效開發中草藥對於骨髓延伸之血管先驅細胞提供資訊，並希望本計畫可使我們更進一步了解血管病變發生時的可能的分子機轉，而能夠在未來應用於防止血管併發症的發生，提供臨床血管疾病新的治療策略。

關鍵詞：動脈粥狀硬化；中草藥；薑黃素；血管先驅細胞；氧化壓力
Abstracts

Atherosclerosis is a chronic inflammatory disease which may cause obstructions of the coronary, cerebral, and peripheral arteries. Accumulating evidence suggests that bone marrow-derived circulating progenitor cells, including endothelial progenitor cells (EPCs) and smooth muscle progenitor cells (SMPCs), contribute to vascular healing and remodeling under pathophysiological conditions. Recent findings obtained from ex vivo cell culture and animal models examining the potential roles of circulating vascular progenitor cells in pulmonary hypertension, vascular healing, and atherogenesis.

Our previous studies found that some Atheroprotective Chinese herbal medicines, such as curcumin, *Ginkgo biloba* extract (GBE), salvianolic acid B, magnolol, and resveratrol, etc., may reduce the risk of cardiovascular diseases. The antioxidative compounds in these herbal medicines appear to interfere with the molecular processes underlying the initiation, progression, and rupture of atherosclerotic plaques. It has been demonstrated that several Chinese herbal medicines, such as Ginkgo biloba, puerarin, and resveratrol, with antioxidant activity, could significantly enhance number and activity of EPCs from peripheral blood, although the underlying mechanisms are mostly unknown.

Curcumin, a major component from *Curcuma longa*, is a yellow pigment of turmeric and is commonly used as a spice and food-coloring agent. It is also used as a cosmetic and in some medical preparations. In our pilot study, we found that curcumin could modulate EPC function and differentiation through its antioxidative and anti-inflammatory properties. In this project, curcumin will be used as a model for investigating the potential roles of Chinese herbal medicines on bone marrow-derived vascular progenitor cells.

We have successfully established *ex vivo* EPC and SMPC culture model and found the benefit effects of curcumin. The new project is divided to three parts, including *ex vivo* SMPC culture model, screening of potential atheroprotective Chinese herbal medicines, and the investigating the mechanisms of herbal medicines on hyperglycemia- or TNF-α-induced progenitor dysfunction. In SMPC culture model, total mononuclear cells will be isolated from peripheral blood by Histopaque 1077 density gradient centrifugation, and then cells will be plated on fibronectin-coated culture dishes. After 4-day cultured, attached cells were treated with curcumin. Cells will be characterized by immunofluorescent staining under a laser scanning confocal microscope. Cell senescence, proliferation, migration, and in vitro vasculogenesis activity will be assayed with β-galactosidase stain, MTT, modified Boyden chamber assay, and in vitro vasculogenesis kit, respectively. The expression of endothelial nitric oxide (eNOS) and intracellular signalings will also be examined. In the second part, the effects of various atheroprotective Chinese herbal medicines (including Ginkgo biloba, magnolol, salvianolic acid B, and resveratrol) on vascular progenitor cells will be compared. In the third part, the effects and underlying mechanisms of various atheroprotective Chinese herbal medicines on hyperglycemia- or TNF-α-induced progenitor dysfunction will be explored.

The findings from this project may hopefully to develop potential Chinese herbal medicines on modulating bone marrow-derived circulating EPCs/SMPCs and understand the molecular mechanisms to provide novel therapeutic strategies for clinical vascular complications.

**Keywords:** Atherosclerosis; Chinese herbal medicines; curcumin; vascular progenitor cells (EPCs); oxidative stress
前言

Atherosclerosis is a chronic inflammatory disease which may cause obstructions of the coronary, cerebral, and peripheral arteries. It is typically multi-factorial, most often dependent on risk factors such as hypercholesterolemia, diabetes, smoking, hypertension, and obesity. Complications of atherosclerosis remain the leading cause of morbidity and mortality in industrialized countries (Katagiri et al., 2007). In addition to regular clinical therapeutic strategy, many evidences from basic researches supporting the used of atheroprotective Chinese herbal medicines, such as curcumin, Ginkgo biloba extract (GBE), salvianolic acid B, magnolol, and resveratrol, etc., may reduce the risk of cardiovascular diseases. The antioxidative compounds in these herbal medicines appear to interfere with the molecular processes underlying the initiation, progression, and rupture of atherosclerotic plaques (Chen et al., 2006a).

