

中國醫藥大學營養學系碩士班

碩士論文

薑黃素和鼠尾草酸下降細胞激素所誘發的人類臍  
靜脈內皮細胞黏附因子表現

Curcumin and Carnosic acid attenuate  
cytokine-induced adhesion molecule expression in  
HUVECs

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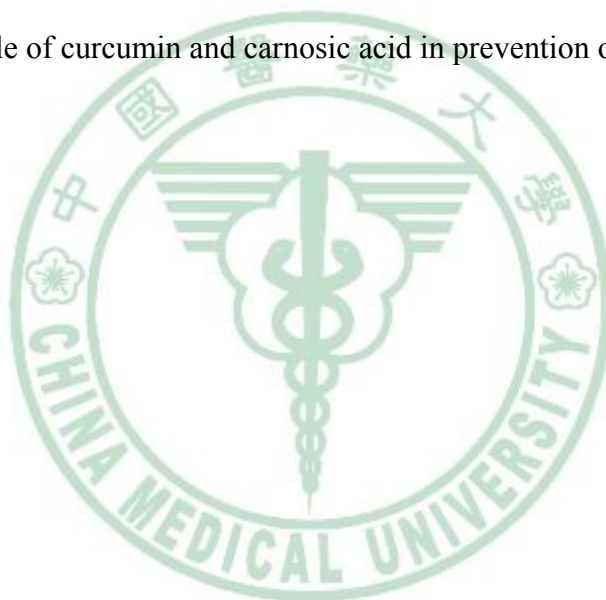
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## 中文摘要

內皮細胞表達細胞黏附因子，以及單核球黏附到內皮細胞上，在動脈粥狀硬化形成的過程中扮演一重要的角色。薑黃素 (curcumin) 是一種存在薑黃中的多酚類化合物，並且是常用在咖哩的辛香料，先前有研究顯示薑黃素具有抗氧化的特性。鼠尾草酸 (carnosic acid) 則是存在於鼠尾草和迷迭香中的多酚類化合物，在過去實驗指出鼠尾草酸也是一種抗氧化劑。在此次實驗中，主要探討薑黃素和鼠尾草酸個別對於 IL-1  $\beta$  所誘導人類臍靜脈內皮細胞黏附因子的表現之影響，以及其影響作用的機制。結果顯示，薑黃素和鼠尾草酸在體外試驗均有良好的抗氧化能力，並且薑黃素和鼠尾草酸都可以顯著降低 IL-1  $\beta$  所誘導之人類單核球細胞 (U937) 之黏附。藉由西方點墨法實驗顯示，薑黃素和鼠尾草酸可降低 IL-1  $\beta$  所誘導之 ICAM-1、VCAM-1 和 E-selectin 蛋白質表現。薑黃素和鼠尾草酸也能減低或阻斷 IL-1  $\beta$  所誘導之核轉錄因子 NF- $\kappa$ B p50、p65 的活化，同時發現薑黃素和鼠尾草酸亦能減少 IL-1  $\beta$  所誘導之細胞中 ROS 的生成。此研究結果證實薑黃素和鼠尾草酸可以降低黏附因子的表現與單核球細胞的黏附，是藉由抑制 NF- $\kappa$ B P50, P65 的活化。因此，薑黃素和鼠尾草酸具有抗發炎的特性，並且在預防動脈粥狀硬化中扮演一重要角色。



關鍵字：薑黃素 (curcumin)、鼠尾草酸(carnosic acid)、黏附因子、動脈  
粥狀硬化，核轉錄因子- $\kappa$ B，活性氧成分。



## Abstract

Expression of cell adhesion molecules by the endothelium and the attachment of monocytes to endothelium may play a major role in the early atherogenic process. Curcumin, which is consumed daily by millions of people, is a polyphenol derived from the plant Turmeric (*Curcuma longa*) and is used as a spice to give a specific flavor and yellow color to curry. Previous studies indicated that curcumin possessed antioxidant activity *in vitro*. Carnosic acid, an antioxidant polyphenol derived from Sage (*Salvia officinalis*) and Rosemary (*Rosmarinus officinalis*), is a lipophilic antioxidant that scavenges singlet oxygen, hydroxyl radicals, and lipid peroxy radicals, thus prevents lipid peroxidation and the disruption of biological membranes. In this study, we investigated the antioxidative effects of curcumin and carnosic acid using the TEAC assay, the inhibition of LDL oxidation and DPPH radicals scavenging ability. The effects of curcumin and carnosic acid on the adhesion of U937 cells to IL-1  $\beta$ -treated human umbilical vein endothelial cells (HUVECs), as well as the expression of adhesion molecules were also examined. In addition, we observed the effect of curcumin and carnosic acid on NF- $\kappa$ B p50, p65 and the formation of intracellular ROS induced by IL-1  $\beta$  in HUVECs. Our data showed that both of curcumin and carnosic acid had antioxidative effect *in vitro*. We also found that curcumin and carnosic acid inhibited the adhesion of U937 cells, abolished the expression of ICAM-1, VCAM-1 and E-selectin, attenuated or block nuclear translocation of NF- $\kappa$ B p50, p65 and decreased the production of ROS in IL-1  $\beta$  treated HUVECs. In conclusion, curcumin and carnosic acid inhibits IL-1  $\beta$ -induced nuclear translocation of p65 and p50, thereby suppressing the expression of ICAM-1, VCAM-1 and E-selectin, resulting in decreased monocyte adhesion. Thus, curcumin and carnosic have anti-inflammatory properties and may play

an important role in the prevention of atherosclerosis.

Key word: curcumin, carnolic acid, adhesion molecule, atherosclerosis, NF- $\kappa$ B, ROS



# ***Chapter 1***

## ***Introduction***

### ***1.1 Atherosclerosis***

Atherosclerosis is the most common cause of death in the Western world, and it is a complex, multifarious disease (Glass & Witztum, 2001). Recent insights into the pathogenesis of atherosclerosis suggest that it may be viewed as a chronic inflammatory disease and inflammatory processes are instrumental in all stages of this disease (Medford, 1995; Offermann & Medford, 1994; Ross, 1995).

Ross, 1999 showed that atherosclerosis has four stages. Stage 1: Endothelial dysfunction in atherosclerosis. This stage is the earliest changes that precede the formation of lesions of atherosclerosis take place in the endothelium. These changes include 1.) increased endothelial permeability to lipoproteins and other plasma constituents, which is mediated by nitric oxide, prostacyclin, platelet-derived growth factor (PDGF), angiotensin II, and endothelin; 2.) the up-regulation of leukocyte adhesion molecules, including L-selectin, integrins, and platelet-endothelial-cell adhesion molecule 1 (PECAM-1), and the up-regulation of endothelial adhesion molecules including E-selectin, P-selectin, intercellular adhesion molecule 1 (ICAM-1), and vascular-cell adhesion molecule 1 (VCAM-1); 3.) and the migration of leukocytes into the artery wall, which is mediated by oxidized low-density lipoprotein (ox-LDL), monocyte chemoattractant protein 1 (MCP-1), interleukin-8 (IL-8), platelet-derived growth factor (PDGF),

macrophage colony-stimulating factor (MCSF), and osteopontin (Fig 1-1).

Stage 2 : Fatty-streak formation in atherosclerosis. Fatty streaks initially consist of lipid-laden monocytes and macrophages (foam cells) together with T lymphocytes. Later they are joined by various numbers of smooth muscle cells. These steps include 1.) smooth muscle migration, which is stimulated by PDGF, fibroblast growth factor 2, and transforming growth factor  $\beta$  (TGF- $\beta$ ); 2.) T-cell activation, which is mediated by tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), interleukin-2 (IL-2), and granulocyte-macrophage colony-stimulating factor (GM-CSF); 3.) foam cell formation, which is mediated by ox-LDL, MCSF, TNF- $\alpha$ , and interleukin-1 (IL-1); and 4.) platelet adherence and aggregation, which are stimulated by integrins, P-selectin, fibrin, thromboxane A<sub>2</sub>, tissue factors, and the factors described as responsible for the adherence and migration of leukocytes (Fig 1-2).

Stage 3 : Formation of an advanced, complicated lesion of atherosclerosis. As fatty streaks progress to intermediate and advanced lesions, they tend to form a fibrous cap that walls off the lesion from the lumen. This represents a type of healing or fibrous response to the injury. The fibrous cap covers a mixture of leukocytes, lipid, and debris, which may form a necrotic core. These lesions expand at their shoulders by means of continued leukocyte adhesion and entry caused by the same factors as stage 1 and 2. The principal factors associated with macrophage accumulation include MCSF, MCP-1, and ox-LDL. The necrotic core represents the results of apoptosis and necrosis, increased proteolytic activity, and lipid accumulation. The fibrous cap forms as a result of increased activity of PDGF, TGF- $\beta$ , IL-1, TNF- $\alpha$ , and osteopontin and decreased connective tissue degradation (Fig 1-3).

Stage 4 : Unstable fibrous plaques in atherosclerosis. Rupture of the fibrous cap or

ulceration of the fibrous plaque can rapidly lead to thrombosis and usually occurs at sites of thinning of the fibrous cap that covers the advanced lesion. Thinning of the fibrous cap is apparently due to the continuing influx and activation of macrophages, which release metalloproteinases and other proteolytic enzymes at these sites. These enzymes cause degradation of the matrix, which can lead to hemorrhage from the vasa vasorum or from the lumen of the artery and can result in thrombus formation and occlusion of the artery (Fig 1-4).

## ***1.2 Adhesion molecules and atherosclerosis***

Cellular adhesion molecules are, according to the definition of the Gene Ontology Consortium (<http://www.geneontology.org/>) “molecules expressed on the surface of a cell that mediate the adhesion of the cell to other cells or to the extracellular matrix”. These proteins are playing numerous crucial functions at the interface of a cell and its environment, whether this environment is another cell from a similar or different cell type (Blankenberg *et al.*, 2003).

In the cardiovascular system, the adhesion of cells to each other and to the proteins of the extracellular matrix provides a stable environment for cell growth, differentiation, and migration. Adhesive interactions are also involved in the pathogenesis of cardiovascular disease, regulating thrombus formation, facilitating leukocyte infiltration, mediating the migration and proliferation of smooth muscle cells, and enabling the deposition of fibrotic tissue (Hillis, 1998). Three main groups of adhesion molecules have been implicated in cardiovascular pathology: integrins, selectins, and members of the immunoglobulin superfamily (Table 1-1).

Integrins are glycoproteins composed of two subunits, the  $\alpha$  and  $\beta$  chains. As their name suggests they mediate the adhesion of cells to each other and to surround substrates, “integrating” the cell with its environment (Hynes, 1987). In addition, they straddle the cell membrane and play an important role in initiating intracellular events in response to extracellular stimuli (Hynes, 1992). Usually integrins have multiple ligands (Table 1-1). Among these ligands, many extracellular matrix proteins like laminins, collagens, fibronectin, etc., are known to interact with one or more integrins.

Selectins are a family of three different glycoproteins sharing a conserved structure and these proteins contain an N-terminal C-type lectin domain (Blankenberg *et al.*, 2003). Unlike other adhesion receptors, selectins bind to carbohydrates and glycopeptides rather than protein ligands (Hillis, 1998) The three members of the selectin family are named according to their main expression site: L-selectin is expressed in leukocytes, E-selectin is specific to endothelial cells and P-selectin is mainly found on platelets but is also expressed in endothelial cells (Bevilacqua & Nelson, 1993). All selectins participate in different, though overlapping, ways to the early steps of leukocyte recruitment at the endothelial surface under shear forces: leukocyte rolling and tethering (Hillis, 1998 ; Blankenberg *et al.*, 2003) (Fig 1-5).

The immunoglobulin gene superfamily such as the ICAM-1, ICAM-2 and ICAM-3; VCAM-1 and PECAM-1 are principally involved in the adhesion of leucocytes (Hillis, 1998) (table 1-1). Immunoglobulin molecules contain a series of immunoglobulin-like domains of 90 to 100 amino acids that function as endothelial ligands for the integrins expressed on leukocytes and platelets (Muller *et al.*, 1993). ICAM-1 to 3 molecules mediate adhesion of leukocytes to activated endothelium by establishing strong bonds with integrins and inducing firm arrest of inflammatory cells at the vascular surface, and

participate to leukocyte extravasation (Fig 1-5). VCAM-1 participates to the recruitment of blood cells by activated endothelium by favoring their firm adhesion (Blankenberg *et al.*, 2003) (Fig 1-5). Previous studies have shown that the first step in atherosclerosis development is the adhesion of monocytes to the vascular endothelium (Ross, 1999). The importance of the inflammatory response and the cellular interactions mediated by adhesion molecules in the development of the fatty streak and the fibrous plaque is also evident from experimental data and pathological observations (Daniel *et al.*, 1999). Proinflammatory cytokines such as TNF- $\alpha$  and IL-1  $\beta$ , commonly found in atherosclerotic lesions, can induce chemotactic factors, other cytokines, and cell adhesion molecules, all of which contribute to the inflammatory process (Zibara *et al.*, 2000; Ross, 1999; Springer, 1994). Previous report has indicated that E-selectin is synthesized by endothelial cells in response to IL-1  $\beta$  and TNF- $\alpha$  (Bevilacqua *et al.*, 1985). ICAM expression is induced by cytokines, such as TNF- $\alpha$  and IL-1 $\beta$ , or by adherence to extracellular matrix (Dustin *et al.*, 1986; Pober *et al.*, 1986; Daniel *et al.*, 1999) and VCAM-1 is similarly upregulated by IL-1  $\beta$  and TNF- $\alpha$  (Pober *et al.*, 1986; Marui. *et al.*, 1993).

### ***1.3 NF- $\kappa$ B and atherosclerosis***

Nuclear factor kappa-B (NF- $\kappa$ B) is a redox-sensitive transcription factor regulating a battery of inflammatory genes; it has been implicated as an important factor for initiation and progression of pathogenesis in atherosclerosis, inflammatory bowel disease, autoimmune arthritis, glomerulonephritis, asthma, lung fibrosis, septic shock, carcinogenesis and AIDS (Barnes & Adcock, 1997; Chen *et al.*, 1999; Ghosh *et al.*,



1998 ).

NF- $\kappa$ B was first identified as a regulator of the expression of the kappa light-chain gene in murine B lymphocytes (Sen & Baltimore, 1986). The NF- $\kappa$ B/Rel family includes NF- $\kappa$ B1 (p50/p105), NF- $\kappa$ B2 (p52/p100), p65 (RelA), RelB, and c-Rel NF- $\kappa$ B (Chen *et al.*, 1999), and most members of this family (RelB being one exception) can be homodimers or heterodimers with each other. The activated form of NF- $\kappa$ B is a heterodimer, which usually consists of two proteins, a p65 (also called relA) subunit and a p50 subunit. Other subunits, such as rel, relB, v-rel, and p52, may also be part of activated NF- $\kappa$ B, and it is likely that the different forms of NF- $\kappa$ B may activate different sets of target genes. In unstimulated cells, NF- $\kappa$ B exists in the cytoplasm in an inactive form and is bound to I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$ , which prevent it from entering the nuclei (Baldwin, 1996). Agents that activate NF- $\kappa$ B induce specific phosphorylation events on I $\kappa$ B via I $\kappa$ B kinase activity, which direct I $\kappa$ B to a ubiquitination/proteosomal degradation pathway. Degradation of I $\kappa$ B thus unmasks the nuclear localization sequence of NF- $\kappa$ B and allows NF- $\kappa$ B to enter the nucleus and bind to specific DNA containing the sequence that activates gene transcription (5'-GGGPuNNPyPyCC-3'), where Pu = purine, N = any base; and Py = pyrimidine (Baeuerle & Henkel, 1996) (Fig. 1-6).

Many stimuli activate NF- $\kappa$ B, including reactive oxygen intermediates, hypoxia/anoxia, hyperoxia, cytokines, protein kinase C activators, mitogen-activated protein kinase (MAPK) activators, bacterial or viral products, such as lipopolysaccharide (LPS), dsRNA, or the human T-cell leukemia virus type 1 Tax protein, and by UV-irradiation (Barnes & Adcock, 1997; Li *et al.*, 1997; Chen *et al.*, 1999; Ghosh *et al.*, 1998; Thurberg & Collins, 1998; Li & Karin, 1999; Li *et al.*, 1999). NF- $\kappa$ B regulates the expression of many genes involved in both innate and adaptive immunity, among them

pro-inflammatory cytokines, chemokines, leukocyte adhesion molecules and inflammatory enzymes (Barnes & Adcock, 1997; Chen *et al.*, 1999; Ghosh *et al.*, 1998; Thurberg & Collins, 1998).

It is well established that NF- $\kappa$ B in the development of atherosclerotic injury, mediates cell migration, endothelial cell activation and controls the balance between cell proliferation and apoptosis (Tak & Firestein, 2001). Several of the cytokines and growth factors found in the atherosclerotic lesion, such as TNF- $\alpha$  and IL-1 $\beta$ , activate NF- $\kappa$ B in vitro in relevant cell types such as macrophages, SMCs, ECs, and lymphocytes. The activation of NF- $\kappa$ B in endothelial cells is associated with the activation of genes responsible of an increased transcription of adhesion molecules, cytokines and chemokines, which function in regulating critical processes in atherogenesis (True *et al.*, 2000; Valen *et al.*, 2001; Thornburg *et al.*, 2003; Hatada *et al.*, 2003)

#### ***1.4 Oxidative Stress and atherosclerosis***

The "response to injury" hypothesis developed by Russell Ross in the late 1970s suggested that atherosclerosis resulted from an initial injury to endothelial cells, leading to impaired endothelial function and subsequent macrophage infiltration and smooth muscle dysfunction. More recently, it has become apparent that reactive oxygen species (ROS) play central roles in the pathogenesis of endothelial dysfunction and atherosclerosis (Harrison *et al.*, 2003), the most important of which are nitric oxide (NO $\bullet$ ), superoxide (O $_2^-$  $\bullet$ ), hydrogen peroxide (H $_2$ O $_2$ ) and peroxynitrite (ONOO $^-$  $\bullet$ ). Recent studies have begun to implicate ROS in the cellular responses to inflammatory cytokines such as TNF- $\alpha$ , IL-1  $\beta$  (Chua *et al.*, 1998; Simon *et al.*, 1998), whereas other studies have

demonstrated that ROS participates in the intracellular signaling initiated during physiological hypoxia (Chandel *et al.*, 1998; Duranteau *et al.*, 1998). ROS in both of these cellular responses suggests that cytokines and hypoxia may interact in the regulation of endothelial barrier function during inflammation (Charles & Russell, 1999).

Previous study indicated that the activation mechanism of I $\kappa$ B degradation and NF- $\kappa$ B nuclear translocation have been suggested to involve ROS (Schreck *et al.*, 1991, 1992; Suzuki *et al.*, 1993). Other studies have demonstrated that ROS plays a role as second messengers to regulate signal transduction pathways that ultimately control gene expression and posttranslational modifications of proteins (Charles & Russell, 1999).

## **1.5 Curcumin**

Curcumin (Fig 1-7), which is consumed daily by millions of people, is a polyphenol derived from the plant turmeric (*Curcuma longa*) (Fang *et al.*, 2005) and is used as a spice to give a specific flavor and yellow color to curry. In the Indian subcontinent and Southeast Asia, turmeric has traditionally been used as a treatment for inflammation, skin wounds, and tumors (Christopher *et al.*, 2002). Curcumin exhibits a variety of pharmacological effects including antitumor, anti-inflammatory, anti-infectious activities and is currently in clinical trials for AIDS patients (Mazumder *et al.*, 1995; Ruby *et al.*, 1995; Surh, 2002). Molecular actions of curcumin to elucidate these therapeutic effects have been associated with a variety of signaling pathways such as NF- $\kappa$ B, AP-1, and the early growth response gene (EGR-1) (Huang *et al.*, 1995; Pendurthi & Rao, 2000; Grandjean *et al.*, 2002). Curcumin has been shown to inhibit the expression of a sequence of inflammatory cytokines such as TNF- $\alpha$ , IL-1, or IL-8 (Surh,

2002). Previous study also indicated that supplementation with curcuma longa reduced oxidative stress and attenuated the development of fatty streaks in rabbits fed a high cholesterol diet (Quiles *et al.*, 2002).

## **1.6 Carnosic acid**

Sage (*Salvia officinalis*) and Rosemary (*Rosmarinus officinalis*), popular Labiatae herbs, are increasingly used as food additives with very high antioxidant activity, they have been proposed as important human dietary factors and inhibitors of skin tumorigenesis (Singletary & Nelshoppen, 1991; Schwarz *et al.*, 1992; Huang *et al.*, 1994). Carnosic acid (Fig 1-8), an antioxidant polyphenol derived from these plants, is a lipophilic antioxidant that scavenges singlet oxygen, hydroxyl radicals, and lipid peroxy radicals, thus preventing lipid peroxidation and disruption of biological membranes (Aruoma *et al.*, 1992; Haraguchi *et al.*, 1995). Its radical scavenging activity follows a mechanism analogous to those of other antioxidants such as  $\alpha$ -tocopherol and is caused by the presence of two *O*-phenolic hydroxyl groups found at C<sub>11</sub> and C<sub>12</sub> of the molecule (Richheimer *et al.*, 1999). Previous studies indicated that carnosic acid augmented the inhibition of growth and the induction of differentiation of HL60 and U937 leukemia cells by 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> and all-trans-retinoic acid (ATRA) (Danilenko *et al.*, 2000). Previous study also demonstrated that carnosic acid could inhibit plasma triglyceride elevation in olive oil-loaded mice and reduce the gain of body weight and the accumulation of epididymal fat weight in high fat diet-fed mice (Kiyofumi *et al.*, 2004).

## ***1.7 The purpose of this research***

Atherosclerosis, a progressive pathological disorder leading to cardiovascular and cerebrovascular diseases, is still the leading cause of mortality and morbidity in industrialized countries, in spite of improved pharmacological and lifestyle approaches (Ross, 1993). The adhesion of circulating leukocytes to the vascular endothelium is a critical early event in the development of atherosclerosis (Joris *et al.*, 1983; Faggiotto *et al.*, 1984). This process depends on the interaction between cell adhesion molecules expressed on the surface of endothelial cells and their cognate ligands on leukocytes (Price & Loscalzo, 1999). Previous studies have indicated that NF- $\kappa$ B/Rel transcription factors may play an important role in the development of the atherosclerosis (Collins, 1993; Qwarnstrom *et al.*, 1994). The activation of NF- $\kappa$ B in endothelial cells is associated with the activation of genes responsible of an increased transcription of adhesion molecules, cytokines and chemokines (True *et al.*, 2000; Valen *et al.*, 2001; Thornburg *et al.*, 2003; Hatada *et al.*, 2003).

