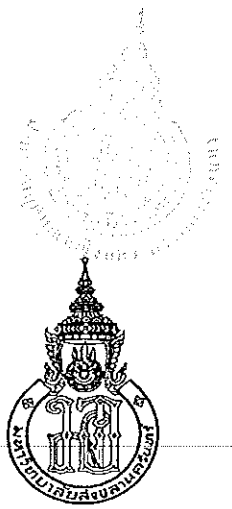

Study on Anti-Atherosclerotic Mechanisms of Morelloflavone from *Garcinia dulcis* Leaves

Decha Pinkaew

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biochemistry

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Thesis Title	Study on Anti-Atherosclerotic Mechanisms of Morelloflavone from *Garcinia dulcis* Leaves
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ชื่อวิทยานิพนธ์	การศึกษากลไกต้านภาวะหลอดเลือดแดงแข็งของ morelloflavone จากใบมะพูด
ผู้เขียน	นายเดชา ปิ่นแก้ว
สาขาวิชา	ชีวเคมี
ปีการศึกษา	2550

บทคัดย่อ

ในปัจจุบันโรคหลอดเลือดหัวใจถูกจัดเป็นสาเหตุสำคัญของการตายในประชากรของประเทศต่างๆ ทั่วโลกและความพยายามแสวงหาปัจจัยที่จะช่วยชลอภาวะความผิดปกติดังกล่าวกำลังดำเนินไปอย่างไม่หยุดยั้ง ผลจากการทดลองที่ผ่านมาแสดงให้เห็นว่าสารประกอบโพลีฟีนอลิก (polyphenolics) จากพืชนานับชนิดมีศักยภาพในการยับยั้งโรคหลอดเลือดหัวใจได้เนื่องจากสามารถต้านออกซิเดชันได้ดี ทำให้สารประกอบกลุ่มดังกล่าวได้รับความสนใจอย่างยิ่ง ดังนั้นในการศึกษานี้จึงได้นำ morelloflavone ที่สกัดได้จากใบมะพูดมาทดสอบฤทธิ์ต้านภาวะหลอดเลือดตีบซ้ำ (restenosis) และภาวะหลอดเลือดแดงแข็ง (atherosclerosis) ในหนูถีบจักร 2 ชนิดที่มียีนบกพร่องได้แก่ หนู apoE-KO กับหนู apoBEC-1/LDLR-DKO ตามลำดับ พบว่าความหนาของชั้น intima ที่เกิดขึ้นมาใหม่ (neointimal hyperplasia) ในผนังหลอดเลือดแดงของหนู apoE-KO ที่ได้รับ morelloflavone (0.15% โดยน้ำหนัก) มีค่าน้อยกว่า (4,804 ± 5,019 μm^2) ของหนูในกลุ่มควบคุมที่ไม่ได้รับสารทดสอบ (17,333 ± 13,465 μm^2) อย่างมีนัยสำคัญ ซึ่งเป็นผลจากความสามารถยับยั้งการเคลื่อนที่ของเซลล์กล้ามเนื้อเรียบในผนังหลอดเลือด (vascular smooth muscle cell) รวมทั้งการเกิด lamellipodia ซึ่งใช้ในการเคลื่อนที่ของเซลล์ชนิดดังกล่าวของ morelloflavone นั่นเอง จากการศึกษากลไกภายในเซลล์พบหลักฐานที่แสดงว่า morelloflavone หยุดการเคลื่อนที่ของเซลล์ผ่านขั้นตอนยับยั้งการทำงานที่เกี่ยวข้องของโปรตีน 4 ชนิด ได้แก่ FAK, c-Src, ERK และ RhoA โดยไม่ได้ทำให้วัฏจักรของเซลล์หยุดชงักลง (cell cycle arrest) หรือก่อให้เกิดการตายของเซลล์แบบ apoptosis แต่อย่างใด