Accumulating evidence suggests that bone marrow-derived circulating progenitor cells, including endothelial progenitor cells (EPCs) and smooth muscle progenitor cells (SMPCs), contribute to vascular healing and remodeling under pathophysiological conditions (Roberts et al., 2005). Recent findings obtained from ex vivo cell culture and animal models examining the potential roles of circulating vascular progenitor cells in pulmonary hypertension, vascular healing, and atherogenesis. Although there are several therapeutic and diagnostic applications of bone marrow-derived stem/progenitor cells, there are concerns that transplanted bone marrow cells or precursors may participate in the pathogenesis of unfavorable diseases such as cancer, retinopathy, and atherosclerosis (Yao et al., 2006).

Curcumin, a major component from Curcuma longa, is a yellow pigment of turmeric and is commonly used as a spice and food-coloring agent. It is also used as a cosmetic and in some medical preparations. The desirable preventive or putative therapeutic properties of curcumin have also been considered to be associated with its antioxidant and anti-inflammatory properties (Hsu and Cheng, 2007). Because oxidative stress-mediated damages are believed to be associated with a variety of chronic pathological complications such as cancer, neurodegenerative diseases, and atherosclerosis, curcumin is thought to play a vital role against these pathological conditions (Miriyala et al., 2007). In our pilot study, we found that curcumin could modulate EPC function and differentiation through its antioxidative and anti-inflammatory properties. The development of potential Chinese herbal medicines on modulating bone marrow-derived circulating EPCs/SMPCs and understating the molecular mechanisms may provide novel therapeutic strategies for clinical vascular complications.

研究目的

Our pilot study demonstrated that curcumin, a major component from Curcuma longa, modulated EPC function and differentiation through its antioxidative and anti-inflammatory properties. Thus, this proposal will further investigate the anti-atherogenesis contribution of curcumin on EPCs/SMPCs and examine underlying mechanisms. By using ex vivo human vascular progenitor cell culture, we will focus on the mechanisms for the cardiovascular benefic effects of curcumin and analyze the properties at multiple levels, such as differentiation of EPCs/SMPCs, EPC function, SMPC proliferation and apoptosis, intracellular PI3K/Akt signalings (Chen et al., 2007), and atheroprotective endothelial NO synthase (eNOS) expression (Thum et al., 2007). This study will also screen other Chinese herbal medicines (GBE, magnolol, salvianolic acid B, and resveratrol) to explore the relationship with reactive oxygen species (ROS)- and NO-related underlying mechanisms, and investigate how the
regulation of these activities by Chinese herbal medicines can lead to a prevention of vascular complications. The projection goals are to elucidate new concepts of alternative medicines on vascular progenitors that contribute to the regulation of the vascular diseases and associated inflammatory effects.

文獻探討

Putative Circulating EPC Discovery

The integrity of the endothelium of the vasculature is essential for vascular homeostasis and normal function. Endothelial injury or dysfunction is assumed as an early event in the development of atherosclerosis (Schulz et al., 2004). Recent evidence suggests that adult peripheral blood contains EPCs that were successfully incorporated into the site of angiogenesis under physiological and pathological conditions (Asahara et al., 1999). EPCs were also shown to participate in re-endothelialization after vascular injury (Gunsilius et al., 2000; Sata et al., 2002). After the discovery of putative EPCs, numerous studies have been done for therapeutic applications of EPCs to treat various cardiovascular diseases (Sata, 2006).

Potential of EPCs to Accelerate Re-endothelialization

Seeding of autologous EPCs dramatically improved graft patency in vascular grafts (Kaushal et al., 2001) and inhibited neointimal hyperplasia in prosthetic grafts (Griese et al., 2003). Local delivery of cultured EPCs to the balloon-injured carotid artery was associated with accelerated re-endothelialization, enhanced endothelium-dependent vasoreactivity, and reduced neointimal formation (Gulati et al., 2003). It was hypothesized that transplanted EPCs might secrete several proangiogenic cytokines that stimulated migration and proliferation of adjacent endothelial cells in a paracrine manner. Atheroprotective effects of bone marrow-derived EPCs were also demonstrated in hyperlipidemia-induced atherosclerosis (Rauscher et al., 2003). These results suggest that local or systemic administration of EPCs or bone marrow cells may prevent vascular diseases by accelerating restoration of the endothelium and maintenance of vascular homeostasis.