Plant polyphenols are a large group of naturally-occurring antioxidants and epidemiological studies have suggested that higher polyphenol intake from fruits and vegetables is associated with decreased risk for cardiovascular disease (Ilja & Peter, 2005). Previous studies showed that polyphenolic compounds such as dietary flavonoids or red wine polyphenols could prevent atherosclerosis by inhibiting adhesion molecules expression in endothelial cell (Murase *et al.*, 1999; Silvina & Balz, 2006). Curcumin, which is consumed daily by millions of people, is a polyphenol derived from the plant turmeric (*Curcuma longa*) (Fang *et al.*, 2005) and is used as a spice to give a specific flavor and yellow color to curry. Curcumin exhibits a variety of pharmacological effects

including antitumor, anti-inflammatory, anti-infectious activities and is currently in clinical trials for AIDS patients (Mazumder *et al.*, 1995; Ruby *et al.*, 1995; Surh, 2002). Previous study also indicated that supplementation with *curcuma longa* reduced oxidative stress and attenuated the development of fatty streaks in rabbits fed a high cholesterol diet (Quiles *et al.*, 2002). Carnosic acid, an antioxidant polyphenol derived from Sage (*Salvia officinalis*) and Rosemary (*Rosmarinus officinalis*), is a lipophilic antioxidant that scavenges singlet oxygen, hydroxyl radicals, and lipid peroxy radicals, thus preventing lipid peroxidation and disruption of biological membranes (Aruoma *et al.*, 1992; Haraguchi *et al.*, 1995). Previous study also demonstrated that carnosic acid can inhibit plasma triglyceride elevation in olive oil-loaded mice and reduce the gain of body weight and the accumulation of epididymal fat weight in high fat diet-fed mice (Kiyofumi *et al.*, 2004). Therefore, we designed to examine the inhibitory effects of curcumin and carnosic acid on monocyte adhesion to cultured human endothelial cells and the expression of adhesion molecules (VCAM-1, ICAM-1 and E-selectin) by IL-1  $\beta$  in HUVECs and elucidate its possible mechanism of action.

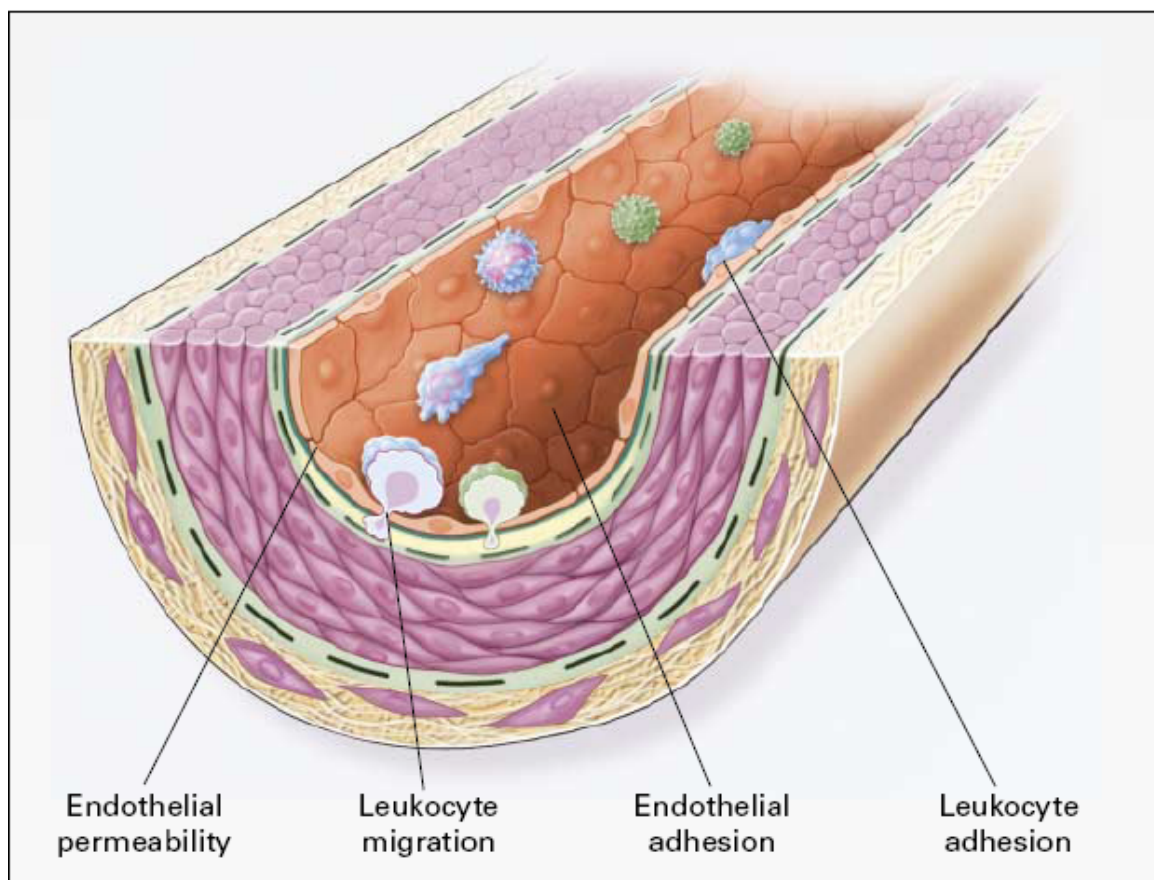


Fig. 1-1. Endothelial dysfunction in atherosclerosis (Ross, 1999).

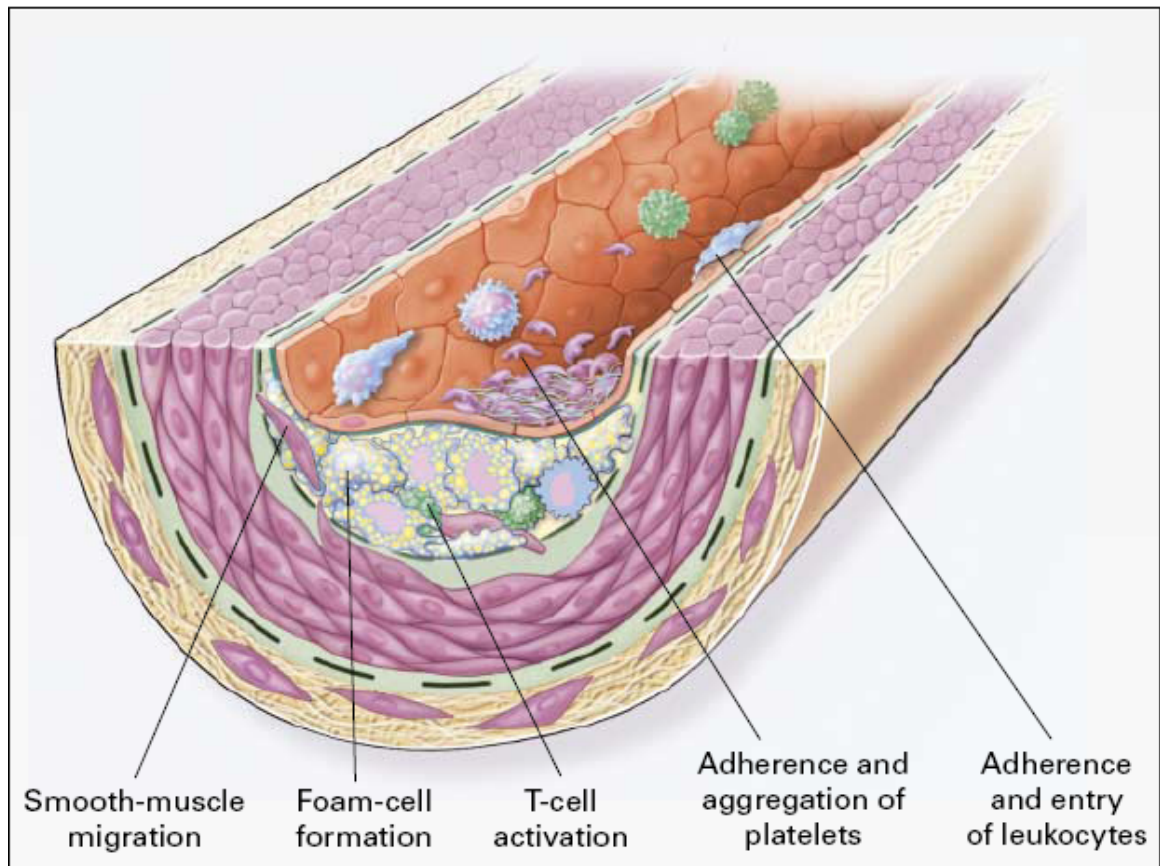


Fig. 1-2. Fatty-streak formation in atherosclerosis (Ross, 1999).



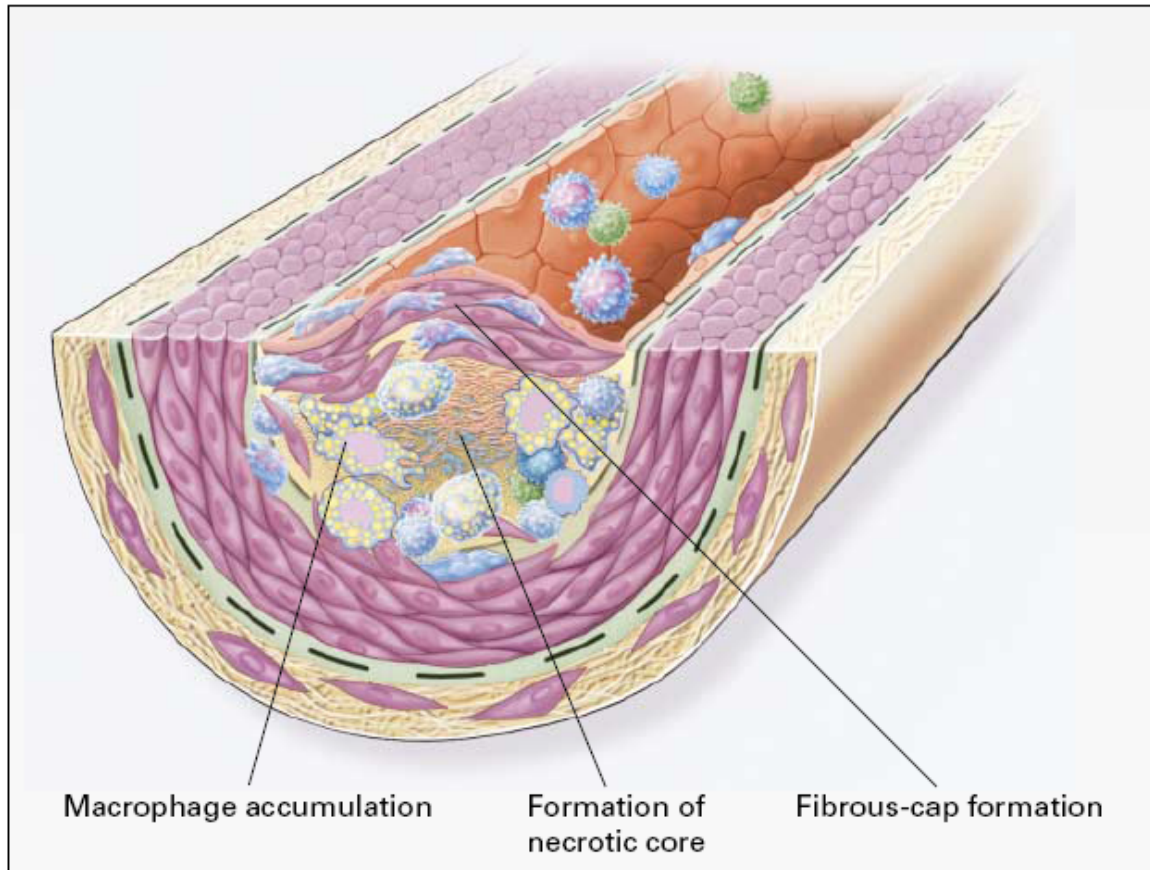


Fig. 1-3. Formation of an advanced, complicated lesion of atherosclerosis (Ross, 1999).

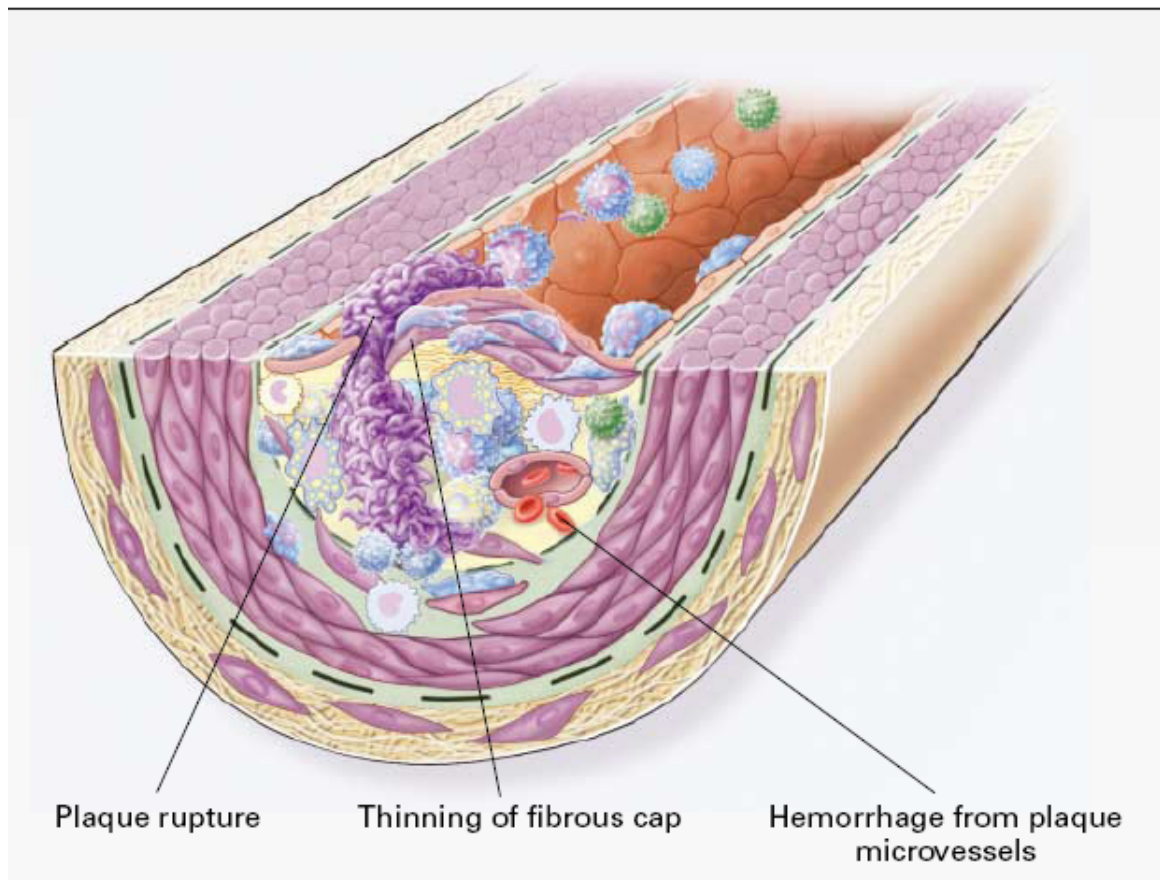


Fig. 1-4. Unstable fibrous plaques in atherosclerosis (Ross, 1999).

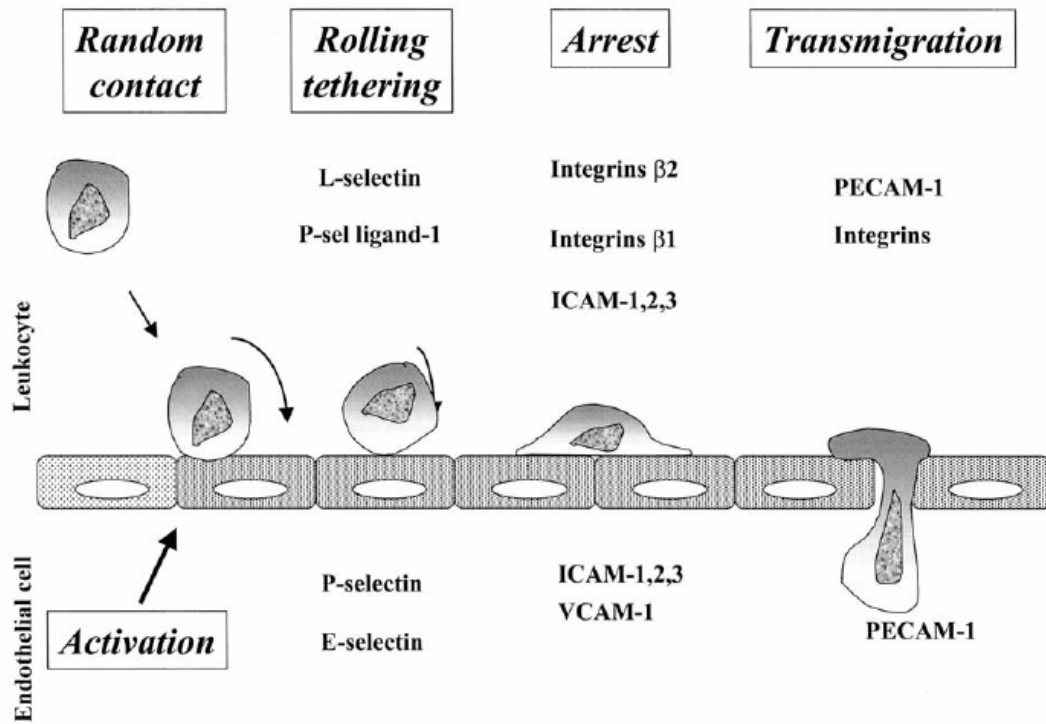


Fig. 1-5. Role of the different adhesion molecule in atherosclerosis. (Blankenberg *et al.*, 2003)

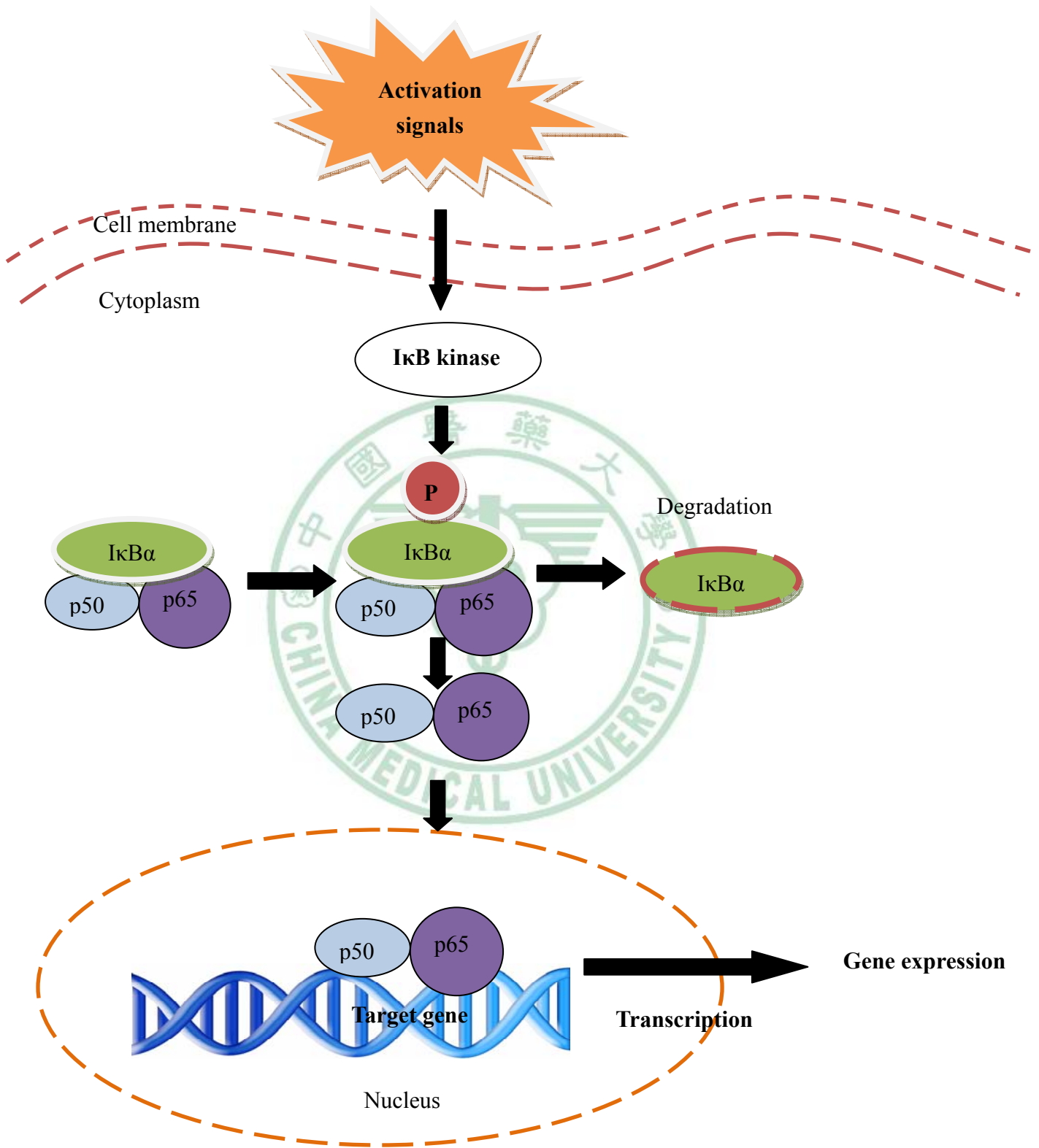


Fig. 1-6. The signal transduction pathway of NF- $\kappa$ B activation (Baeuerle & Henkel, 1996).