ผลจากการทดสอบฤทธิ์ต้านภาวะหลอดเลือดแดงแข็งในหนู apoBEC-1/LDLR-DKO พบว่า morelloflavone (0.003% โดยน้ำหนัก) ไม่ได้เปลี่ยนแปลงระดับของไขมันต่างๆ ในเลือด แต่ลดการเกิดภาวะหลอดเลือดแดงแข็งอย่างมีนัยสำคัญเมื่อเปรียบเทียบกับปริมาณ plaques ที่พบในผนังหลอดเลือดแดงของหนูกลุ่มควบคุม จากการวิเคราะห์ทั้งแบบ *en face* (ค่าของกลุ่มทดสอบและกลุ่มควบคุมเท่ากับ 24.86 ± 6.87% และ 33.76 ± 5.88% ตามลำดับ) และแบบตัดตามขวาง (ค่าของกลุ่มทดสอบและกลุ่มควบคุมเท่ากับ 7.64 ± 1.30

$x10^3$ μm^2 และ 5.65 ± 1.05 $x10^3$ μm^2 ตามลำดับ) นอกจากนี้ยังพบว่า ในขณะที่จำนวนเซลล์กล้ามเนื้อเรียบในผนังหลอดเลือดแดงของหนูที่ได้รับ morelloflavone ลดลงอย่างมีนัยสำคัญ แต่ไม่พบการเปลี่ยนแปลงของจำนวนเซลล์ทั้งหลายที่กำลังแบ่งตัว (proliferating cells) เซลล์ตาย (apoptotic cells) รวมทั้งเม็ดเลือดขาวชนิด macrophages ในบริเวณที่มีรอยโรค (lesions) ปรากฏอยู่ บ่งชี้ว่า morelloflavone ชะลอภาวะหลอดเลือดแดงแข็งในหนูทดลองที่นำมาศึกษาโดยยับยั้งการเคลื่อนที่ของเซลล์กล้ามเนื้อเรียบเข้าสู่บริเวณรอยโรคนั่นเอง

ผลจากการศึกษานี้แสดงให้เห็นว่า morelloflavone เป็นสารสำคัญชนิดหนึ่งจากพืชที่มีศักยภาพซึ่งควรค่าแก่การนำไปพัฒนาต่อไปเป็นยาต้านภาวะหลอดเลือดตีบซ้ำ รวมทั้งชะลอภาวะหลอดเลือดแดงแข็งที่ไม่ก่อให้เกิดความเป็นพิษและหยุดการเจริญของเซลล์

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Major Program	Biochemistry
Academic Year	2007

ABSTRACT

Cardiovascular disease is currently the leading cause of death worldwide, and a search for factors limiting its occurrence is ongoing. Accumulated data, from *in vitro* and animal studies, have indicated beneficial cardiovascular effects of various plant-derived polyphenolic compounds, mostly relating to their anti-oxidant activities. In the present study, morelloflavone, the major biflavonoid in *Garcinia dulcis*, was investigated for its potential to retard atherosclerosis in aortas of apolipoprotein B mRNA editing catalytic polypeptide-1 and low density lipoprotein receptor genes double knockout (apoBEC-1/LDLR-DKO) mice and restenosis after angioplasty in carotid arteries of apolipoprotein E gene knockout (apoE-KO) mice. In restenosis model, oral morelloflavone (0.15% w/w) significantly decreased the degree of neointimal hyperplasia after carotid artery endothelial denudation in apoE-KO mice (control group *vs* morelloflavone treated group, 17,333 ± 13,465 μm^2 *vs* 4,804 ± 5,019 μm^2, respectively). At the cellular level, morelloflavone robustly inhibited vascular smooth muscle cell (VSMC) migration as shown by both scratch wound and invasion assays. In addition, morelloflavone prevented VSMCs from forming lamellipodia, VSMC migration apparatus. Mechanistically, the inhibition by morelloflavone of VSMC migration was through its negative regulatory effects on several migration-related kinases, including FAK, Src, ERK, and RhoA. Strikingly, morelloflavone did not affect VSMC cell cycle progression or induce apoptosis, as shown by flow cytometric and Brd-U assays as well as by MTT and DNA fragmentation assays. These data suggest that morelloflavone blocks injury-induced neointimal hyperplasia *via* the inhibition of VSMC migration, without inducing apoptosis or cell cycle arrest.