Dysfunction of EPC and Vascular Diseases

The reduced number and function of EPCs have been implicated in the pathogenesis of vascular diseases (Goldschmidt-Clermont et al., 2005; Karra et al., 2005). Human EPCs from type II diabetic patients exhibited impaired proliferation, adhesion, and incorporation into vascular structures (Tepper et al., 2002). In human cardiac recipients, decrease in circulating EPCs was associated with allograft vasculopathy (Simper et al., 2003). These results suggest that circulating endothelial progenitors normally repair and rejuvenate the arteries and that progressive progenitor cell deficits and consequent delayed vascular healing may account for the pathogenesis of atherosclerosis (Sata, 2006).

研究方法

Study Protocol

In the first part (year) of the study, human EPCs isolated from peripheral blood will be treated with medium containing various concentrations of curcumin. EPC number and activity will be examined for screening the effects of curcumin on EPCs. The in vitro cell proliferation, migration, and vasculogenesis represented the neovascularization and re-endothelialization abilities of EPCs will be also investigated. Western blot will be examined to explore the role of PI3K/Akt signalings and iNOS or eNOS expression in EPCs. Changes will also be detected for ROS generation and activation of ROS-sensitive transcription factors, NF-κB and AP-1,
whose activities have been previously shown to alter in oxidative stress-stimulated cells.

**Experimental Design**

To demonstrate a concentration-dependent effect of curcumin (Sigma) on EPCs, cells will be incubated with 0.1, 1, 10, and 100 µM for 4 days, respectively. To determine reaction time course, cells will be treated with various concentrations of curcumin for 1, 3, 5, and 7 days.

**Isolation and Cultivation of Human EPCs & SMPCs**

EPCs will be cultured according to previously described techniques (Hill et al., 2003; Kalka et al., 2000). Briefly, total mononuclear cells (MNCs) will be isolated from peripheral blood (40 ml from donor’s vein) of healthy young human volunteers (~50 person-times) by density gradient centrifugation (Histopaque 1077). Cells will be plated on culture dishes coated with human fibronectin (Chemicon) and maintained endothelial cell basal medium-2 (Clonetics) supplemented with EGM-2 MV single aliquots consisting of 5% FBS, vascular endothelial growth factors (VEGF), fibroblast growth factor-2, epidermal growth factor, insulin-like growth factor-1, and ascorbic acid. The medium will be replaced at day 4, and all assays will be performed by using cells harvested on day 7 with PBS plus 5 mM EDTA.

**EPC & SMPC Characterization**

Endothelial identity will be confirmed by Immunofluorescent staining with antibodies recognizing human vascular endothelium (VE)-cadherin (Chemicon), CD34 (Pharmingen), CD31 (Pharmingen), and vWF (Chemicon). All antibodies will be added for 30 minutes at 25°C, and a FITC-conjugated anti-mouse antibody (Vector) will be added for staining. After the staining, samples will be viewed with an inverted fluorescent microscope (Leica) and further demonstrated by laser scanning confocal microscope (Leica).

**EPC Senescence Assay.**

The cellular aging was determined with a Senescent Cells Staining Kit (Sigma). Briefly, after washing with PBS, both early and late EPCs were fixed for 6 min in 2% formaldehyde and 0.2% glutaraldehyde in PBS, and then incubated for 12 h at 37°C without CO2 with fresh X-gal staining solution (1 mg/ml X-gal, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 2 mM MgCl₂; pH 6). After staining, blue-stained cells and total cells were counted and the percentage of β-galactosidase-positive cells was calculated.

**EPC Migration Assay**

EPC migration will be evaluated by using a modified Boyden chamber assay. In brief, isolated EPCs will be detached using 0.25% trypsin, harvested by centrifugation, resuspended in 500 µl medium, and counted; then 2×10^4 EPCs will be placed in the upper chamber of a modified Boyden chamber. VEGF in serum-free medium will be placed in the lower compartment of the chamber. After 24 hours incubation at 37°C, the lower side of the filter will be washed with PBS and fixed with 2% paraformaldehyde. For quantification, cells will be stained with Giemsa solution. Cells migrating into the lower chamber will be counted manually in 3 random microscopic fields (Vasa et al., 2001).

**EPC and SMPC Proliferation Assay**

The effect of curcumin on EPC proliferation will be determined by MTT assay. After being cultured for 7 days, EPCs will be digested with 0.25% trypsin and then cultured in medium in 96-well culture plate (200 µl/well). Each concentration included 6 wells. After being cultured for 24 hours, EPCs will be supplemented with 10 µl MTT (5 g/l, Sigma) and incubated for another 6 hours. Then the supernatant
will be discarded by aspiration and the EPC preparation will be shaken with 200 µl dimethyl sulfoxide (DMSO) for 10 minutes, before the OD value will be measured at 540/690 nm (Chen et al., 2004b).