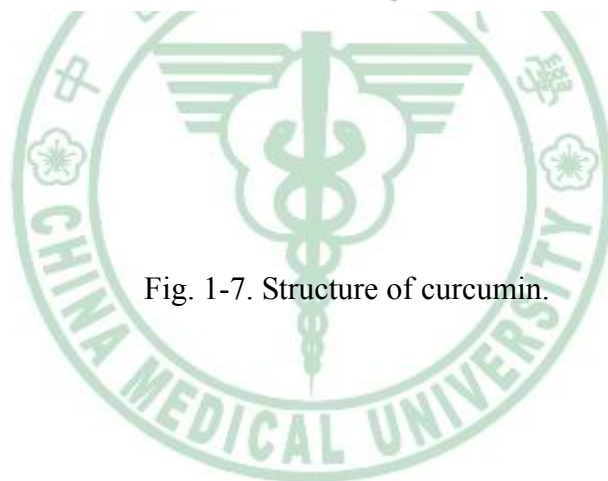
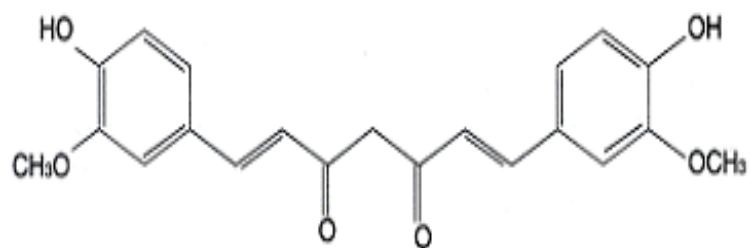


Fig. 1-7. Structure of curcumin.

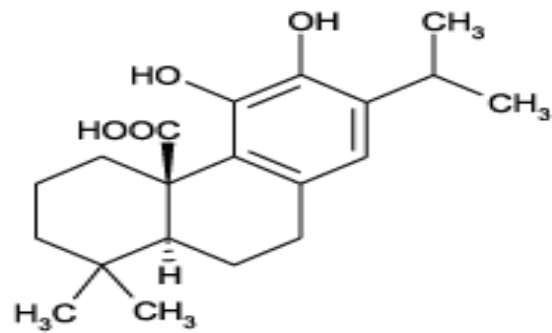
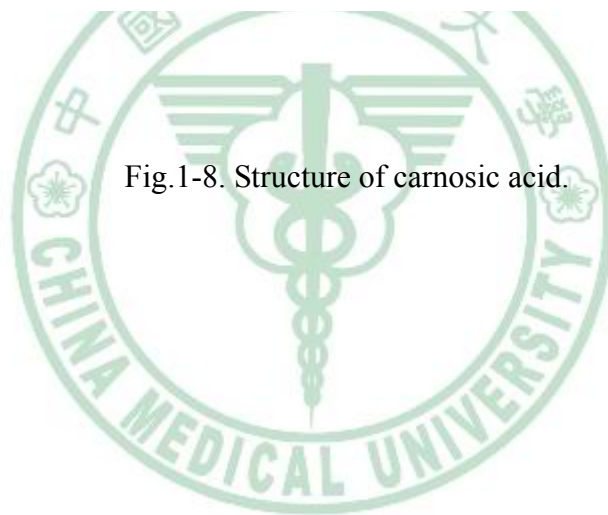


Fig.1-8. Structure of carnosic acid.



**Table 1-1**

Description of adhesion molecules involved in atherosclerosis, their main ligands, their functions, and their tissue distribution

Adhesion molecules	Other names	Ligands	Functions	Tissue distribution		
				Endoth	Leuko	Platelets
<i>Selectins/ligands</i>						
P-selectin	CD62P, GMP140	PSGL-1, Lewis X, CD24	Rolling/tethering	+		+
E-selectin	CD62E, ELAM1	ESL-1, Lewis X, PSGL-1, L-set	Rolling/tethering	+		
L-selectin	CD62L	Lewis X, CD34, PSGL-1, GlyCAM	Rolling/tethering		+	
E-selectin ligand 1	ESL-1	E-selectin	Rolling/tethering		+	
P-selectin ligand 1	CD162, PSGL-1	P-, L-, E-selectin	Rolling/tethering		+	
<i>Immunoglobulins</i>						
ICAM-1	CD54	$\alpha$ L $\beta$ 2, $\alpha$ M $\beta$ 2, $\alpha$ X $\beta$ 2	Firm adhesion	+	+	
ICAM-2	CD102	$\alpha$ L $\beta$ 2, $\alpha$ M $\beta$ 2	Firm adhesion	+	+	+
ICAM-3	CD50	$\alpha$ L $\beta$ 2, $\alpha$ D $\beta$ 2, DC-SIGN	Firm adhesion	+	+	
VCAM-1	CD106	$\alpha$ 4 $\beta$ 1, $\alpha$ 4 $\beta$ 7, $\alpha$ D $\beta$ 2	Firm adhesion	+		
PECAM-1	CD31	PECAM-1, $\alpha$ V $\beta$ 3	Endothelial integrity, leukocyte extravasation	+	+	+
<i>Integrins</i>						
Integrin $\alpha$ 2/ $\beta$ 1	CD49b/CD29, VLA2	Collagen, laminin	Platelet receptor			+
Integrin $\alpha$ 4/ $\beta$ 1	CD49d/CD29, VLA4	VCAM-1, FN	Firm adhesion	+		
Integrin $\alpha$ L/ $\beta$ 2	CD11a/CD18, LFA1	ICAMs	Firm adhesion		+	
Integrin $\alpha$ M/ $\beta$ 2	CD11b/CD18, Mac1	ICAMs, iC3b, FX, FG	Firm adhesion		+	
Integrin $\alpha$ X/ $\beta$ 2	CD11c/CD18	ICAM-1, FG, iC3b, CD23	Firm adhesion		+	
Integrin $\alpha$ D/ $\beta$ 2	CD11d/CD18	ICAM-3, VCAM-1	Firm adhesion		+	
Integrin $\alpha$ 2B/ $\alpha$ 3	GPIIb/IIIa	vWF, FN, FG, VN, thrombospondin	Platelet receptor			+
Integrin $\alpha$ V/ $\beta$ 3	VNR, CD51/CD61	PECAM-1, VN, FN, FG, vWF	Proliferation, migration	+		+
Integrin $\alpha$ V/ $\beta$ 5		VN	Proliferation, migration	+		

( Blankenberg *et al.*, 2003 )

## *Chapter 2*

### **Experimentation I**

Curcumin attenuates the expression of adhesion molecules by

IL-1  $\beta$  in HUVECs

薑黃素下降介白素-1  $\beta$  所誘發的人類臍靜脈內皮細胞黏附  
因子表現





## ***2.1 Prolegomenon***

Atherosclerosis, a progressive pathological disorder leading to cardiovascular and cerebrovascular diseases, is still the leading cause of mortality and morbidity in industrialized countries, in spite of improved pharmacological and lifestyle approaches (Ross, 1993). The adhesion of circulating leukocytes to the vascular endothelium is a critical early event in the development of atherosclerosis (Joris *et al.*, 1983; Faggiotto *et al.*, 1984). This process depends on the interaction between cell adhesion molecules expressed on the surface of endothelial cells and their cognate ligands on leukocytes (Price & Loscalzo, 1999). Previous studies have indicated that NF- $\kappa$ B/Rel transcription factors may play an important role in the development of the atherosclerosis (Collins, 1993; Qwarnstrom *et al.*, 1994). The activation of NF- $\kappa$ B in endothelial cells is associated with the activation of genes responsible of an increased transcription of adhesion molecules, cytokines and chemokines (True *et al.*, 2000; Valen *et al.*, 2001; Thornburg *et al.*, 2003; Hatada *et al.*, 2003). Plant polyphenols are a large group of naturally-occurring antioxidants and epidemiological studies have suggested that higher polyphenol intake from fruits and vegetables is associated with decreased risk for cardiovascular disease (Ilja & Peter, 2005). Previous studies showed that polyphenolic compounds such as dietary flavonoids or red wine polyphenols could prevent atherosclerosis by inhibiting adhesion molecules expression in endothelial cell (Murase *et al.*, 1999; Silvina & Balz, 2006). Curcumin, which is consumed daily by millions of people, is a polyphenol derived from the plant turmeric (*Curcuma longa*) (Fang *et al.*, 2005) and is used as a spice to give a specific flavor and yellow color to curry. Curcumin exhibits a variety of pharmacological effects including antitumor, anti-inflammatory,

anti-infectious activities and is currently in clinical trials for AIDS patients (Mazumder *et al.*, 1995; Ruby *et al.*, 1995; Surh, 2002). Previous study also indicated that supplementation with *curcuma longa* reduced oxidative stress and attenuated the development of fatty streaks in rabbits fed a high cholesterol diet (Quiles *et al.*, 2002). Therefore, we designed to examine the inhibitory effects of curcumin on monocyte adhesion to cultured human endothelial cells and the expression of adhesion molecules (VCAM-1, ICAM-1 and E-selectin) by IL-1  $\beta$  in HUVECs and elucidate its possible mechanism of action.



## 2.2 Materials and Methods

### 2.2.1 Instruments

CO <sub>2</sub> incubator	NUAIRE, MN, USA
Laminar flow	NUAIRE, MN, USA
Microscope	Nikon, Japan
pH meter	HANNA, RI, USA
Stirrer/Hotplate	Corning, Taiwan
Waterbath tank	TKS, Taiwan
Haemocytometer	Boeco, Germany
Eppendorf centrifugator	Hamburg, Germany
Pipetman	Gilson, France
Spectrophotometer	HITACHI, Japan
Spectrophotometer	Beckman Coulter, CA, USA
MicroPlate fluorescence reader	Bio-Tek, VT, USA
Shaking incubator	Orbital, VA, USA
ELISA plate reader	Bio-Tek, VT, USA
PCR thermocycler	Bio-Rad, CA, USA
Electrophoresis tank	Bio-Rad, CA, USA
Transfer system	Bio-Rad, CA, USA
Electrophoresis chamber	Bio-Rad, CA, USA
Power supply	Hoefler, CA, USA



## 2.2.2 Chemicals

ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid))	Sigma, MO, USA
BSA (Bovine serum albumin)	Sigma, MO, USA
Bradford reagent	Bio-Rad, CA, USA
CHCl <sub>3</sub> (Chloroform)	BDH, Poole, England
Curcumin	Sigma, MO, USA
CuSO <sub>4</sub> (Cupric sulfate)	Sigma, MO, USA
DCFH-DA (2', 7'-dichlorofluorescein diacetate)	Molecular Probe, Oregon, USA
DEPC (Diethyl pyrocarbonate)	Sigma, MO, USA
DMSO (Dimethyl sulfoxide)	Sigma, MO, USA
DPPH (2, 2-diphenyl-1-picrylhydrazyl)	Sigma, MO, USA
DTT (1, 4-Dithio-D,L-threitol)	Bio-Rad, CA, USA
ECG (Endothelial cell growth supplement)	Sigma, MO, USA
EDTA-Disodium (Ethylenediamine Teraacetate Disodium Salt)	Bio-Rad, CA, USA
FBS (Fetal Bovine serum)	Gibco, NY, USA
Gelatin (2% solution Type B)	Sigma, MO, USA
Glucose	Sigma, MO, USA
HBSS (Hank's balanced salts modified)	Sigma, MO, USA
HCl (Hydrochloric acid)	Wako, Osaka, Japan
Heparin	Sigma, MO, USA
Hepes buffer solution (1 mol/l)	Gibco, NY, USA
H <sub>2</sub> O <sub>2</sub> (Hydrogen Peroxide)	Wako, Osaka, Japan
KBr (potassium bromide)	SHOWA, Tokyo, Japan

KCl (potassium chloridem)	SHOWA, Tokyo, Japan
KH <sub>2</sub> PO <sub>4</sub> (potassium dihydrogenphosphate)	SHOWA, Tokyo, Japan
L-Glutamine (200 mmol/l)	Gibco, NY, USA
MDA (malonaldehyde bis-(dimethyl acetal))	Alderich, WI, USA
MTT (3-[4, 5-Dimethylthiazol-2-yl]-2, 5-diphenyl-terazoliumbromide)	Sigma, MO, USA
Medium 199	Sigma, MO, USA
Methanol	TEDIA, OH, USA
NaCl (sodium chloride)	Sigma, MO, USA
NaHCO <sub>3</sub> (sodium bicarbonate)	Sigma, MO, USA
Na <sub>2</sub> HPO <sub>4</sub> (Disodium hydrogenphosphate)	SHOWA, Tokyo, Japan
NF-κB kit	TransAM, Active Motif, CA, USA
Nuclear Extract kit	TransAM, Active Motif, CA, USA
Penicillin-Streptomycin	Gibco, NY, USA
Peroxidase (Type VI-A)	Sigma, MO, USA
Recombinant human interleukin-1 beta	ProSpec-Tany, Rehovot, Isreal
RPMI-1640	Gibco, NY, USA
TBA (2-Thiobarbituric acid)	Sigma, MO, USA
TCA (Trichloroacetic acid)	Sigma, MO, USA
Trolox (6-Hydroxy-2,5,7,8-treamethylchroman-2-carboxylic acid)	Alderich, WI, USA
Trypsin-EDTA (0.25 %)	Gibco, NY, USA
SDS (Sodium dodecyl sulfat)	Bio-Rad, CA, USA
SYBR Green suoermix	Bio-Rad, CA, USA

2-propanol	Sigma, MO, USA
95 % Ethanol	Echo Chemical, Taichung, Taiwan
Mouse anti-human $\beta$ -actin	Abcam, Cambridge, UK
Mouse anti-human ICAM-1	Abcam, Cambridge, UK
Mouse anti-human VCAM-1	Abcam, Cambridge, UK
Mouse anti-human E-selectin	Abcam, Cambridge, UK
Sheep anti-mouse IgG antibody	Abcam, Cambridge, UK

### 2.2.3 Antioxidative capacities of curcumin *in vitro*

#### 2.2.3.1 DPPH scavenging assay

Free radical scavenging effect was determined using the free radical generator DPPH (2, 2-diphenyl-1-picrylhydrazyl) by a similar method to Yamaguchi *et al.* 1998. Briefly, the reaction mixture contained 500  $\mu$ l of curcumin concentrations (0–200  $\mu$ mol/l) and 500  $\mu$ l of DPPH (0.5 mmol/l in methanolic solution). The DPPH radical scavenging activity was evaluated by measuring the decrease of DPPH radical detected at 517 nm and by determining the difference in the peak area between control and reaction mixture. Inhibition % was calculated via Eq.

$$\text{Inhibition \%} = (B_0 - B_1 / B_0) \times 100$$

(where B<sub>0</sub>: is the absorbance of control. B<sub>1</sub>: is the absorbance of reaction mixture.)

The decoloration was plotted against the sample extract concentration in order to calculate the IC<sub>50</sub> values (inhibitory concentration 50  $\mu$ mol/l), which is the amount of sample needed to decrease the absorbance of DPPH by 50%.

### 2.2.3.2 Trolox equivalent antioxidant capacity (TEAC) assay

The assay was carried out using a Spectronic Genesys 5 spectrophotometer by the improved ABTS<sup>•+</sup> method as described by Miller *et al.*, (1993) with slight modification. Briefly, ABTS<sup>•+</sup> radical cation was generated by a reaction of 500 µmol/l 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and 450 µmol/l hydrogen peroxide. The reaction mixture was allowed to stand in the dark for 6 min at room temperature and used within 2 days. All samples were diluted appropriately to provide 20–80% inhibition of the blank absorbance. Fifty microliters of the diluted sample were mixed with 1.9 ml of diluted ABTS<sup>•+</sup> solution. The assay with the mixture was carried out in triplicate, the mixture was allowed to stand for 6 min at room temperature and the absorbance was immediately recorded at 734 nm (Li *et al.*, 2007). Trolox solution (final concentration 0~15 µmol/l) was used as a reference standard. A value of 1 TEAC in a sample is defined as a concentration equivalent to 1 µmol/l Trolox, a water-soluble analog of  $\alpha$ -tocopherol.

### 2.2.3.3 Inhibition of LDL Oxidation

Blood was collected from healthy donors after a 12 h overnight fasting using EDTA as anticoagulant. Plasma was obtained after low-speed centrifugation of the blood, adjusted to a density of 1.21 g/ml with KBr and a discontinuous density gradient was made by overlaying the plasma solution with a 10 mmol/l PBS and 1 mmol/l EDTA pH 7.4. LDL were isolated after ultracentrifugation as described previously (Vieira *et al.*, 1996). The LDL fraction was exhaustively dialyzed against PBS buffer pH 7.4 without

EDTA in the dark, filtered through a 0.45  $\mu\text{m}$  filter, stored at 4  $^{\circ}\text{C}$  under nitrogen and used in 24-72 h. LDL were oxidised using the classical copper-induced LDL auto-oxidation (Wallin *et al.*, 1993). Briefly, 0.9 mg/ml of LDL total cholesterol was incubated in PBS in the presence of  $\text{CuSO}_4$  (50  $\mu\text{mol/l}$ ). Increasing concentrations of curcumin (0–10  $\mu\text{mol/l}$ ) dissolved in DMSO were present in the incubation media. After incubation, 150  $\mu\text{L}$  EDTA (2 mmol/l) was added. A 100  $\mu\text{l}$  portion of the mixture was then transferred to an eppendorf tube containing 0.9 ml of 2-propanol. The precipitates were removed by centrifugation. The concentration of conjugated diene in the supernatant was determined by absorption at 234 nm.

#### 2.2.4 Cell Culture

Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cords using collagenase type II (Jaffe, 1973), and cultured on 0.1 % gelatin-coated culture dishes in medium M199 (Sigma, MO, USA) supplemented with 10 % FBS, 1 % Antibiotic -Antimycotic, Glutamine (2 mmol/l), Heparin (10 U/ml), HEPES (10 mmol/l), Endothelial cell growth supplement (ECG) (12.5  $\mu\text{g/ml}$ ) at 37 $^{\circ}\text{C}$  in a humidified atmosphere of 5 %  $\text{CO}_2$  and 95 % air. After 3 days, the medium was replaced by fresh medium and subcultured at a 1: 4 ratio one time per week. All experiments were performed with HUVEC from passages two to five.

#### 2.2.5 Cell viability assay (MTT test)

The viability of the cells was assessed by MTT (3-[4,5-dimethylthiazol-2-yl] -2,5



-diphenyl-tetrazolium bromide) assay (Mosmann, 1983), which is based on the reduction of MTT by the mitochondrial dehydrogenase of intact cells to a blue formazan product. Briefly,  $1 \times 10^4$  cells/well were dispensed within 96-well culture plates and incubated with various concentrations of curcumin (which was dissolved in dimethyl sulphoxide) for 24 h. Four hours before the end of the treatment 10  $\mu$ l MTT (5 mg/ml) was added to each well. At the end of the treatment the incubation medium was removed and the formazan crystals were dissolved in 100  $\mu$ l of solution of DMSO. MTT reduction was quantified by measuring the light absorbance with a ELISA plate reader ( $\mu$ QUANT, Bio-Tek, USA) at 590 nm. The reduction in optical density caused by curcumin was used as a measurement of cell viability, normalized to cells incubated in medium with DMSO only, which were considered to be 100 % viable.

### **2.2.6 Adhesion of U937 cells to endothelial cells**

Adhesion was evaluated using the human leukemia pro-monocytic U937 cells which were labeled with calcein AM (10 nmol/l; Molecular Probe; Invitrogen) (Yu *et al.*, 2007). HUVECs ( $2 \times 10^5$ ) were distributed into 6-well plates before the assay and allowed to reach confluence. Then the growth medium was supplemented with 10 and 20  $\mu$ mol/l curcumin for 18 h, followed by incubating 10 ng/ml IL-1  $\beta$  for 6 h in the continued presence of curcumin. U937 cells were grown in RPMI 1640 medium (Gibco, New York, USA) containing 10 % FBS and subcultured at a 1:5 ratio three times per week. U937 cells were incubated with 10 nmol/l calcein AM in RPMI 1640 medium for 30 min at 37°C, then washed with PBS to remove free dye and resuspended in M-199 medium containing 10 % FBS. Labeled U937 cells ( $2 \times 10^5$ ) were added to each

HUVEC-containing well and incubation continued for 30 min. Non-adherent cells were removed by two gentle washes with PBS, then the number of bound U937 cells was determined by a fluorescence plate reader at an excitation wavelength of 485 nm and emission at 530 nm; HUVECs cell monolayers with DMSO only were served as the control.

### **2.2.7 Bradford assay**

The Bradford assay (Bradford, 1976), a colorimetric protein assay, is based on an absorbance shift in the dye Coomassie when bound to arginine and hydrophobic amino acid residues present in protein. The anionic (bound) form of the dye is blue and has an absorption spectrum maximum historically held to be at 595 nm. The cationic (unbound) forms are green and red. The increase of absorbance at 595 nm is proportional to the amount of bound dye, and thus to the amount (concentration) of protein present in the sample. Standard solutions contain a range of 0 to 25 micrograms protein (BSA) in 800  $\mu$ l H<sub>2</sub>O, followed by adding 200  $\mu$ l dye reagent and incubate 5 min. 1  $\mu$ l of sample solution add into 799  $\mu$ l H<sub>2</sub>O, followed by adding 200  $\mu$ l dye reagent and incubated for 5 min. The absorbance was read at 595 nm. The results made a standard curve and the protein concentration of sample was determined by standard curve.

### **2.2.8 Western blot**

For Western blotting,  $3 \times 10^6$  cells were seeded in 10 cm dishes and treated 10 and 20  $\mu$ mol/l curcumin for 18 h, followed by incubating 10 ng/ml IL-1  $\beta$  for 6 h in the

continued presence of curcumin, afterwards cells were scraped with a rubber policeman in PBS and centrifuged at 1,200 rpm for 10 min. Cells were lysed for 1 h at 4 °C with lysis buffer (0.32 mol/l Sucrose, 10 mmol/l Tris, 5 mmol/l EDTA, 2 mmol/l DTT, 1 % Triton X-100, and 1 mmol/l PMSF) and centrifuged at 12,000 g for 30 min at 4 °C. The protein concentration of cell extracts was determined with a Bradford-based assay (Bradford, 1976). Cell extracts were loaded per lane, resolved by 10 % SDS -PAGE and transferred at room temperature by blotting to polyvinylidene difluoride (PVDF) membrane (Shishodia *et al.*, 2003). Non-specific binding was blocked by soaking the membrane in PBS-Tween 20 buffer containing 50 g/L fat-free milk and separately incubated for 1 h at room temperature with mouse anti-human-VCAM-1, ICAM-1, and E-selectin antibodies. Subsequently, the membrane was incubated with a sheep anti-mouse IgG antibody. The protein levels were determined with the enhanced chemiluminescence (Upstate, USA) and High performance chemiluminescence film (Amersham biosciences, USA). Incubation with mouse anti-human  $\beta$ -actin antibody was also performed as an internal control. Results were quantified with scanning densitometer using an image analysis system with software.