In order to examine whether morelloflavone could reduce atherosclerosis, apoBEC-1/LDLR-DKO mice were fed a regular diet or morelloflavone-containing diet for 8 months. Treatment with morelloflavone (0.003% w/w) had no effect on the plasma lipids profile, but the progression of atherosclerosis was significantly reduced compared with control by both *en face* analysis (control *vs* morelloflavone, 33.76 ± 5.88% *vs* 24.86 ± 6.87%, respectively) and cross-sectioned analysis (control *vs* morelloflavone, 7.64 ± 1.30 x10^3 μm^2 *vs* 5.65 ± 1.05 x10^3 μm^2, respectively). Furthermore, the atherosclerotic lesions in morelloflavone-treated mice had a significant decrease in VSMCs, whereas the proliferating cells and apoptotic cells including macrophages were not markedly affected by morelloflavone. These observations suggest that the ability of morelloflavone to prevent atherosclerotic lesion development might be associated with its function in VSMC migration in this animal model.

The findings from this study thus indicate a potential for morelloflavone as a novel agent for the prevention of restenosis and as a therapeutic compound against atherosclerosis without compromising effects on cytotoxicity and cell cycle arrest.

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Decha Pinkaew

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LIST OF ABBREVIATIONS

ApoB	=	Apolipoprotein B
ApoBEC-1	=	ApoB mRNA editing catalytic polypeptide-1
ApoE	=	Apolipoprotein E
CABG	=	Coronary artery bypass graft
CAD	=	Coronary artery disease
CE	=	Cholesterol ester
DES	=	Drug-eluting stents
EC	=	Endothelial cell
HDL	=	High density lipoprotein
HIV-1 RT	=	HIV-1 reverse transcriptase
HMG-CoA	=	3-Hydroxy-3-methylglutaryl coenzyme A
HPLC	=	High performance liquid chromatography
ICAM-I	=	Intracellular cell-adhesion molecule-I
LDL	=	Low density lipoprotein
LDLR	=	LDL receptor
M-CFS	=	Macrophage colony-stimulating factor
MTT	=	(3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide)
NEFA	=	Non-esterified fatty acids
NO	=	Nitric oxide
PCI	=	Percutaneous coronary intervention
PDGF	=	Platelet derived growth factor
PL	=	Phospholipids
PLA_2	=	Phospholipase A_2
PTA	=	Percutaneous transluminal angioplasty
VSMCs	=	Vascular smooth muscle cells
VCAM-I	=	Vascular cell adhesion molecule-I

LIST OF ABBREVIATIONS (CONTINUED)

VLDL	=	Very low density lipoprotein
SDS-PAGE	=	Sodium dodecylsulphate polyacrilamide gel electrophoresis
TC	=	Total cholesterol
TG	=	Triglycerides
TLC	=	Thin-layer chromatography
TPA	=	12-*O*-tetradecanoylphorbol 13-acetate
TUNEL	=	Terminal deoxynucleotidyl transferase deoxyuridine nick-end labeling
VVG	=	Verhoeff–van Gieson
WBCs	=	White blood cells

CHAPTER 1

INTRODUCTION

1.1 Overview

Atherosclerosis is a disease of arteries resulting in more than half of all mortality in developed countries and the leading cause of death in the United States (Jemal *et al.*, 2005). It is also known by several other names – arteriosclerosis, hardening of the arteries, cholesterol deposits in the arteries, and arterial blockages. Several clinical risk factors that contribute to the development of atherosclerosis, some of which can be controlled, and some that cannot. Risk factors include hyperlipidemia, hypertension, high blood homocysteine, diabetes, obesity, smoking, stress, and physical inactivity (Roeters van Lennep *et al.*, 2002).

The main features of this disease involve the building up of deposits of fatty substances so called plaque (atheroma), decaying of vascular muscle cells, and accumulations of cholesterol, calcium and other substances in the inner lining of an artery. The earliest visible atherosclerotic lesion is the fatty streak, which comprises an area of intimal thickening containing macrophages overloaded with lipid droplets known as "foam cells", lymphocytes and vascular smooth muscle cells (VSMCs). Plaques develop as a result of the accumulation of oxidatively-modified low-density lipoproteins (LDL) in the sub-endothelial space mediated by over-expression of scavenger receptors, followed by the diapedesis of leukocytes and formation of foam cells, proliferation of VSMCs and production of connective tissue. Plaque can grow and can considerably narrow the artery, so the artery becomes constricted and the elasticity is reduced. Therefore the amount of blood able to travel through it is reduced (Latifoglua *et al.*, 2007). Plaque rupture and thrombus formation are the last stages of the atherosclerotic process, leading to clinical events involving coronary, cerebral and peripheral arteries (Libby *et al.*, 2001; Munteanu *et al.*, 2007).