**Statistical Analysis**

All data are presented as mean ± SEM. Differences between group means will be assessed by Student’s t test for single comparisons and by ANOVA for multiple comparisons. Values of $P < 0.05$ will be considered significant.

**結果與討論**

As shown in [Figure 1](#), the mononuclear cells isolated from healthy young subjects were cultured in the fibronectin-coated plate. The morphology and characterization of EPCs are shown. [Figure 2](#) shows the representative photos for the tube formation (vasculogenesis), migration, and cellular senescence in EPCs. As shown in [Figures 3](#), the preliminary results of EPC proliferation test, with curcumin-treated EPCs have been obtained. As shown in [Figure 4](#), the mononuclear cells isolated from healthy young subjects were cultured in the fibronectin-coated plate. The morphology of smooth muscle-like cells is shown. The characterization of SMPCs will be performed in our next plan.

To further understand the relationships and mechanisms between Chinese herbal medicines and vascular progenitor cells, additional studies in this project should be performed.

![Figure 1](image1.png)

**Figure 1.** Morphology and Characterization of EPCs. Human MNCs were isolated and plated on culture dish at first day (A). Seven days after plating, adherent early EPCs with spindle shape were shown (B). Twenty days after plating, late EPCs with cobblestone-like morphology were selected, reseeded and grown to confluence (C). Immunofluorescence detection (green) of VE-cadherin (D), vWF (E), and PECAM-1 (CD-31) (F). EPCs were also shown to simultaneously bind FITC-UEA-1 for lectin staining (green) (G) and endocytose Dil-acLDL (red) (H). Merged image (I) shows that most cells are dual-positive. Scale bar, 50 µm.
Figure 2. Curcumin dose-dependently increases EPC proliferation activity. Human MNCs were isolated from healthy subjects and incubated with different concentrations of curcumin for 4 days. MTT assay was performed for EPC proliferative activity. Data are expressed as mean (SEM; n=3, *P < 0.05 vs. untreated control).

Figure 3. Confluent smooth muscle-like cells derived from peripheral mononuclear cells with “hill and valley” morphology.

Figure 4. Curcumin dose-dependently decreases SMPC proliferation activity. Human MNCs were isolated from healthy subjects and incubated with different concentrations of curcumin for 4 days. MTT assay was performed for SMPC proliferative activity. Data are expressed as mean (SEM; n=3, *P < 0.05 vs. untreated control.)
Figure 4. Curcumin dose-dependently decreases MMP-9 expression. Human PMPCs were isolated from healthy subjects and incubated with different concentrations of curcumin. Western blot assay was performed. Data are expressed as mean (SEM; n=3, *P < 0.05 vs. untreated control).

Curcumin, a major component from *Curcuma longa*, is used as a cosmetic and in some medical preparations. The desirable preventive or putative therapeutic properties of curcumin have also been considered to be associated with its antioxidant and anti-inflammatory properties. Because oxidative stress-mediated damages are believed to be associated with a variety of chronic pathological complications such as cancer, neurodegenerative diseases, and atherosclerosis, curcumin is thought to play a vital role against these pathological conditions. Moreover, it has been demonstrated that several Chinese herbal medicines, such as *Ginkgo biloba*, puerarin, and resveratrol, with antioxidant activity, could significantly enhance number and activity of EPCs from peripheral blood, although the underlying mechanisms are mostly unknown. In the presenting project, we hypothesized that curcumin and other herbal medicines could modulate function and differentiation of both EPCs and SMPCs through its antioxidative and anti-inflammatory properties. Therefore, the development of potential Chinese herbal medicines on modulating bone marrow-derived circulating EPCs/SMPCs and understating the molecular mechanisms may provide novel therapeutic strategies for clinical vascular complications.

計畫成果自評
研究內容與原計劃方向相符, 達成預期目標, 其成果可供學術界參考, 為後來也將在學術期刊中發表。並且根據此年度的研究成果, 繼續深入探討薑黃素調控之分子機制，扮演保護 EPCs 與抑制 SMPCs 的效果。

目前由此國科會計畫支持，發表於國際 SCI 之相關論文如下，特致謝忱。


Chen WC, Chen SY, Chen CH, Chen HY, Lin YW, Ho TJ, Huang YC, Shen JL, Tsai FJ, Chen YH. Lack of association between transient receptor potential cation channel 6 polymorphisms and primary membranous glomerulonephritis. *Ren Fail.* 2010. [Epub ahead of print]. (NSC 97-2320-B-039-022-MY3)