### **2.2.9 Nuclear extract preparation**

Nuclear protein extracts were prepared using a nuclear extract kit (TransAM nuclear extract kit, CA, USA) from HUVECs to assay the NF- $\kappa$ B activity. Nuclear protein were prepared as described previously (Dschietzig *et al.*, 2001).  $3 \times 10^6$  cells were seeded in 10 cm dishes and treated 10 and 20  $\mu$ mol/l curcumin for 18 h, followed by incubating 10 ng/ml IL-1  $\beta$  for 6 h in the continued presence of curcumin, afterwards

cells were scraped with a rubber policeman in PBS, collected and centrifuged at 1,200 rpm for 10 min. Cells were lysed in hypotonic buffer and centrifuge suspension for 30 seconds at 14,000×g in a microcentrifuge pre-cooled at 4 °C. Then resuspend nuclear pellet in 50 µl complete lysis buffer containing 10 mmol/l DTT, lysis buffer AM1, and protease inhibitor cocktail by pipetting up and down. The suspension was incubated for 30 min on ice, and centrifuged for 10 min at 14,000×g in a microcentrifuge pre-cooled at 4 °C. Transfer supernatant and stored at -80 °C. The protein concentration was determined with a Bradford-based assay (Bradford, 1976).

### **2.2.10 Measurement of NF-κB activity**

For analysis of NF-κB activation with the TransAM NF-κB Family kit (TransAM, Active Motif, CA, USA) (Yu *et al.*, 2007). The oligonucleotide containing the NF-κB consensus binding site (5'-GGGACTTCC-3') specific for the active form of NF-κB was immobilized to a 96-well plate and the well was filled with 10 µg of nuclear extract. After 1 hour incubation and three washings, the primary antibody against the active form of NF-κB recognizing an epitope on p65, p50 that is accessible only when NF-κB is activated and bound to its target DNA was added for 1 hour. After washing, the secondary antibody conjugated to horseradish peroxidase was added to achieve a sensitive readout by spectrophotometry at 450 nm and the blank was subtracted from all measurements.

### **2.2.11 Measurement of reactive oxygen species**

ROS generation in cells was assessed using the probe 2,7-dichlorofluorescein (DCF) (Molecular Probes Europe BV, Leiden, The Netherlands) according to the method reported by Wang and Joseph (1999). Confluent HUVECs ( $1 \times 10^4$  cells/well) in 96-well plates were pretreated with 10 and 20  $\mu\text{mol/l}$  curcumin for 18 h, followed by incubation of 10 ng/ml IL-1  $\beta$  for 6 h in the continued presence of curcumin. After the removal of curcumin or carnosic acid from wells, cells were incubated with 10  $\mu\text{mol/l}$  DCFH-DA for 30 minutes. The fluorescence intensity (relative fluorescence units) was measured at 485-nm excitation and 530-nm emission using a fluorescence microplate reader.

### **2.2.12 Statistical analysis**

Results are shown as mean  $\pm$  S.D. Statistical analyses of MTT were performed using One-way ANOVA followed by Dunnett's test and others were performed using One-way ANOVA followed by Duncan's Multiple Range Test. A value of  $P < 0.05$  was considered statistically significant.

## **2.3 Results**

### **2.3.1 Antioxidative capacities of curcumin *in vitro***

Free radical scavenging effect was determined using the free radical generator DPPH (2, 2-diphenyl-1-picrylhydrazyl) and the IC<sub>50</sub> of DPPH assay was 45.84±0.14 µmol/l. In the inhibition of LDL oxidation assay, LDL were oxidized using the classical copper-induced LDL auto-oxidation and the IC<sub>50</sub> of inhibition of LDL oxidation was 2.56±0.12 µmol/l. The TEAC assay is based on the reduction of the ABTS (2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid)) radical cation by antioxidants and the TEAC value of curcumin was 2.88±0.04 (Table 2-1). A value of 1 TEAC in a sample is defined as a concentration equivalent to 1 µmol/l Trolox, a water-soluble analog of α-tocopherol.

### **2.3.2 Cell viability of curcumin for HUVECs**

Cell viability was determined by the MTT test. After 24 h incubation with 10 and 20, 30, 40 and 50 µmol/l curcumin, cell viability was 97.1 ± 5.5, 93.4 ± 3.2, 58.7 ± 4.3, 51.5 ± 3.3 and 48.5 ± 2.8 % of control levels, respectively, with the three highest concentrations causing a significant reduction in cell viability (Fig 2-1). Therefore, according to the MTT test we chose 10 and 20 µmol/l curcumin to do all the following experiments.

### **2.3.3 Curcumin inhibited the adhesion of U937 cells to IL-1 $\beta$ -stimulated HUVECs**

In order to determine the effect of curcumin in the adhesion of U937 cells to endothelial cells, HUVECs were treated with 10 and 20  $\mu\text{mol/l}$  curcumin for 18 h, followed by incubation of 10 ng/ml IL-1  $\beta$  for 6 h and the percentage of cell adhesion was evaluated by the quantification of calcein AM (Fig. 2-2 (B)). The adhesion of U937 cells to HUVECs also was photographed (Fig. 2(A)). The control group showed that minimal binding of HUVECs to U937 cells, but adhesion significantly increased when the HUVECs were treated with IL-1  $\beta$  (Fig 2-2 A & B). Pretreatment with 10 and 20  $\mu\text{mol/l}$  curcumin could significantly reduce the number of U937 cells adhering to IL-1  $\beta$ -stimulated HUVECs. The concentration of 20  $\mu\text{mol/l}$  curcumin was more effective on cell adhesion than 10  $\mu\text{mol/l}$  curcumin (Fig 2-2 A & B). These results indicate that curcumin could inhibit monocytes adhesion to endothelial cells.

### **2.3.4 Curcumin inhibits IL-1 $\beta$ -induced cell protein expression of VCAM-1, ICAM-1 and E-selectin in HUVECs**

To determine if the inhibition of cell adhesion by curcumin was due to inhibit the expression of adhesion molecules, HUVECs were pretreated for 18 h with 10 and 20  $\mu\text{mol/l}$  curcumin before the addition of 10 ng/ml IL-1  $\beta$ . The expression of ICAM-1, VCAM-1 and E-selectin was increased after IL-1  $\beta$  stimulation (Fig 2-3, 2-4, 2-5). Pretreatment of HUVECs with 10  $\mu\text{mol/l}$  curcumin significantly inhibited the expression of ICAM-1 and VCAM-1, but not E-selectin (Fig 2-3, 2-4, 2-5). Pretreatment of

HUVECs with 20  $\mu\text{mol/l}$  curcumin could significantly inhibit the expression of ICAM-1, VCAM-1 and E-selectin. Therefore, 20  $\mu\text{mol/l}$  curcumin was more effective than 10  $\mu\text{mol/l}$  curcumin on the inhibition of cell adhesion molecular expression (Fig 2-3, 2-4, 2-5).

### **2.3.5 Curcumin decrease activation of NF- $\kappa$ B and nuclear translocation of NF- $\kappa$ B p65 and p50 in IL-1 $\beta$ -stimulated HUVECs**

To examine whether the inhibitory effect of curcumin on the cytokine-induced expression of adhesion molecules was mediated via NF- $\kappa$ B, we measured the nuclear translocation of p65 and p50 protein of the NF- $\kappa$ B family. Incubation of IL-1  $\beta$  (10 ng/ml) for 6 h induced the nuclear translocation of p65 and p50 (Fig. 2-6, 2-7). Pretreatment of HUVECs with 10 and 20  $\mu\text{mol/l}$  curcumin significantly decreased the nuclear translocation of p65 and p50 (Fig. 2-6, 2-7). In addition, pretreatment of HUVECs with 20  $\mu\text{mol/l}$  curcumin was more effective on decreasing the nuclear translocation of p65 and p50 than that of 10  $\mu\text{mol/l}$  curcumin.

### **2.3.6 Curcumin inhibits IL-1 $\beta$ -induced ROS production in HUVECs**

To study the effect of curcumin on IL-1  $\beta$ -induced ROS production in HUVECs, cells were pretreated with 10 and 20  $\mu\text{mol/l}$  curcumin for 18 h and then stimulated by IL-1  $\beta$  (10 ng/ml) for 6 h. HUVECs were labeled with H<sub>2</sub>O<sub>2</sub>-sensitive fluorescent probe and detected by fluorescence microplate reader. The production of ROS induced by IL-1  $\beta$  was increased and significantly decreased after pretreatment with 10 and 20  $\mu\text{mol/l}$



curcumin (Fig. 2-8 A & B). In addition, pretreatment of HUVECs with 20  $\mu\text{mol/l}$  curcumin was more effective on decreasing the production of ROS than 10  $\mu\text{mol/l}$  curcumin.



## 2.4 Discussion

The expression of cell adhesion molecules by the endothelium and the attachment of monocytes to endothelium may play a major role in the early atherogenic process. Curcumin is the principal curcuminoid of the Indian curry spice turmeric. The curcuminoids are polyphenols and are responsible for the yellow color of turmeric. Previous studies indicated that curcumin exhibits a variety of pharmacological effects including antitumor, anti-inflammatory and anti-infectious activities (Mazumder *et al.*, 1995; Ruby *et al.*, 1995; Surh, 2002). In the present study, we found that curcumin had antioxidative effect *in vitro*. In addition, we also found that curcumin could suppress IL-1  $\beta$ -induced intracellular ROS production, the activation of redox-sensitive transcription factors NF- $\kappa$ B p50, p65, the expression of VCAM-1, ICAM-1 and E-selectin; and the adhesiveness to a human monocytic cell line (U937) in HUVECs. These results demonstrated that curcumin had inhibitory effect on proatherosclerotic mechanism *in vitro*.

Curcumin is the most active component of turmeric which contains 2 to 5 % of curcumin. Commercial curcumin is usually isolated from the rhizome of turmeric which contains three major curcuminoids (approximately 77 % curcumin, 17 % demethoxy-curcumin, and 3 % bisdemethoxycurcumin) (Bharat *et al.*, 2005). There are several good sources of curcumin with purity ranging from 60 to 98 % (Bharat *et al.*, 2005). Previous study indicated that the serum concentration was  $1.77 \pm 1.87 \mu\text{mol/l}$  after the intake of 8 g curcumin in human (Cheng *et al.*, 2001), therefore, curcumin was absorbable in digestive tract in human. In the present study, we found that  $20 \mu\text{mol/l}$  curcumin did not have any significant effect on the viability of HUVECs from MTT test, therefore, we chose 10 and

20  $\mu\text{mol/l}$  curcumin to do all the experiments.

One of the earliest events in atherogenesis is the adhesion of monocytes to the endothelium, followed by their infiltration and differentiation into macrophages. In the present study, we found that the control group showed minimal binding to U937 cells, but adhesion increased when the HUVECs were treated with IL-1  $\beta$ . Pretreatment with 10 and 20  $\mu\text{mol/l}$  curcumin reduced the number of U937 cells adhering to IL-1  $\beta$ -stimulated HUVECs. A similar result was seen when HUVECs were pretreated with other polyphenolic compound, such as vitamin E (40  $\mu\text{mol/l}$ ), probucol (50  $\mu\text{mol/l}$ ) or tea flavonoid (60  $\mu\text{mol/l}$ ), these polyphenolic compound reduced monocytes adhesion to endothelial cells (Islam *et al.*, 1998; Zapolska-Downar *et al.*, 2001; Ludwig *et al.*, 2004).

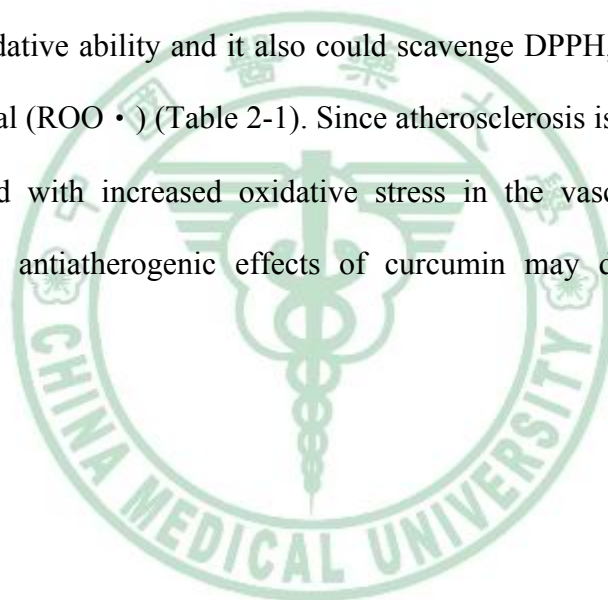
The process by which monocytes become adherent to the endothelium is the result of complex choreography requiring the sequential, yet overlapping, functions of many classes of adhesion molecules (Munro, 1993; Price & Loscalzo, 1999). Tethering and rolling, the first steps of monocyte adhesion to the endothelial surface, appear to depend on the interaction of P- and E- selectin with carbohydrate ligands on leukocytes. Firm adhesion follows if the leukocytes encounter activating signals while rolling along the endothelium, and is facilitated by the interaction of very late antigen-4 (VLA-4) with VCAM-1 or lymphocyte function antigen-1 (LFA-1) with ICAM-1 (Marlin & Springer, 1987; Elices *et al.*, 1990). Previous study showed that adhesion molecules are strong predictors of atherosclerotic lesion development and future cardiovascular events (Blankenberg *et al.*, 2003). It has been reported that expression of adhesion molecules on HUVECs is increased or induced by stimulation with inflammatory cytokines, including TNF- $\alpha$ , IL-1  $\beta$ , IL-4, and IL-13 (Schleimer & Rutledge, 1986). In the present study, we found that the expression of ICAM-1, VCAM-1 and E-selectin was increased by IL-1  $\beta$

and the pretreatment with curcumin could decreased the induction of expression by IL-1  $\beta$  in HUVECs. These results indicated that curcumin could inhibit the rolling, tethering and firm adhesion of the monocytes on the vascular wall.

NF- $\kappa$ B is a redox-sensitive transcription factor which mediates cell migration, endothelial cell activation and the balance between cell proliferation and apoptosis (Tak & Firestein, 2001). The activation of NF- $\kappa$ B in endothelial cells is associated with the activation of genes responsible for an increased transcription of adhesion molecules, cytokines and chemokines (True *et al.*, 2000; Valen *et al.*, 2001; Thornburg *et al.*, 2003; Hatada *et al.*, 2003). The activated form of NF- $\kappa$ B is a heterodimer, which usually consists of two proteins, a p65 (also called relA) subunit and a p50 subunit (Baldwin, 1996). Udalova *et al.*, 2000 described that the p50–p65 heterodimers are involved in enhancing the transcription of adhesion molecules. In the present study, we examined whether the inhibitory effect of curcumin on the cytokine-induced expression of adhesion molecules is mediated via NF- $\kappa$ B, therefore, we measured the nuclear translocation of p65 and p50 protein of the NF- $\kappa$ B family. We found that the nuclear translocation of p65 and p50 induced by IL-1  $\beta$  was increased and the pretreatment with curcumin could decreased the induction of translocation by IL-1  $\beta$  in HUVECs. Previous studies also showed that curcumin could inhibit the activation of NF- $\kappa$ B in human lung epithelial cells and human myelomonoblastic leukemia cells (Sanjaya & Bharat, 1995; Shishodia *et al.*, 2003). Therefore, we confirmed that curcumin has an anti-inflammatory effect through the partial interference of NF- $\kappa$ B activation.

Several studies have indicated that ROS are implicated in the activation of NF- $\kappa$ B (Muller *et al.*, 1997). In the present study, we found that the production of ROS induced by IL-1  $\beta$  was increased and the pretreatment with curcumin could decreased the

induction of expression by IL-1  $\beta$  in HUVECs. Previous studies showed that antioxidants such as PDTC and NAC could inhibit the activation of NF- $\kappa$ B, it is strongly suggested that endogenous ROS may play an important role in these redox-sensitive transcription pathways in atherogenesis (Schreck *et al.*, 1992; Weber *et al.*, 1994). Based on the present results, we proposed that the inhibitory effect of curcumin on adhesion molecules expression and NF- $\kappa$ B activation may be due to its antioxidant and anti-inflammatory properties and that it may act by directly scavenging free radicals. In the present study, we found that curcumin was approximately 2-3-folds more potent than Trolox in antioxidative ability and it also could scavenge DPPH, alkoxy radical (RO  $\cdot$ ) and peroxy radical (ROO  $\cdot$ ) (Table 2-1). Since atherosclerosis is a chronic inflammatory disease associated with increased oxidative stress in the vascular endothelium, it is possible that the antiatherogenic effects of curcumin may due to its antioxidative properties.



## ***2.5 Conclusion***

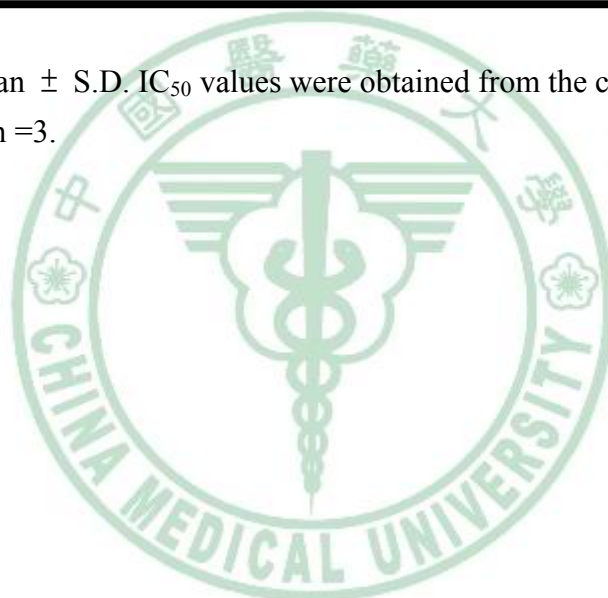
In the present study, we found that curcumin inhibited monocyte adhesion to endothelial cell, the expression of adhesion molecules, the translocation of NF- $\kappa$ B and the production of ROS in HUVECs. These findings may provide a rationale for the *in vitro* antiatherosclerosis effect of curcumin and support its potential use in the prevention of atherosclerosis.



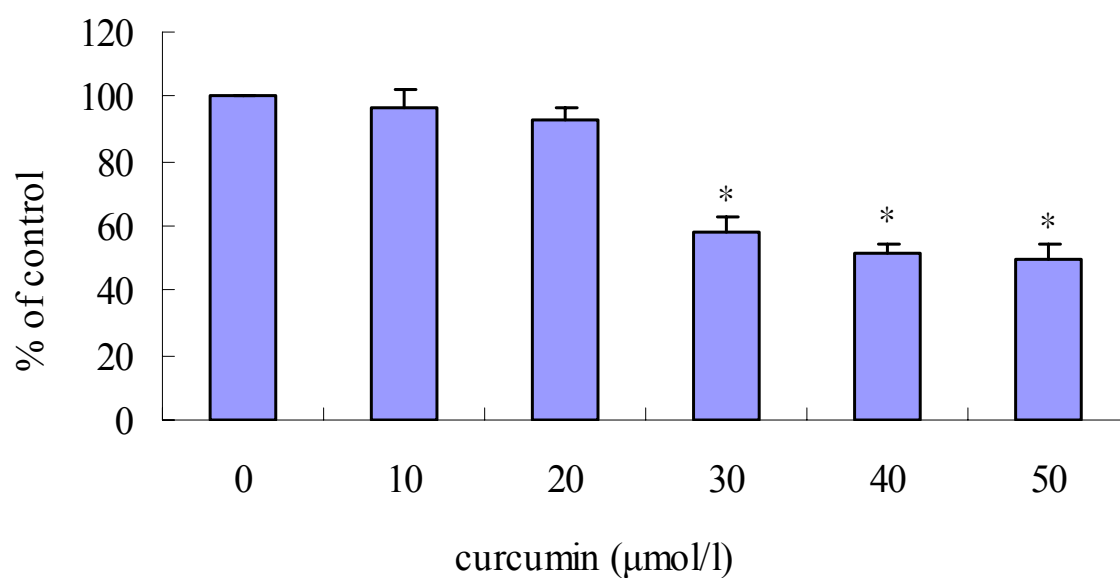
**Table 2-1.** Antioxidative capacities of curcumin *in vitro*.

	Inhibition of LDL Oxidation (IC <sub>50</sub> , μmol/l)	DPPH radicals scavenging ability (IC <sub>50</sub> , μmol/l)	TEAC
curcumin	2.56±0.12	45.8±0.1	2.88±0.04

All values are mean ± S.D. IC<sub>50</sub> values were obtained from the concentration response curves; n =3.



**Fig. 2-1**



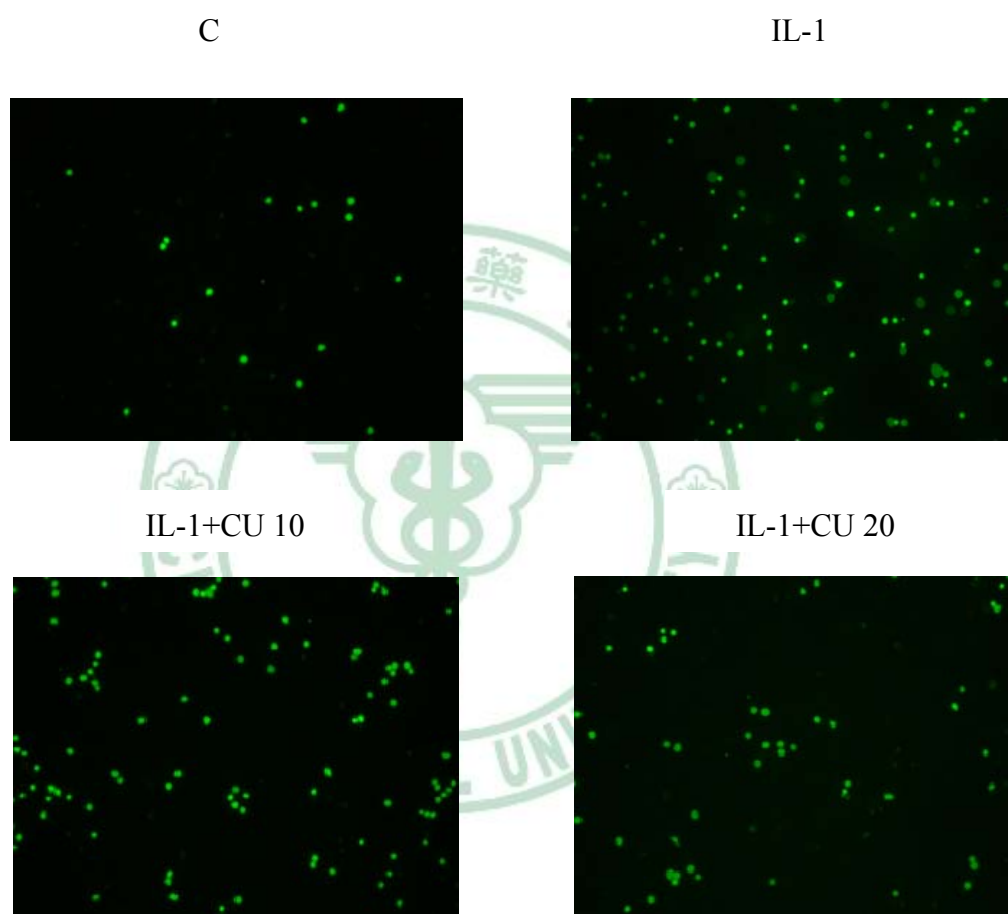
**Figure 2-1. Cytotoxic effect of curcumin on HUVECs with MTT test.**

HUVECs were treated with various concentration (0~50 µmol/l) of curcumin for 24 h in 10 % FBS-M199. Values are mean  $\pm$  S.D., n=4. \* $p < 0.05$  compared with control .