Several approaches have been proposed to encounter progress of atherosclerosis: reduction of those risk factors, use of statins (Beaudry *et al.*, 2007), calcium blockers (Thakur *et al.*, 2002), angiotensin-converting enzyme inhibitors (Kowala *et al.*, 1994), antithrombotic drugs (Helft *et al.*, 2000). Prolonged usage of these synthetic drugs however could be accompanied with some undesirable side effects and toxicity. For example, statins side effects have been the subject of much controversy over the past few decades, as more and more research is revealing serious potential adverse reactions from statins medications. In addition to the common side effects such as headaches, nausea and fever, other statins side effects can be much more serious. Three of the most troubling statins side effects include extreme muscle pain and muscle disease (statins induced myopathy), kidney toxicity, and serious liver problems (Sinzinger *et al.*, 2002; Gotto, 2003; McKenney, 2006)

Flavonoids are natural polyphenolic compounds which exert a broad spectrum of biological activities including antioxidative properties (Rice-Evans *et al.*, 1996). Flavonoids from various sources have been demonstrated *in vitro* to inhibit the oxidation of LDL which is thought to play a key role in triggering pathological events through multiple pathways, leading to atherosclerosis (Kasaoka *et al.*, 2002; Naderi *et al.*, 2003). In addition, oral flavonoid supplementations have been shown to attenuate atherosclerosis development in several animal models (Lucas *et al*, 2003; Enkhmaa *et al.*, 2005; Juźwiak *et al.*, 2005).

Morelloflavone (5, 7, 4', 5", 7", 3"', 4"'-heptahydroxy-[3, 8"]-flavonylflavanone), is a biflavonoid found abundant in plants belonging to the Guttiferae family. Its biological activities have been studied. Li and co-workers showed that morelloflavone inhibited the activity of fatty acid synthase, an antifungal target enzyme (Li *et al.*, 2002) and also strongly inhibited tyrosinase activity, an enzyme that involved in melanization in animal skin (Masuda *et al.*, 2005). Lin and others discovered that morelloflavone inhibited the activity of HIV-1 reverse transcriptase *in vitro* and that it protected leukocytes infected with the virus against cell death in a whole cell assay (Lin *et al.*, 1997). Gil and others reported that morelloflavone inhibited secretory phospholipase A2 and ameliorated 12-O-

tetradecanoylphorbol 13-acetate (TPA)-induced ear inflammation and carrageenan–induced paw edema in mice (Gil *et al.*, 1997). Others have shown that morelloflavone possess a potent anti-oxidant activity and blocks lipid peroxidation *in vitro* (Sanz *et al.*, 1994; Deachathai *et al.*, 2006; Hutadilok-Towatana *et al.*, 2007). Despite these studies and the fact that flavonoids in general are cardioprotective, the exact role of morelloflavone in cardiovascular system remains unknown. This study was then carried out to evaluate the effect of morelloflavone derived from *Garcinia dulcis* Kurz, a tropical fruit plant among Southeast Asian countries, on atherosclerosis. By using a mouse model of atherosclerosis, the effects of morelloflavone isolated from *G. dulcis* leaf, were studied in detail. The experiments were also undertaken to examine the effects of *G. dulcis* morelloflavone on VSMCs and arterial injury in a mouse model of restenosis.

CHAPTER 2

LITERATURE REVIEW

2.1 Atherosclerosis

Atherosclerosis is a special type of thickening and hardening of arteries, leading to the development of myocardial infarction, strokes, peripheral vascular diseases which are all together the major causes of morbidity and mortality worldwide (Wissler, 1980; Lopez and Murray, 1998). Atherosclerosis is a progressive disease characterized by a chronic inflammation and accumulation of lipids in the arteries (Goldstein and Brown, 1977; Hansson *et al.*, 1989). The fatty streaks are the first sign of atherosclerotic lesions and progress into plaques that susceptible to rupture and thrombosis (Hansson, 2005).