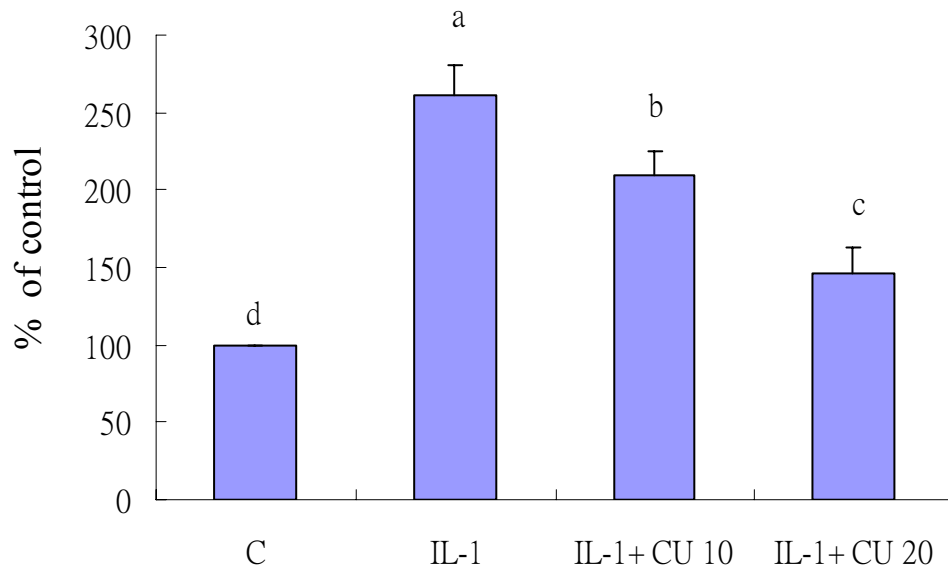
**Fig. 2-2**

(A)



**Fig. 2-2**

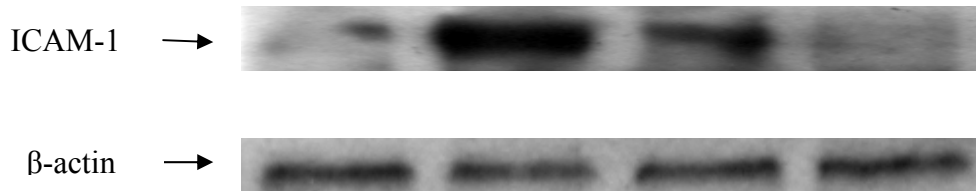
(B)



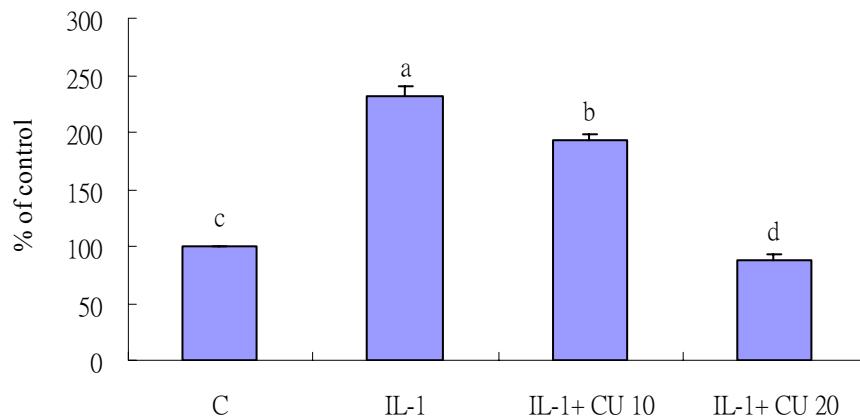
**Figure 2-2. Effect of curcumin on IL-1  $\beta$ -induced adhesion of U-937 cells to HUVECs.** (A) Representative images of the reduction of IL-1  $\beta$ -induced adhesion of U-937 cells to HUVECs monolayers after pretreatment of 10 and 20  $\mu\text{mol/l}$  curcumin for 18 hours. (B) HUVECs were pretreated with 10 and 20  $\mu\text{mol/l}$  curcumin for 18 hours and induced by IL-1  $\beta$  (10 ng/ml) for 6 hours. Fluorescence-labeled U-937 cell were added to the HUVECs monolayer and allowed to adhere for 30 min. Values are mean  $\pm$  S.D., n=3. <sup>a-d</sup> Means with different letters are significantly different at  $p < 0.05$ . Abbreviation: C (control), IL-1 (IL-1  $\beta$ ), CU10 (curcumin 10  $\mu\text{mol/l}$ ), CU20 (curcumin 20  $\mu\text{mol/l}$ ).

**Fig. 2-3**

(A)



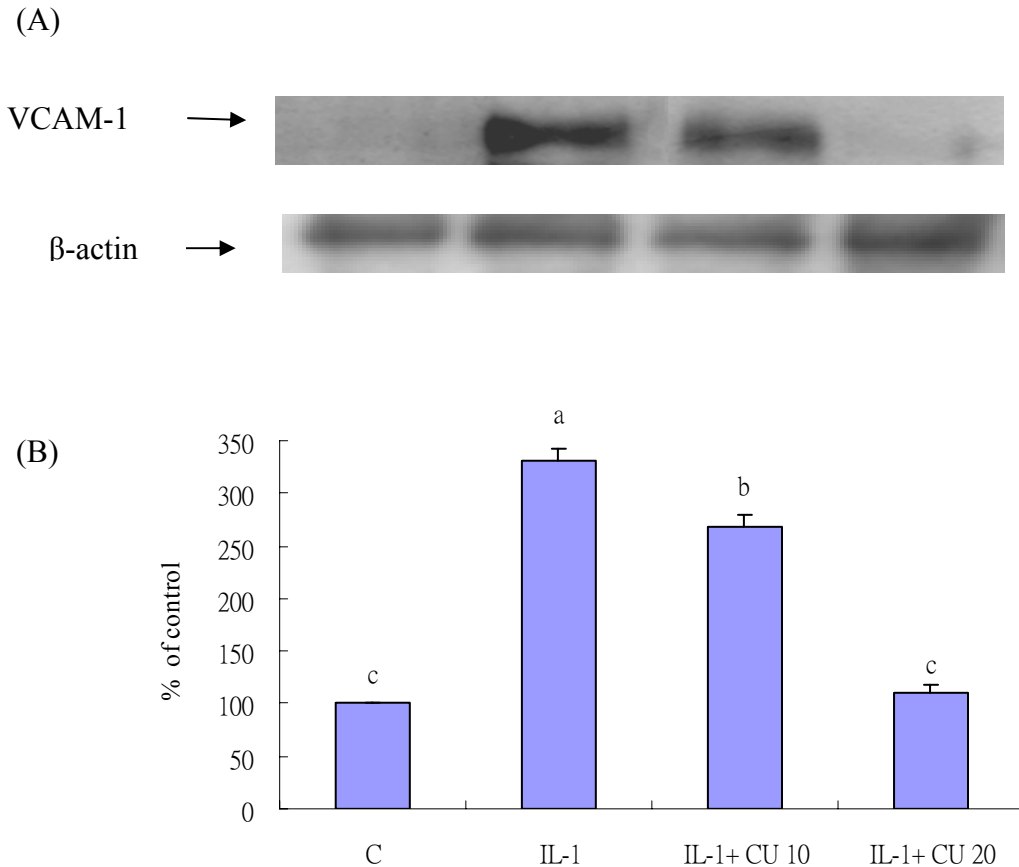
(B)



**Figure 2-3. Effect of curcumin on the protein levels of ICAM-1 in cultured HUVECs.**

HUVECs were pretreated with 10 and 20  $\mu\text{mol/l}$  curcumin for 18 hours and induced by IL-1  $\beta$  (10 ng/ml) for 6 hours. (A) Representative images of the reduction of IL-1  $\beta$ -induced the expression of ICAM-1 by curcumin in HUVECs. (B) Densitometric analysis was conducted with image analysis system software to quantify Western blot data. The summarized data (mean  $\pm$  S.D.) from 3 separate experiments is shown in the bar graph. <sup>a-d</sup>Means with different letters are significantly different at  $p < 0.05$ . Abbreviation: C (control), IL-1 (IL-1  $\beta$ ), CU10 (curcumin 10  $\mu\text{mol/l}$ ), CU20 (curcumin 20  $\mu\text{mol/l}$ ).

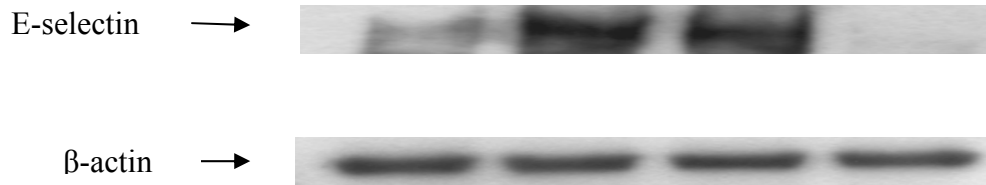
**Fig. 2-4**



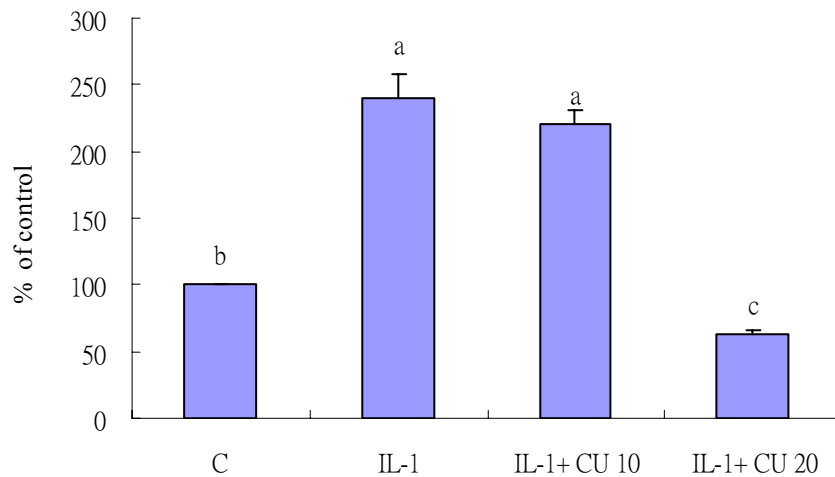
**Figure 2-4. Effect of curcumin on the protein levels of VCAM-1 in cultured HUVECs.** HUVECs were pretreated with 10 and 20  $\mu\text{mol/l}$  curcumin for 18 hours and induced by IL-1  $\beta$  (10 ng/ml) for 6 hours. (A) Representative images of the reduction of IL-1  $\beta$ -induced the expression of VCAM-1 by curcumin in HUVECs. (B) Densitometric analysis was conducted with image analysis system software to quantify Western blot data. The summarized data (mean  $\pm$  S.D.) from 3 separate experiments is shown in the bar graph. <sup>a-c</sup>Means with different letters are significantly different at  $p < 0.05$ . Abbreviation: C (control), IL-1 (IL-1  $\beta$ ), CU10 (curcumin 10  $\mu\text{mol/l}$ ), CU20 (curcumin 20  $\mu\text{mol/l}$ ).

**Fig. 2-5**

(A)

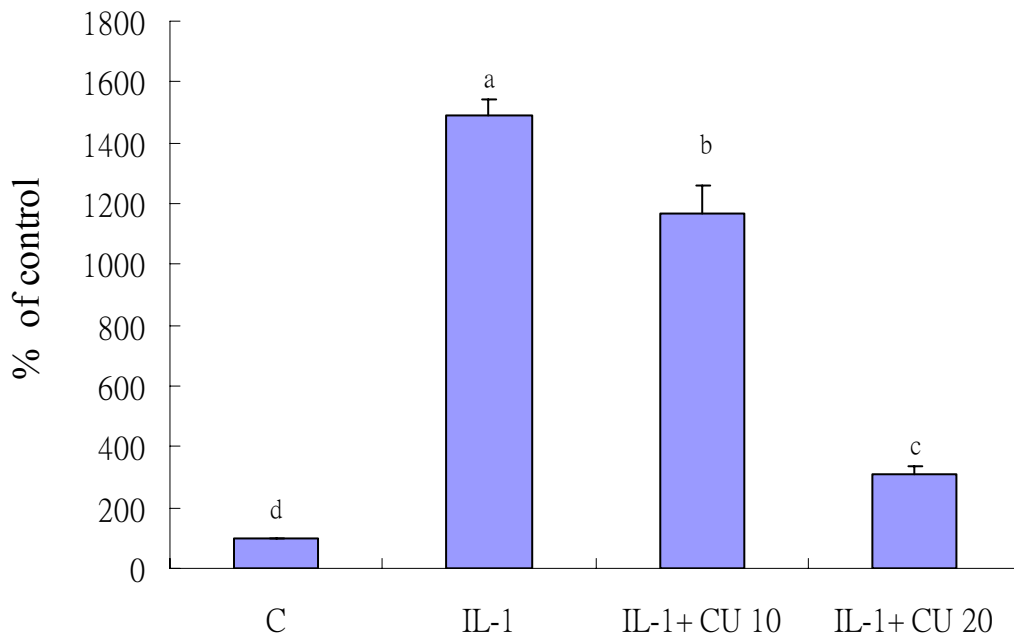


(B)



**Figure 2-5. Effect of curcumin on the protein levels of E-selectin in cultured HUVECs.** HUVECs were pretreated with 10 and 20  $\mu\text{mol/l}$  curcumin for 18 hours and induced by IL-1  $\beta$  (10 ng/ml) for 6 hours. (A) Representative images of the reduction of IL-1  $\beta$ -induced the expression of E-selectin by curcumin in HUVECs. (B) Densitometric analysis was conducted with image analysis system software to quantify Western blot data. The summarized data (mean  $\pm$  S.D.) from 3 separate experiments is shown in the bar graph. <sup>a-c</sup>Means with different letters are significantly different at  $p < 0.05$ . Abbreviation: C (control), IL-1 (IL-1  $\beta$ ), CU10 (curcumin 10  $\mu\text{mol/l}$ ), CU20 (curcumin 20  $\mu\text{mol/l}$ ).

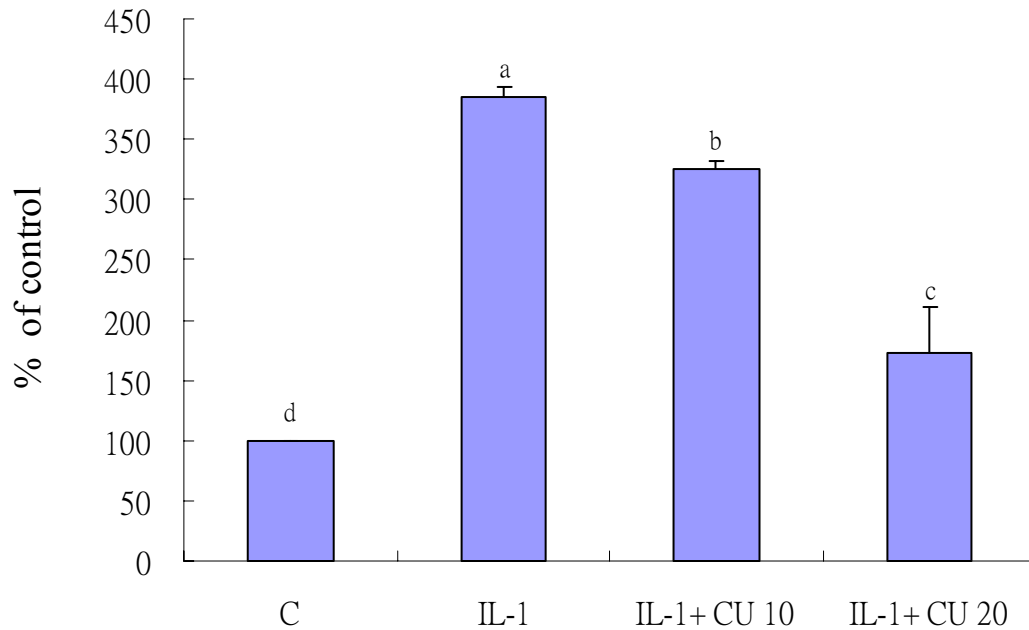
**Fig. 2-6**



**Figure 2-6. Effect of curcumin on IL-1  $\beta$ -induced activation of NF- $\kappa$ B p65.**

HUVECs were pretreated with 10  $\mu$ mol/l and 20  $\mu$ mol/l curcumin for 18 hours and then induced by IL-1  $\beta$  (10 ng/ml) for 6 hours. Nuclear extractes were prepared and analyzed for the activation of NF- $\kappa$ B. Ten micrograms of nuclear protein was used in each experiment. Values are mean  $\pm$  S.D., n=3. <sup>a-d</sup> Means with different letters are significantly different at  $p < 0.05$ . Abbreviation: C (control), IL-1 (IL-1  $\beta$ ), CU10 (curcumin 10  $\mu$ mol/l), CU20 (curcumin 20  $\mu$ mol/l).

**Fig. 2-7**

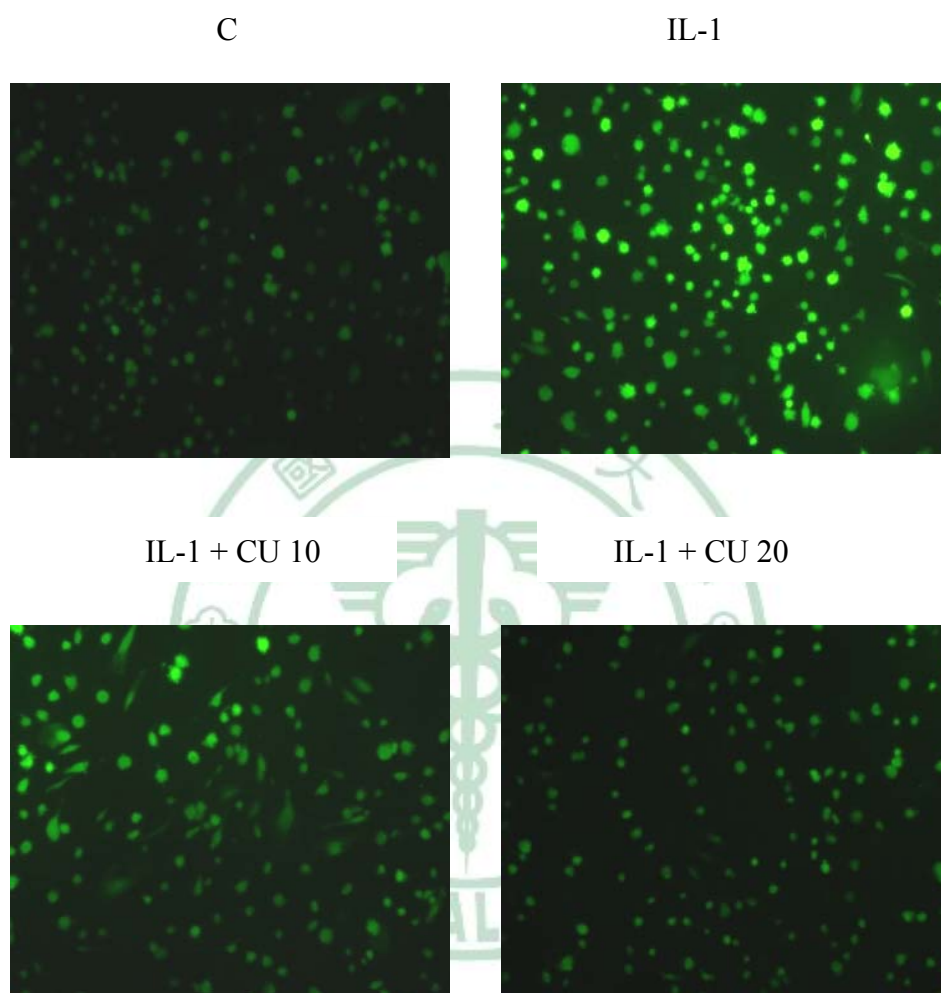


**Figure 2-7. Effect of curcumin on IL-1  $\beta$ -induced activation of NF- $\kappa$ B p50.**

HUVECs were pretreated with 10  $\mu$ mol/l and 20  $\mu$ mol/l curcumin for 18 hours and then induced by IL-1  $\beta$  (10ng/ml) for 6 hours. Nuclear extractes were prepared and analyzed for activation of NF- $\kappa$ B. Ten micrograms of nuclear protein was used in each experiment. Values are mean  $\pm$  S.D., n=3. <sup>a-d</sup> Means with different letters are significantly different at  $p < 0.05$ . Abbreviation: C (control), IL-1 (IL-1  $\beta$ ), CU10 (curcumin 10  $\mu$ mol/l), CU20 (curcumin 20  $\mu$ mol/l).

**Fig. 2-8**

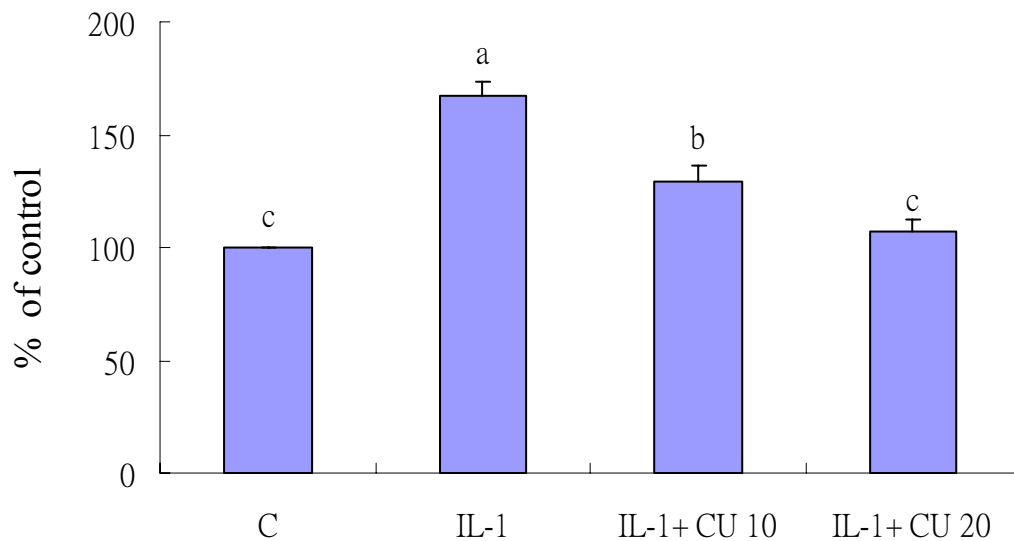
(A)





**Fig. 2-8**

(B)



**Figure 2-8. Effect of curcumin on IL-1  $\beta$ -induced ROS production in HUVECs.**

(A) Fluorescent images showed the reduction of IL-1  $\beta$ -induced ROS production in HUVECs after pretreatment of 10 and 20  $\mu\text{mol/l}$  curcumin for 18 hours. (B) HUVECs were pretreated with 10  $\mu\text{mol/l}$  and 20  $\mu\text{mol/l}$  curcumin for 18 hours and then induced by IL-1  $\beta$  (10ng/ml) for 6 hours. HUVECs were labeled with  $\text{H}_2\text{O}_2$ -sensitive fluorescent probe and detected by fluorescence microplate reader. Values are mean  $\pm$  S.D.,  $n=3$ . <sup>a-c</sup> Means with different letters are significantly different at  $p < 0.05$ . Abbreviation: C (control), IL-1 (IL-1  $\beta$ ), CU10 (curcumin 10  $\mu\text{mol/l}$ ), CU20 (curcumin 20  $\mu\text{mol/l}$ ).

## *Chapter 3*

### **Experimentation II**

Carnosic acid attenuates the expression of adhesion molecules by

IL-1  $\beta$  in HUVECs

鼠尾草酸下降介白素-1  $\beta$  所誘發的人類臍靜脈內皮細胞黏  
附因子表現



### ***3.1 Prolegomenon***

Atherosclerosis, a progressive pathological disorder leading to cardiovascular and cerebrovascular diseases, is still the leading cause of mortality and morbidity in industrialized countries, in spite of improved pharmacological and lifestyle approaches (Ross, 1993). The adhesion of circulating leukocytes to the vascular endothelium is a critical early event in the development of atherosclerosis (Joris *et al.*, 1983; Faggiotto *et al.*, 1984). This process depends on the interaction between cell adhesion molecules expressed on the surface of endothelial cells and their cognate ligands on leukocytes (Price & Loscalzo, 1999). Previous studies have indicated that NF- $\kappa$ B/Rel transcription factors may play an important role in the development of the atherosclerosis (Collins, 1993; Qwarnstrom *et al.*, 1994). The activation of NF- $\kappa$ B in endothelial cells is associated with the activation of genes responsible of an increased transcription of adhesion molecules, cytokines and chemokines (True *et al.*, 2000; Valen *et al.*, 2001; Thornburg *et al.*, 2003; Hatada *et al.*, 2003). Plant polyphenols are large group of naturally-occurring antioxidants and epidemiologic studies have suggested that higher polyphenol intake from fruits and vegetables is associated with decreased risk for cardiovascular disease (Ilja & Peter, 2005). Previous studies showed that polyphenolic compounds such as dietary flavonoids or red wine polyphenols could prevent atherosclerosis by inhibiting adhesion molecules expression in endothelial cell (Murase *et al.*, 1999; Silvina & Balz, 2006). Carnosic acid, an antioxidant polyphenol derived from Sage (*Salvia officinalis*) and Rosemary (*Rosmarinus officinalis*), is a lipophilic antioxidant that scavenges singlet oxygen, hydroxyl radicals, and lipid peroxy radicals, thus preventing lipid peroxidation and disruption of biological membranes (Aruoma *et al.*,

1992; Haraguchi *et al.*, 1995). Previous study also demonstrated that carnosic acid can inhibit plasma triglyceride elevation in olive oil-loaded mice and reduce the gain of body weight and the accumulation of epididymal fat weight in high fat diet-fed mice (Kiyofumi *et al.*, 2004). Therefore, we designed to examine the effect of carnosic acid on monocyte adhesion to cultured human endothelial cells and the expression of adhesion molecules (VCAM-1, ICAM-1 and E-selectin) and to elucidate its possible mechanism.



## 3.2 Materials and Methods

### 3.2.1 Instruments

CO <sub>2</sub> incubator	NUAIRE, MN, USA
Laminar flow	NUAIRE, MN, USA
Microscope	Nikon, Japan
pH meter	HANNA, RI, USA
Stirrer/Hotplate	Corning, Taiwan
Waterbath tank	TKS, Taiwan
Haemocytometer	Boeco, Germany
Eppendorf centrifugator	Hamburg, Germany
Pipetman	Gilson, France
Spectrophotometer	HITACHI, Japan
Spectrophotometer	Beckman Coulter, CA, USA
MicroPlate fluorescence reader	Bio-Tek, VT, USA
Shaking incubator	Orbital, VA, USA
ELISA plate reader	Bio-Tek, VT, USA
PCR thermocycler	Bio-Rad, CA, USA
Electrophoresis tank	Bio-Rad, CA, USA
Transfer system	Bio-Rad, CA, USA
Electrophoresis chamber	Bio-Rad, CA, USA
Power supply	Hoefler, CA, USA



### 3.2.2 Chemicals

ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid))	Sigma, MO, USA
BSA (Bovine serum albumin)	Sigma, MO, USA
Bradford reagent	Bio-Rad, CA, USA
Carnosic acid	Sigma, MO, USA
CHCl <sub>3</sub> (Chloroform)	BDH, Poole, England
CuSO <sub>4</sub> (Cupric sulfate)	Sigma, MO, USA
DCFH-DA (2', 7'-dichlorofluorescein diacetate)	Molecular Probe, Oregon, USA
DEPC (Diethyl pyrocarbonate)	Sigma, MO, USA
DMSO (Dimethyl sulfoxide)	Sigma, MO, USA
DPPH (2, 2-diphenyl-1-picrylhydrazyl)	Sigma, MO, USA
DTT (1, 4-Dithio-D,L-threitol)	Bio-Rad, CA, USA
ECG (Endothelial cell growth supplement)	Sigma, MO, USA
EDTA-Disodium (Ethylenediamine Teraacetate Disodium Salt)	Bio-Rad, CA, USA
FBS (Fetal Bovine serum)	Gibco, NY, USA
Gelatin (2 % solution Type B)	Sigma, MO, USA
Glucose	Sigma, MO, USA
HBSS (Hank's balanced salts modified)	Sigma, MO, USA
HCl (Hydrochloric acid)	Wako, Osaka, Japan
Heparin	Sigma, MO, USA
Hepes buffer solution (1 mol/l)	Gibco, NY, USA
H <sub>2</sub> O <sub>2</sub> (Hydrogen Peroxide)	Wako, Osaka, Japan
KBr (potassium bromide)	SHOWA, Tokyo, Japan

KCl (potassium chloridem)	SHOWA, Tokyo, Japan
KH <sub>2</sub> PO <sub>4</sub> (potassium dihydrogenphosphate)	SHOWA, Tokyo, Japan
L-Glutamine (200 mmol/l)	Gibco, NY, USA
MDA (malonaldehyde bis-(dimethyl acetal))	Alderich, WI, USA
MTT (3-[4, 5-Dimethylthiazol-2-yl]-2, 5-diphenyl-terazoliumbromide)	Sigma, MO, USA
Medium 199	Sigma, MO, USA
Methanol	TEDIA, OH, USA
NaCl (sodium chloride)	Sigma, MO, USA
NaHCO <sub>3</sub> (sodium bicarbonate)	Sigma, MO, USA
Na <sub>2</sub> HPO <sub>4</sub> (Disodium hydrogenphosphate)	SHOWA, Tokyo, Japan
NF-κB kit	TransAM, Active Motif, CA, USA
Nuclear Extract kit	TransAM, Active Motif, CA, USA
Penicillin-Streptomycin	Gibco, NY, USA
Peroxidase (Type VI-A)	Sigma, MO, USA
Recombinant human interleukin-1 beta	ProSpec-Tany, Rehovot, Isreal
RPMI-1640	Gibco, NY, USA
TBA (2-Thiobarbituric acid)	Sigma, MO, USA
TCA (Trichloroacetic acid)	Sigma, MO, USA
Trolox (6-Hydroxy-2,5,7,8-treamethylchroman-2-carboxylic acid)	Alderich, WI, USA
Trypsin-EDTA (0.25 %)	Gibco, NY, USA
SDS (Sodium dodecyl sulfata)	Bio-Rad, CA, USA
SYBR Green suoermix	Bio-Rad, CA, USA

2-propanol	Sigma, MO, USA
95 % Ethanol	Echo Chemical, Taichung, Taiwan
Mouse anti-human $\beta$ -actin	Abcam, Cambridge, UK
Mouse anti-human ICAM-1	Abcam, Cambridge, UK
Mouse anti-human VCAM-1	Abcam, Cambridge, UK
Mouse anti-human E-selectin	Abcam, Cambridge, UK
Sheep anti-mouse IgG antibody	Abcam, Cambridge, UK

### 3.2.3 Cell Culture

Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cords using collagenase type II (Jaffe, 1973), and cultured on 0.1 % gelatin-coated culture dishes in medium M199 (Sigma, MO, USA) supplemented with 10 % FBS, 1 % Antibiotic -Antimycotic, Glutamine (2 mmol/l), Heparin (10 U/ml), Heps (10 mmol/l), Endothelial cell growth supplement (ECG) (12.5  $\mu$ g/ml) at 37°C in a humidified atmosphere of 5 % CO<sub>2</sub> and 95 % air. After 3 days, the medium was replaced by fresh medium and subcultured at 1: 4 ratio one time per week. All experiments were performed with HUVEC from passages two to five.

### 3.2.4 Cell viability assay (MTT test)

The viability of the cells was assessed by MTT (3-[4,5-dimethylthiazol-2-yl] -2,5-diphenyl-tetrazolium bromide) assay (Mosmann, 1983), which is based on the reduction of MTT by the mitochondrial dehydrogenase of intact cells to a blue formazan product.



Briefly,  $1 \times 10^4$  cells/well were dispensed within 96-well culture plates and incubated with various concentrations of carnosic acid (which was dissolved in dimethyl sulphoxide) for 24 h. Four hours before the end of the treatment 10  $\mu$ l MTT (5 mg/ml) was added to each well. At the end of the treatment the incubation medium was removed and the formazan crystals were dissolved in 100  $\mu$ l of solution of DMSO. MTT reduction was quantified by measuring the light absorbance with a ELISA plate reader ( $\mu$ QUANT, Bio-Tek, USA) at 590 nm. The reduction in optical density caused by carnosic acid was used as a measurement of cell viability, normalized to cells incubated in medium with DMSO only, which were considered to be 100 % viable.

### **3.2.5 Adhesion of U937 cells to endothelial cells**

Adhesion was evaluated using the human leukemia pro-monocytic U937 cells which were labeled with calcein AM (10 nmol/l; Molecular Probe; Invitrogen) (Yu *et al.*, 2007). HUVEC ( $2 \times 10^5$ ) were distributed into 6-well plates before the assay and allowed to reach confluence. Then the growth medium was supplemented with 10 and 20  $\mu$ mol/l carnosic acid for 18 h, followed by incubation 10 ng/ml IL-1  $\beta$  for 6 h in the continued presence of carnosic acid. U937 cells were grown in RPMI 1640 medium (Gibco, New York, USA) containing 10 % FBS and subcultured at a 1:5 ratio three times per week. U937 cells were incubated with 10 nmol/l calcein AM in RPMI 1640 medium for 30 min at 37°C, then washed with PBS to remove free dye and resuspended in M-199 median containing 10 % FBS. Labeled U937 cells ( $2 \times 10^5$ ) were added to each HUVEC-containing well and incubation continued for 30 min. Non-adherent cells were removed by two gentle washes with PBS, then the number of bound U937 cells was

determined by a fluorescence plate reader at an excitation wavelength of 485 nm and emission at 530 nm; HUVEC cell monolayers with DMSO only were served as the blank.

### **3.2.6 Bradford assay**

The Bradford assay (Bradford, 1976), a colorimetric protein assay, is based on an absorbance shift in the dye Coomassie when bound to arginine and hydrophobic amino acid residues present in protein. The anionic (bound) form of the dye is blue and has an absorption spectrum maximum historically held to be at 595 nm. The cationic (unbound) forms are green and red. The increase of absorbance at 595 nm is proportional to the amount of bound dye, and thus to the amount (concentration) of protein present in the sample. Standard solutions contain a range of 0 to 25 micrograms protein (BSA) in 800  $\mu$ l H<sub>2</sub>O, followed by adding 200  $\mu$ l dye reagent and incubate 5 min. 1  $\mu$ l of sample solution add into 799  $\mu$ l H<sub>2</sub>O, followed by adding 200  $\mu$ l dye reagent and incubated for 5 min. The absorbance was read at 595 nm. The results made a standard curve and the protein concentration of sample was determined by standard curve.

### **3.2.7 Western blot**

For Western blotting,  $3 \times 10^6$  cells were seeded in 10 cm dishes and treated 10 and 20  $\mu$ mol/l carnosic acid for 18 h, followed by incubation 10 ng/ml IL-1  $\beta$  for 6 h in the continued presence of carnosic acid, afterwards cells were scraped with a rubber policeman in PBS and centrifuged at 1,200 rpm for 10 min. Cells were lysed for 1 h at 4

°C with lysis buffer (0.32 mol/l Sucrose, 10 mmol/l Tris, 5 mmol/l EDTA, 2 mmol/l DTT, 1 % Triton X-100, and 1 mmol/l PMSF) and centrifuged at 12,000 g for 30 min at 4 °C. The protein concentration of cell extracts was determined with a Bradford-based assay (Bradford, 1976). Cell extracts were loaded per lane, resolved by 10 % SDS -PAGE and transferred at room temperature by blotting to polyvinylidene difluoride (PVDF) membrane (Shishodia *et al.*, 2003). Non-specific binding was blocked by soaking the membrane in PBS-Tween 20 buffer containing 50 g/L fat-free milk and separately incubated for 1 h at room temperature with mouse anti-human-VCAM-1, ICAM-1, and E-selectin antibodies. Subsequently, the membrane was incubated with a sheep anti-mouse IgG antibody. The protein levels were determined with the enhanced chemiluminescence (Upstate, USA) and High performance chemiluminescence film (Amersham biosciences, USA). Incubation with mouse anti-human  $\beta$ -actin antibody was also performed as an internal control. Results were quantified with scanning densitometer using an image analysis system with software.

### **3.2.8 Nuclear extract preparation**

Nuclear protein extracts were prepared using a nuclear extract kit (TransAM nuclear extract kit, CA, USA) from HUVECs to assay the NF- $\kappa$ B activity. Nuclear protein were prepared as described previously (Dschietzig *et al.*, 2001).  $3 \times 10^6$  cells were seeded in 10 cm dishes and treated 10 and 20  $\mu$ mol/l carnosic acid for 18 h, followed by incubating 10 ng/ml IL-1  $\beta$  for 6 h in the continued presence of carnosic acid, afterwards cells were scraped with a rubber policeman in PBS, collected and centrifuged at 1,200 rpm for 10 min. Cells were lysed in hypotonic buffer and centrifuge suspension for 30

seconds at 14,000×g in a microcentrifuge pre-cooled at 4 °C. Then resuspend nuclear pellet in 50 µl complete lysis buffer containing 10 mmol/l DTT, lysis buffer AM1, and protease inhibitor cocktail by pipetting up and down. The suspension was incubated for 30 min on ice, and centrifuged for 10 min at 14,000×g in a microcentrifuge pre-cooled at 4 °C. Transfer supernatant and stored at -80 °C. The protein concentration was determined with a Bradford-based assay (Bradford, 1976).

### **3.2.9 Measurement of NF-κB activation**

For analysis of NF-κB activation with the TransAM NF-κB Family kit (TransAM, Active Motif, CA, USA) (Yu *et al.*, 2007). The oligonucleotide containing the NF-κB consensus binding site (5'-GGGACTTCC-3') specific for the active form of NF-κB was immobilized to a 96-well plate and the well was filled with 10 µg of nuclear extract. After 1 hour incubation and three washings, the primary antibody against the active form of NF-κB recognizing an epitope on p65, p50 that is accessible only when NF-κB is activated and bound to its target DNA was added for 1 hour. After washing, the secondary antibody conjugated to horseradish peroxidase was added to achieve a sensitive readout by spectrophotometry at 450 nm and the blank was subtracted from all measurements.

### 3.2.10 Measurement of reactive oxygen species

ROS generation in cells was assessed using the probe 2,7-dichlorofluorescein (DCF) (Molecular Probes Europe BV, Leiden, The Netherlands) according to the method reported by Wang and Joseph (1999). Confluent HUVECs ( $1 \times 10^4$  cells/well) in 96-well plates were pretreated with 10 and 20  $\mu\text{mol/l}$  carnosic acid for 18 h, followed by incubation of 10 ng/ml IL-1  $\beta$  for 6 h in the continued presence of carnosic acid. After the removal of curcumin or carnosic acid from wells, cells were incubated with 10  $\mu\text{mol/L}$  DCFH-DA for 30 minutes. The fluorescence intensity (relative fluorescence units) was measured at 485-nm excitation and 530-nm emission using a fluorescence microplate reader.

### 3.2.11 Statistical analysis

Results are shown as mean  $\pm$  S.D. Statistical analyses of MTT were performed using One-way ANOVA followed by Dunnett's test and others were performed using One-way ANOVA followed by Duncan's Multiple Range Test. A value of  $P < 0.05$  was considered statistically significant.

### **3.3 Results**

#### **3.3.1 Cell viability of carnosic acid for HUVECs**

Cell viability was determined by the MTT test. After 24h incubation with 5, 10 and 20, 40 and 60 $\mu\text{mol/l}$  carnosic acid, cell viability was  $105.1 \pm 2.5$ ,  $106.3 \pm 5.4$ ,  $99.1 \pm 5.2$ ,  $88.2 \pm 3.3$  and  $71.5 \pm 3.8$  % of control levels, respectively, the two highest concentrations causing a significant reduction in cell viability (Fig 3-1). Therefore, according to the MTT test we chose 10 and 20  $\mu\text{mol/l}$  carnosic acid to do all the following experiments.

#### **3.3.2 Carnosic acid inhibited the adhesion of U937 cells to IL-1 $\beta$ -stimulated HUVECs**

In order to determine the effect of carnosic acid on the adhesion of U937 cells to endothelial cells, HUVECs were treated with 10 and 20  $\mu\text{mol/l}$  carnosic acid for 18 h, followed by incubation of 10 ng/ml IL-1  $\beta$  for 6 h and the percentage of cell adhesion was evaluated by the quantification of calcein AM (Fig. 3-2 (B)). The adhesion of U937 cells to HUVECs also was photographed (Fig. 3-2 (A)). The control group showed that minimal binding of HUVECs to U937 cells, but adhesion significantly increased when the HUVECs were treated with IL-1  $\beta$  (Fig 3-2 A & B). Pretreatment with 10 and 20  $\mu\text{mol/l}$  carnosic acid could significantly reduce the number of U937 cells adhering to IL-1  $\beta$ -stimulated HUVECs. The concentration of 20  $\mu\text{mol/l}$  carnosic acid was more effective on cell adhesion than 10  $\mu\text{mol/l}$  carnosic acid (Fig 3-2 A & B). These results indicated

that carnosic acid could inhibit monocytes adhesion to endothelial cells.

### **3.3.3 Carnosic acid inhibits IL-1 $\beta$ -induced cell protein expression of VCAM-1, ICAM-1 and E-selectin in HUVECs**

To determine if the inhibition of cell adhesion by carnosic acid was due to inhibit the expression of adhesion molecules, HUVECs were pretreated for 18 h with 10 and 20  $\mu\text{mol/l}$  carnosic acid before the addition of 10 ng/ml IL-1  $\beta$ . The expression of VCAM-1, ICAM-1 and E-selectin was increased after IL-1  $\beta$  stimulation (Fig 3-3, 3-4, 3-5). Pretreatment of HUVECs with 10  $\mu\text{mol/l}$  carnosic acid significantly inhibited the expression of VCAM-1 and E-selectin, but not ICAM-1 (Fig 3-3, 3-4, 3-5). Pretreatment of HUVECs with 20  $\mu\text{mol/l}$  carnosic could significantly inhibit the expression of ICAM-1, VCAM-1 and E-selectin. Therefore, 20  $\mu\text{mol/l}$  carnosic could was more effective than 10  $\mu\text{mol/l}$  carnosic acid on the inhibition of cell adhesion molecular expression (Fig 3-3, 3-4, 3-5).

### **3.3.4 Carnosic acid decrease activation of NF- $\kappa$ B and nuclear translocation of NF- $\kappa$ B p65 and p50 in IL-1 $\beta$ -stimulated HUVECs**

To examine whether the inhibitory effect of carnosic acid on the cytokine-induced expression of adhesion molecules was mediated via NF- $\kappa$ B, we measured the nuclear translocation of p65 and p50 protein of the NF- $\kappa$ B family. Incubation of IL-1  $\beta$  (10 ng/ml) for 6 h induced the nuclear translocation of p65 and p50 (Fig. 3-6, 3-7). Pretreatment of HUVECs with 10 and 20  $\mu\text{mol/l}$  carnosic acid significantly decreased the nuclear

translocation of p65 and p50 (Fig. 3-6, 3-7). In addition, pretreatment of HUVECs with 20  $\mu\text{mol/l}$  carnosic acid was more effective on decreasing the nuclear translocation of p65 and p50 than that of 10  $\mu\text{mol/l}$  carnosic acid.