Atherosclerotic lesions are thought to be initiated by accumulation of low density lipoproteins (LDL), adhesion of monocytes into the arterial endothelium, emigration of monocytes into the arterial intima, possibly in response to chemotactic stimuli provided either directly or indirectly by oxidized lipoproteins, and accumulation of cholesterol within macrophage (Quinn *et al.*, 1987; Ross, 1993; Clair, 1997). Growth factors, cytokines, and other vasoactive substances secreted by macrophages, smooth muscle cells, and endothelial cells influence the further progression of atherosclerosis (Ross, 1993; Clair, 1997). At later stages of atherosclerosis, smooth muscle cells migrate from the arterial media into the intima, proliferate, and accumulate cholesterol (Ross, 1993; Stary *et al.*, 1994; Clair, 1997).

The accumulation of LDL in subendothelial matrix is primary initiating event in atherosclerosis. LDL diffuses through endothelial cell junctions passively, and is trapped in extracellular matrix. The retention of LDL seems to involve interactions between the apolipoprotein B of LDL and matrix proteoglycans (Boren *et al.*, 1998). The trapped LDL subsequently undergoes modification, including oxidation. Oxidation of LDL can be induced by endothelial cells (ECs)

(Parthasarathy *et al.*, 1989), smooth muscle cells (Witztum and Steinberg, 1991), macrophages (Rankin *et al.*, 1991), heavy metal ion (Witztum and Steinberg, 1991), lipoxygenase (Parthasarathy *et al.*, 1989; Rankin *et al.*, 1991) or reactive oxygen species (Witztum and Steinberg, 1991), leading to initiation of lipid peroxidation in the LDL. An oxidized LDL activates the overlying ECs to produce several pro-inflammatory molecules, growth factors, including adhesion molecules, resulting in the recruitment of monocytes to the vessel wall (Lusis, 2000). The macrophage colony-stimulating factor (M-CSF) stimulates the proliferation and differentiation of monocytes, and activates monocyte-derived macrophages to express scavenger receptors (Lusis, 2000; Komada, *et al.*, 1990). The oxidized LDL is recognized by SR-A, CD36, and CD68 scavenger receptors, rapidly taken up by macrophage and subsequently become a foam cells as shown in Fig. 1 (Suzuki, *et al.*, 1997; Febbraio *et al.*, 2000).

In advanced atherosclerotic lesions, cholesterol is present extracellularly as cholesterol crystals, and evidence of calcification, necrosis, and hemorrhage can be found (Stary *et al.*, 1995). The most common cause of an acute heart attack or stroke is a sudden blockage of a coronary or cerebral artery, respectively, due to thrombosis at sites of rupture of an atherosclerotic plaque (Clair, 1997; Felton *et al.*, 1997; Schwenke, 1998).

The atherosclerotic lesion can be classified into six types (Table 1) based on its histological composition and structure according to the American Heart Association, (Stary *et al.*, 1995).