### **3.3.5 Carnosic acid inhibits IL-1 $\beta$ -induced ROS in HUVECs**

To study the effect of carnosic acid on IL-1  $\beta$ -induced ROS production in HUVECs, cells were pretreated with 10 and 20  $\mu\text{mol/l}$  carnosic acid for 18 h and then stimulated by IL-1  $\beta$  (10 ng/ml) for 6 h. HUVECs were labeled with  $\text{H}_2\text{O}_2$ -sensitive fluorescent probe and detected by fluorescence microplate reader. The production of ROS induced by IL-1  $\beta$  was increased and significantly decreased after pretreatment with 10 and 20  $\mu\text{mol/l}$  carnosic acid (Fig. 3-8 A & B). In addition, pretreatment of HUVECs with 20  $\mu\text{mol/l}$  carnosic acid was more effective on decreasing the production of ROS than 10  $\mu\text{mol/l}$  carnosic acid.



### 3.4 Discussion

Expression of cell adhesion molecules (CAM) by the endothelium and the attachment of monocytes to endothelium may play a major role in the early atherogenic process. Carnosic acid is an antioxidant polyphenol derived from Sage (*Salvia officinalis*) and Rosemary (*Rosmarinus officinalis*). Previous studies showed that Carnosic acid is a lipophilic antioxidant that scavenges singlet oxygen, hydroxyl radicals, and lipid peroxy radicals, therefore, it suggested that carnosic acid could prevent the peroxidation of lipid and disruption of biological membranes (Aruoma *et al.*, 1992; Haraguchi *et al.*, 1995). In the present study, we found that carnosic acid had antioxidative effect *in vitro*. In addition, we also found that carnosic acid significantly suppressed IL-1  $\beta$ -induced intracellular ROS production, the activation of redox-sensitive transcription factors NF- $\kappa$ B p50, p65, the expression of ICAM-1, VCAM-1 and E-selectin; and the adhesiveness to a human monocytic cell line (U937). These results demonstrated that carnosic acid had inhibitory effect on proatherosclerotic mechanism *in vitro*.

Previous studies showed that sage and rosemary extracts contained 0.29 % and 0.1-0.5 % of carnosic acid, respectively (Huang *et al.*, 1994; Kiyofumi *et al.*, 2004). In the present study, we found that 20  $\mu$ mol/l carnosic acid did not have any significant effect on the viability of HUVECs from MTT test. Previous study also showed that 20  $\mu$ mol/l carnosic acid did not have any significant effect on the viability of HepG2 cells (Costa *et al.*, 2006). Therefore, according to the MTT test, we chose 10 and 20  $\mu$ mol/l carnosic acid to do all the experiments.

Adhesion and transendothelial migration of monocytes into the surrounding tissues are crucial steps in inflammation, immunity, and atherogenesis (Springer, 1994; Li *et al.*,

1993; Jang *et al.*, 1994). Vascular endothelial cells play an active role in this process by expressing cell adhesion molecules which enhance the adhesion of monocytes to the endothelium (Alexiou *et al.*, 2001). In the present study, we found that control group showed minimal binding to U937 cells, but adhesion increased when the HUVECs were treated with IL-1  $\beta$ . Pretreatment with 10 and 20  $\mu\text{mol/l}$  carnosic acid reduced the number of U937 cells adhering to IL-1  $\beta$ -stimulated HUVECs. A similar result was seen when HUVECs were pretreated with other polyphenolic compound, such as vitamin E (40  $\mu\text{mol/l}$ ), probucol (50  $\mu\text{mol/l}$ ) or tea flavonoid (60  $\mu\text{mol/l}$ ), these polyphenolic compound reduced monocytes adhesion to endothelial cells (Islam *et al.*, 1998; Zapolska-Downar *et al.*, 2001; Ludwig *et al.*, 2004).

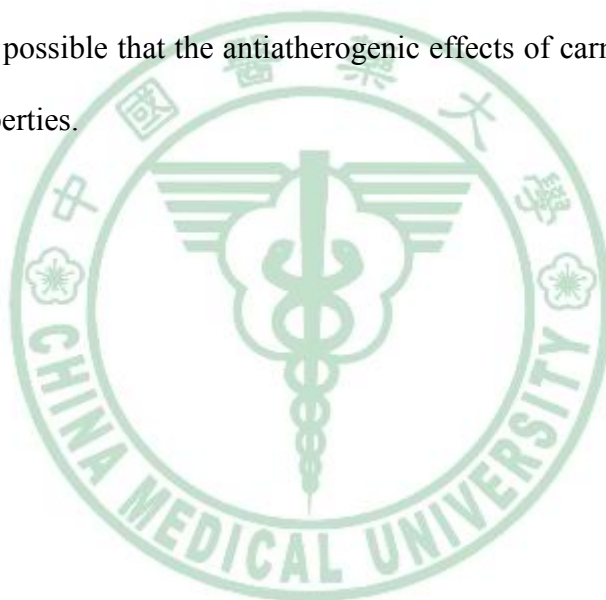
The process by which monocytes become adherent to the endothelium is the result of complex choreography requiring the sequential, yet overlapping, functions of many classes of adhesion molecules (Munro, 1993; Price & Loscalzo, 1999). Tethering and rolling, the first steps of monocyte adhesion to the endothelial surface, appear to depend on the interaction of P- and E- selectin with carbohydrate ligands on leukocytes. Firm adhesion follows if the monocytes encounter activating signals while rolling along the endothelium; this is facilitated by interaction of very late antigen-4 (VLA-4) with VCAM-1 or lymphocyte function antigen-1 (LFA-1) with ICAM-1 (Marlin & Springer, 1987; Elices *et al.*, 1990). Previous study showed that adhesion molecules are strong predictors of atherosclerotic lesion development and future cardiovascular events (Blankenberg *et al.*, 2003). It has been reported that expression of adhesion molecules on HUVECs is increased or induced by stimulation with inflammatory cytokines, including TNF- $\alpha$ , IL-1  $\beta$ , IL-4, and IL-13 (Schleimer & Rutledge, 1986). In the present study, we found that the expression of ICAM-1, VCAM-1 and E-selectin induced by IL-1 $\beta$  were

increased and the pretreatment with carnosic acid could decreased the induction of expression by IL-1  $\beta$  in HUVECs. These results indicated that carnosic acid could inhibit the rolling, tethering and firm adhesion of the monocytes on the vascular wall.

The translocation of the transcription factor, NF- $\kappa$ B, is involved in the signal transduction pathways for IL-1 $\beta$ -induced adhesion molecule expression (Lenardo & Baltimore, 1989). The activated form of NF- $\kappa$ B is a heterodimer, which usually consists of two proteins, a p65 (also called relA) subunit and a p50 subunit (Baldwin, 1996). Udalova *et al.*, 2000 described that the p50–p65 heterodimers are involved in enhancing the transcription of adhesion molecules, cytokines and chemokines. In the present study, we examined whether the inhibitory effect of carnosic acid on the cytokine-induced expression of adhesion molecules is mediated via NF- $\kappa$ B, therefore, we measured the nuclear translocation of p65 and p50 protein of the NF- $\kappa$ B family. We found that the nuclear translocation of p65 and p50 induced by IL-1  $\beta$  were increased and the pretreatment with carnosic acid could decreased the induction of the nuclear translocation by IL-1  $\beta$  in HUVECs. Therefore, we confirmed that carnosic acid has an anti-inflammatory effect through the partial interference of NF- $\kappa$ B activation.

Harrison *et al.*, 2003 described that ROS play a central roles in the pathogenesis of endothelial dysfunction and atherosclerosis. Previous study indicated that the activation mechanisms of NF- $\kappa$ B nuclear translocation has been suggested to involve ROS (Schreck *et al.*, 1991&1992; Suzuki *et al.*, 1993). In the present study, we found that the production of ROS induced by IL-1  $\beta$  were increased and the pretreatment with carnosic acid could decreased the induction of expression by IL-1  $\beta$  in HUVECs. Previous studies showed that the activation of NF- $\kappa$ B could be inhibited by different antioxidants, it is strongly suggested that endogenous ROS may play an important role in these redox-sensitive

transcription pathways in atherogenesis (Manna *et al.*, 1992; Muller *et al.*, 1994). Based on the present results, we proposed that the inhibitory effect of carnosic acid on adhesion molecules expression and NF- $\kappa$ B activation may be due to its antioxidant and anti-inflammatory properties and that it may act by directly scavenging free radicals. In the present study, we also found that carnosic acid was approximately 5-6-folds more potent than Trolox in antioxidative ability and it also could scavenge DPPH, alkoxyl radical (RO $\cdot$ ) and peroxy radical (ROO $\cdot$ ) (Appendix 1). Since atherosclerosis is a chronic inflammatory disease associated with increased oxidative stress in the vascular endothelium, it is possible that the antiatherogenic effects of carnosic acid may due to its antioxidative properties.

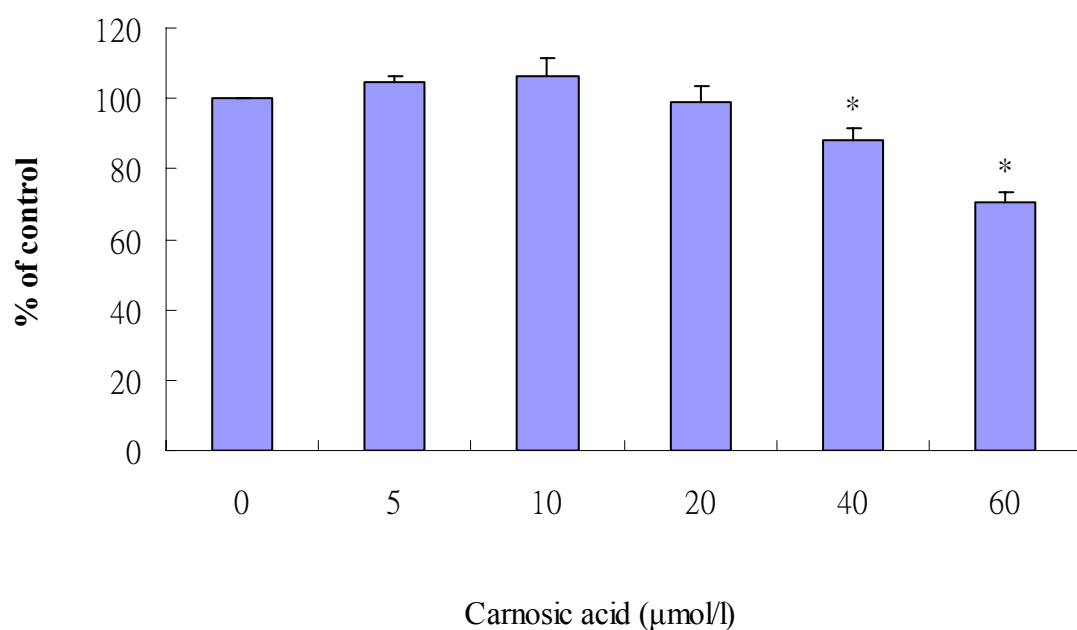


### ***3.5 Conclusion***

In the present study, we found that carnosic acid inhibited monocyte adhesion to endothelial cell, the expression of adhesion molecules, the translocation of NF- $\kappa$ B and the production of ROS in HUVECs. These findings may provide a rationale for the *in vitro* antiatherosclerosis effect of carnosic acid and support its potential use in the prevention of atherosclerosis.



**Fig. 3-1**

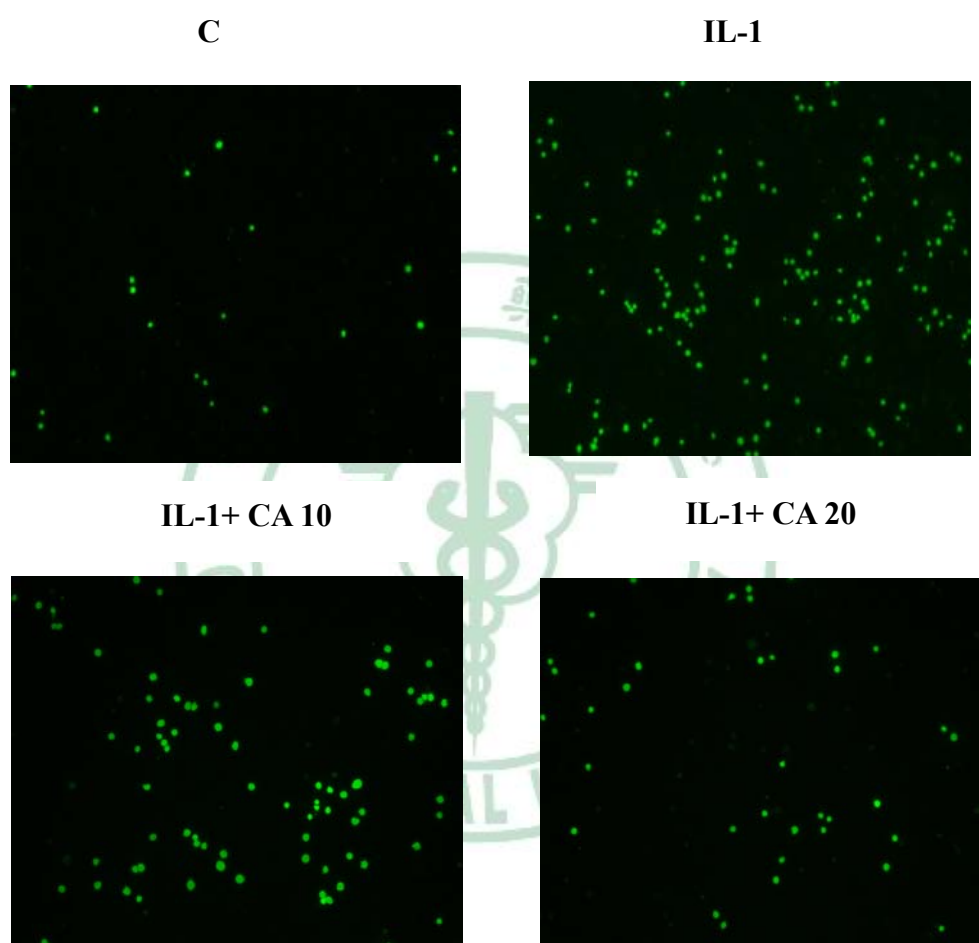


**Figure 3-1. Cytotoxic effect of carnosic acid on HUVECs with MTT test.**

HUVECs were treated with various concentrations (0~60 µmol/l) of carnosic acid for 24 h in 10 % FBS-M199. Results were from four experiments and expressed as mean ± S.D. \* $p < 0.05$  compared with control.

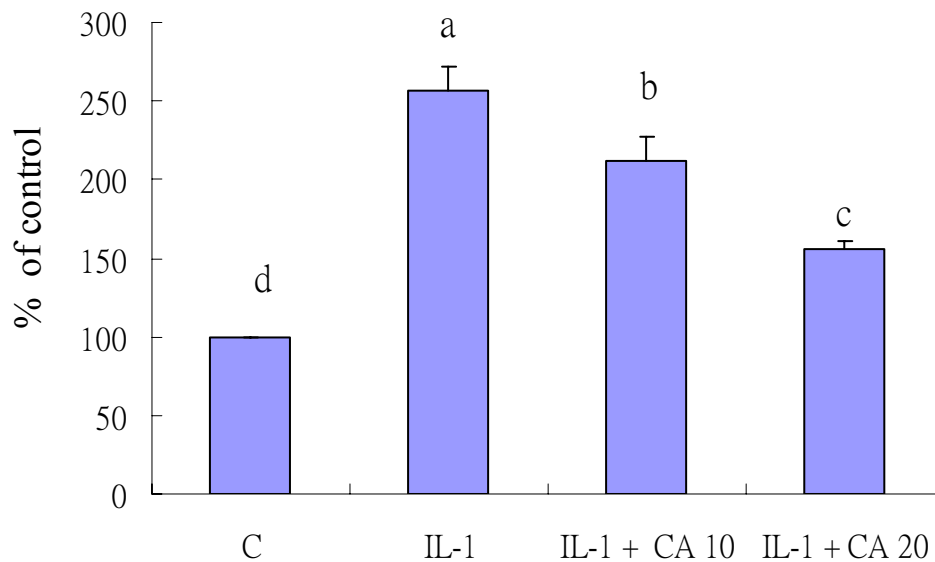
**Fig. 3-2**

(A)



**Fig. 3-2**

(B)

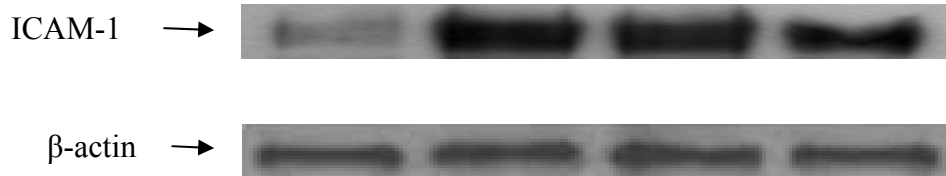


**Figure 3-2. Effect of carnosic acid on IL-1  $\beta$ -induced adhesion of U-937 cells to HUVECs.** (A) Representative images of the reduction of IL-1  $\beta$ -induced adhesion of U-937 cells to HUVECs monolayers after pretreatment of 10 and 20  $\mu\text{mol/l}$  carnosic acid for 18 hours. (B) HUVECs were pretreated with 10 and 20  $\mu\text{mol/l}$  carnosic acid for 18 hours and induced by IL-1  $\beta$  (10 ng/ml) for 6 hours. Fluorescence-labeled U-937 cell were added to the HUVECs monolayer and allowed to adhere for 30 min. Values are mean  $\pm$  S.D.,  $n=3$ . <sup>a-d</sup>Means with different letters are significantly different at  $p < 0.05$ . Abbreviation: C (control), IL-1 (IL-1  $\beta$ ), CA10 (carnosic acid 10  $\mu\text{mol/l}$ ), CA 20 (carnosic acid 20  $\mu\text{mol/l}$ ).

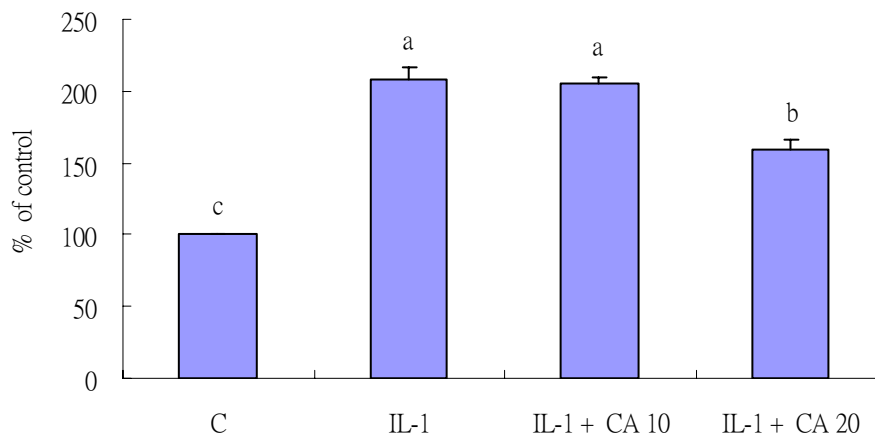


**Fig. 3-3**

(A)

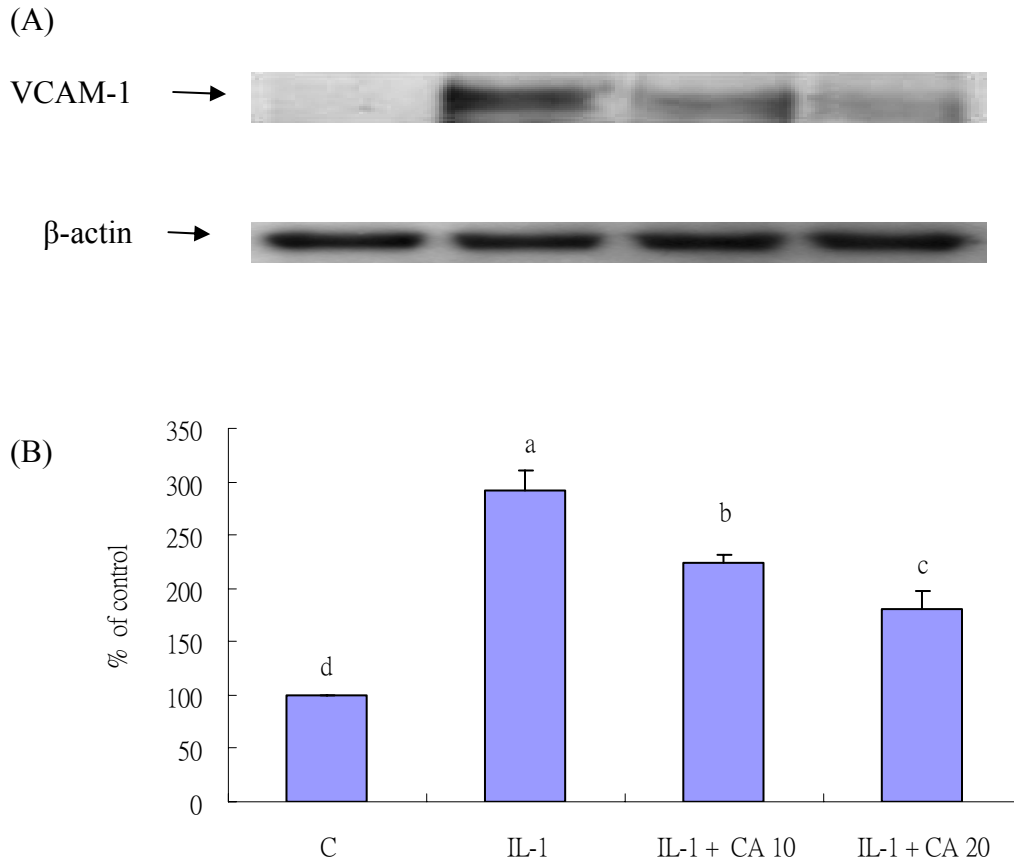


(B)



**Figure 3-3. Effect of carnosic acid on the protein levels of ICAM-1 in cultured HUVECs.** HUVECs were pretreated with 10 and 20  $\mu\text{mol/l}$  carnosic acid for 18 hours and induced by IL-1  $\beta$  (10 ng/ml) for 6 hours. (A) Representative images of the reduction of IL-1  $\beta$ -induced the expression of ICAM-1 by carnosic acid in HUVECs. (B) Densitometric analysis was conducted with image analysis system software to quantify Western blot data. The summarized data (mean  $\pm$  S.D.) from 3 separate experiments is shown in the bar graph. <sup>a-c</sup> Means with different letters are significantly different at  $p < 0.05$ . Abbreviation: C (control), IL-1 (IL-1  $\beta$ ), CA10 (carnosic acid 10  $\mu\text{mol/l}$ ), CA 20 (carnosic acid 20  $\mu\text{mol/l}$ ).

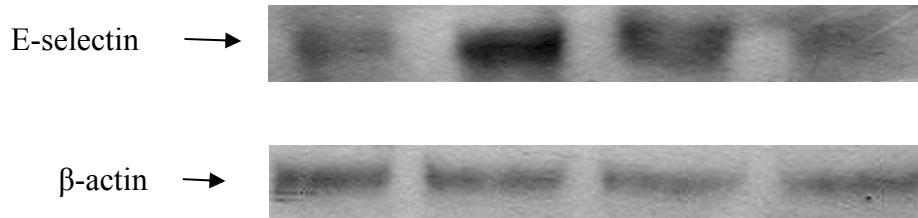
**Fig 3-4**



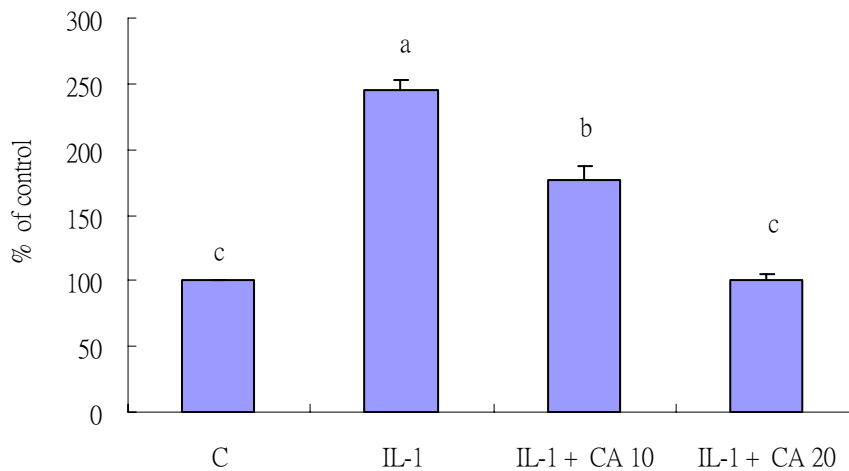
**Figure 3-4. Effect of carnosic acid on the protein levels of VCAM-1 in cultured HUVECs.** HUVECs were pretreated with 10 and 20  $\mu\text{mol/l}$  carnosic acid for 18 hours and induced by IL-1  $\beta$  (10 ng/ml) for 6 hours. (A) Representative images of the reduction of IL-1  $\beta$ -induced the expression of VCAM-1 by carnosic acid in HUVECs. (B) Densitometric analysis was conducted with image analysis system software. to quantify Western blot data. The summarized data (mean  $\pm$  S.D.) from 3 separate experiments is shown in the bar graph. <sup>a-d</sup>Means with different letters are significantly different at  $p < 0.05$ . Abbreviation: C (control), IL-1 (IL-1  $\beta$ ), CA10 (carnosic acid 10  $\mu\text{mol/l}$ ), CA 20 (carnosic acid 20  $\mu\text{mol/l}$ ).

**Fig 3-5**

(A)

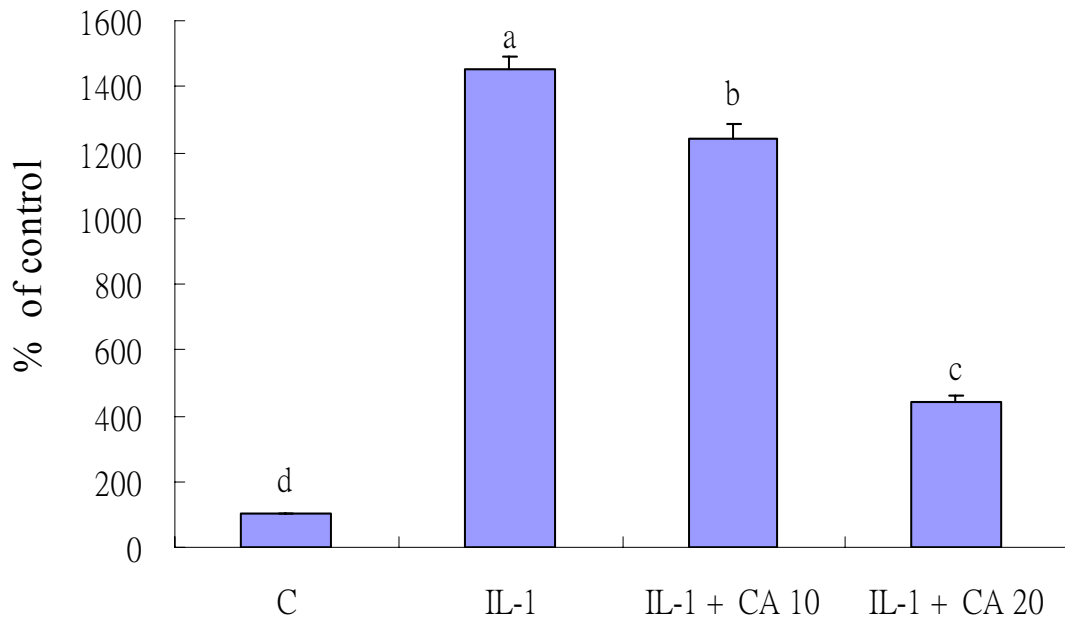


(B)



**Figure 3-5. Effect of carnosic acid on the protein levels of E-selectin in cultured HUVECs.** HUVECs were pretreated with 10 and 20  $\mu\text{mol/l}$  carnosic acid for 18 hours and induced by IL-1  $\beta$  (10 ng/ml) for 6 hours. (A) Representative images of the reduction of IL-1  $\beta$ -induced the expression of E-selectin by carnosic acid in HUVECs. (B) Densitometric analysis was conducted with image analysis system software. to quantify Western blot data. The summarized data (mean  $\pm$  S.D.) from 3 separate experiments is shown in the bar graph. <sup>a-c</sup> Means with different letters are significantly different at  $p < 0.05$ . Abbreviation: C (control), IL-1 (IL-1  $\beta$ ), CA10 (carnosic acid 10  $\mu\text{mol/l}$ ), CA 20 (carnosic acid 20  $\mu\text{mol/l}$ ).

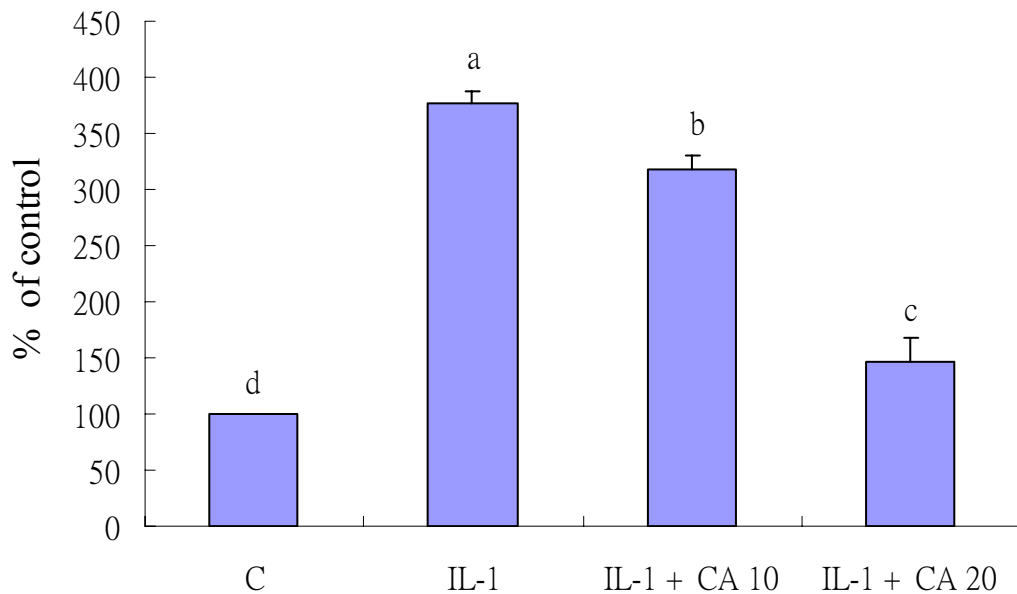
**Fig 3-6**



**Figure 3-6. Effect of carnosic acid on IL-1  $\beta$ -induce activation of NF- $\kappa$ B p65.**

HUVECs were pretreated with 10  $\mu$ mol/l and 20  $\mu$ mol/l carnosic acid for 18 hours and then induced by IL-1  $\beta$  (10ng/ml) for 6 hours. Nuclear extractes were prepared and analyzed for activation of NF- $\kappa$ B. Ten micrograms of nuclear protein was used in each experiment. Values are mean  $\pm$  S.D., n=3. <sup>a-d</sup> Means with different letters are significantly different at  $p < 0.05$ . Abbreviation: C (control), IL-1 (IL-1  $\beta$ ), CA10 (carnosic acid 10  $\mu$ mol/l), CA 20 (carnosic acid 20  $\mu$ mol/l).

**Fig 3-7**

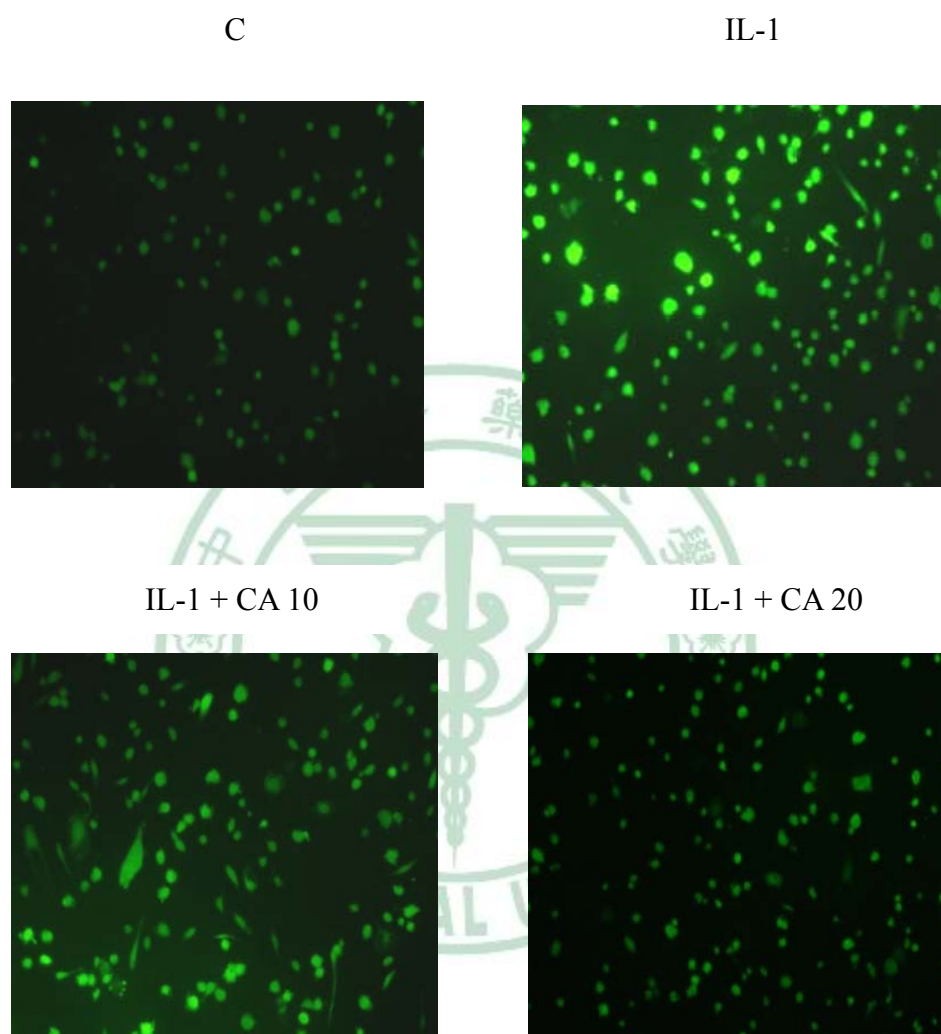


**Figure 3-7. Effect of carnosic acid on IL-1  $\beta$ -induce activation of NF- $\kappa$ B p50.**

HUVECs were pretreated with 10  $\mu$ mol/l and 20  $\mu$ mol/l carnosic acid for 18 hours and then induced by IL-1  $\beta$  (10ng/ml) for 6 hours. Nuclear extractes were prepared and analyzed for activation of NF- $\kappa$ B. Ten micrograms of nuclear protein was used in each experiment. Values are mean  $\pm$  S.D., n=3. <sup>a-d</sup> Means with different letters are significantly different at  $p < 0.05$ . Abbreviation: C (control), IL-1 (IL-1  $\beta$ ), CA10 (carnosic acid 10  $\mu$ mol/l), CA 20 (carnosic acid 20  $\mu$ mol/l).

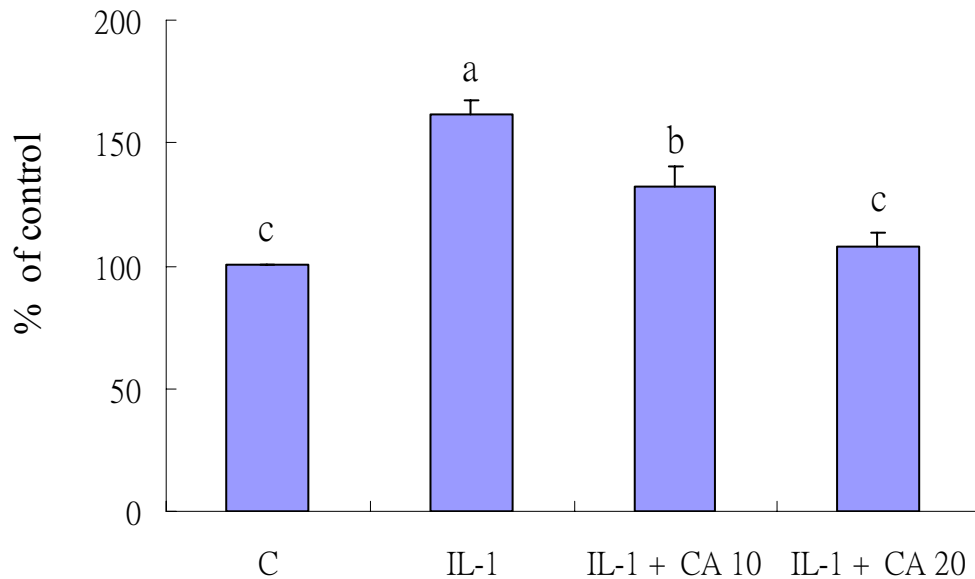
**Fig 3-8**

(A)



**Fig 3-8**

(B)



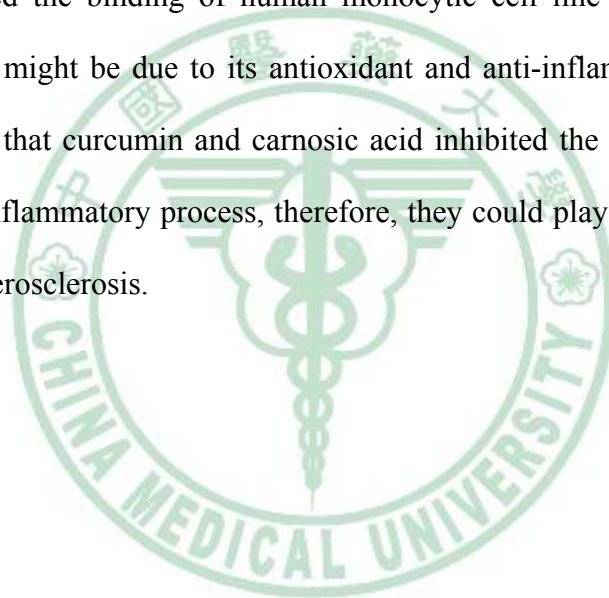
**Figure 3-8. Effect of carnolic acid on IL-1  $\beta$ -induce ROS production in HUVECs.**

(A) Fluorescent images showed the reduction of IL-1  $\beta$ -induced ROS production in HUVECs after pretreatment of 10 and 20  $\mu\text{mol/l}$  carnolic acid for 18 hours. (B) HUVECs were pretreated with 10  $\mu\text{mol/l}$  and 20  $\mu\text{mol/l}$  carnolic acid for 18 hours and then induced by IL-1  $\beta$  (10ng/ml) for 6 hours. HUVECs were labeled with  $\text{H}_2\text{O}_2$ -sensitive fluorescent probe and detected by fluorescence microplate reader. Values are mean  $\pm$  S.D.,  $n=3$ . <sup>a-c</sup> Means with different letters are significantly different at  $p < 0.05$ . Abbreviation: C (control), IL-1 (IL-1  $\beta$ ), CA10 (carnolic acid 10  $\mu\text{mol/l}$ ), CA 20 (carnolic acid 20  $\mu\text{mol/l}$ ).

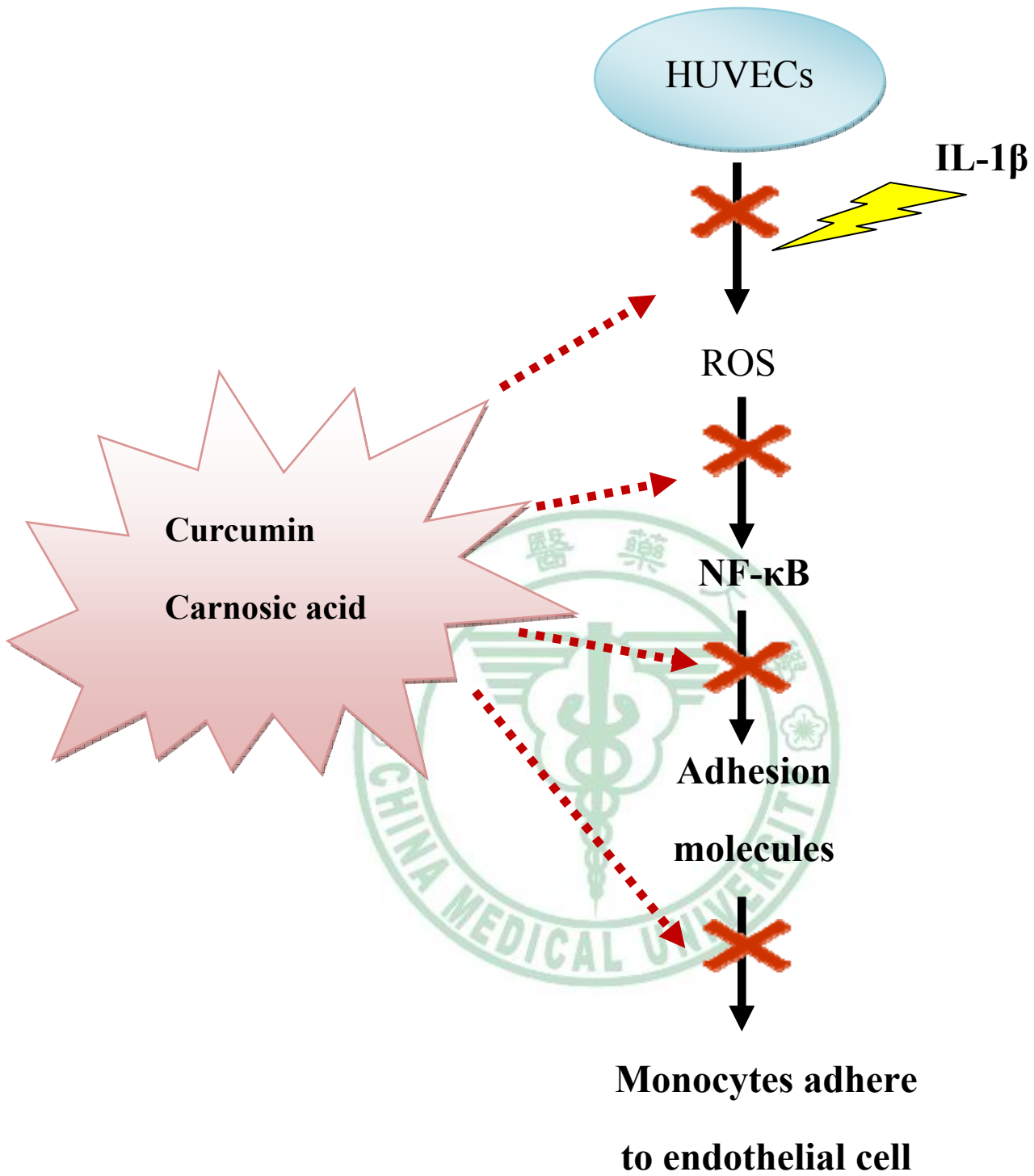
## ***Chapter 4***

### ***Summary***

In summary, curcumin and carnosic acid could inhibit IL-1 $\beta$ -induced ICAM-1, VCAM-1 and E-selectin expression in HUVEC through a mechanism that involves NF- $\kappa$ B. It reduced the binding of human monocytic cell line U937 to IL-1 $\beta$ -induced HUVECs, which might be due to its antioxidant and anti-inflammatory properties. Our results suggested that curcumin and carnosic acid inhibited the expression of molecules involved in the inflammatory process, therefore, they could play an important role in the prevention of atherosclerosis.







**Figure 4-1.** The role of curcumin and carnosic acid in the prevention of atherosclerosis.

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## Appendix 1

Table 1. Antioxidative capacities of carnosic acid *in vitro*.

	Inhibition of LDL Oxidation (IC <sub>50</sub> , μmol/l)	DPPH radicals scavenging ability (IC <sub>50</sub> , μmol/l)	TEAC
carnosic acid	5.63±0.19	35.9±1.7	5.75±0.49

All values are mean ± S.D. IC<sub>50</sub> values were obtained from the concentration response curves; n =3.