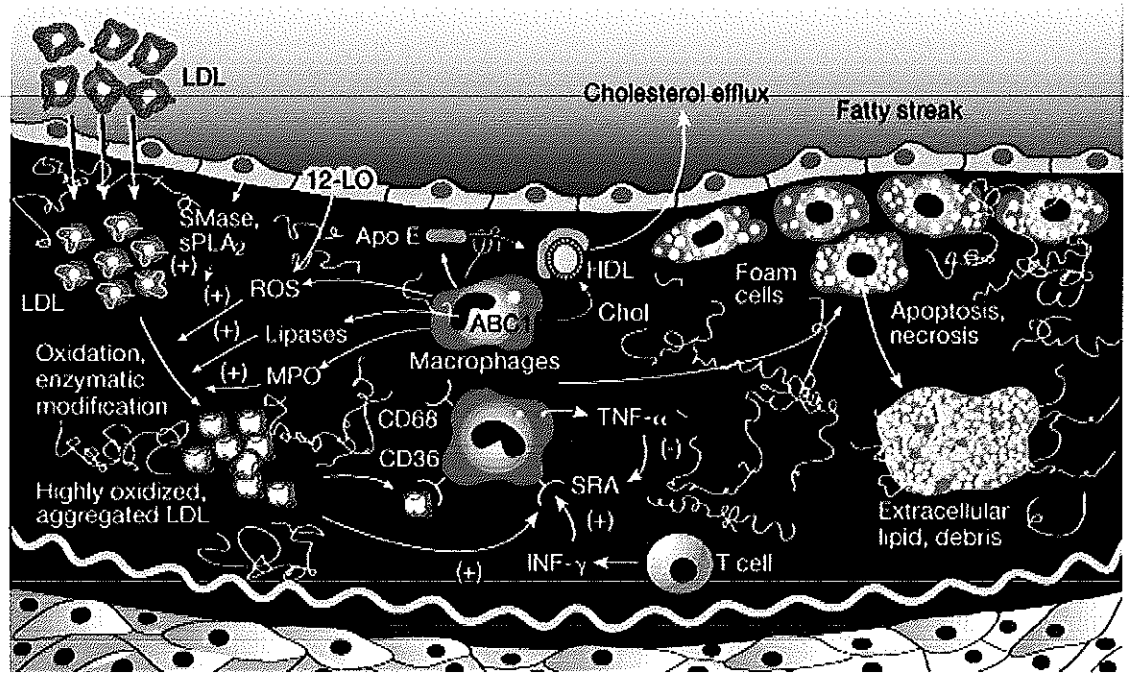


Figure 1. Foam-cell formation. Highly oxidized aggregated LDL is formed in the vessel as a result of the action of reactive oxygen species (ROS) and the enzymes sphingomyelinase (SMase), secretory phospholipase A2 (sPLA2), other lipases, and myeloperoxidase (MPO). The oxidized aggregated LDL is recognized by macrophage scavenger receptors such as SR-A, CD36 and CD68 (Lusis, 2000).

Table 1. Terms used to designate different types of human atherosclerotic Lesions in pathology (Stary *el al.*, 1995)

Term of atherosclerotic lesions	Description
Type I	The lesions contain enough atherogenic lipoprotein to stimulate an increase in macrophages and formation of foam cells
Type II	The lesions consist of foam cells and vascular smooth muscle cells. These lesions are generally known as "fatty streaks".
Type III	The lesions are characterized by pools of extracellular lipid in addition to all the components of type II lesions.
Type IV	The lesions are also known as atheroma and these lesions are characterized by the formation of the "lipid core" and they do not have any increase in the fibrous tissue.
Type V	The lesions are the lesions where fibrous tissue starts to form over the plaque and the calcification of the plaque starts.
Type VI	These type of lesions are "complicated lesions" and are associated with the disruption of the plaque known as the unstable lesions.

2.2 The vessels affected by atherosclerosis

Atherosclerosis is the disease which affects the major conduit arteries of the body. The vulnerability of the arteries for the development of atherosclerotic lesions varies, some parts being more vulnerable than others. The abdominal aorta is

more susceptible to atherosclerosis than the thoracic aorta. In the thoracic aorta, the lesions are predominantly found in the ascending aorta and the aortic arch. In the abdominal segment, the lesions are found between the renal arteries and the iliac bifurcation. Certain medium sized arteries such as coronary arteries and peripheral arteries of the limbs are more severely affected as compared to the others including internal mammary artery and inferior epigastric arteries (Sons *et al.*, 1993).

2.3 Arterial wall

The normal artery wall is composed of different cell types and the connective tissue. These are three tissue layers that observed in arterial wall: intima, media, and adventitia.

2.3.1 Intima

The intima is the innermost layer of arterial wall. It consists of the lining cells of the artery, called endothelial cells which are the boundary between the arterial intima and flowing blood, as well as a subendothelial layer made up of mostly loose connective tissue. They are arranged like paving stones on the inner surface of the arterial wall. They were historically considered to act only as a blood-tissue barrier in blood vessels, but in recent years endothelial cells are important for determining arterial tone and size and for causing inflammation.

2.3.2 Media

The most prominent is the middle layer, called the media, which composed of tightly packed smooth muscle cells and surrounded by extracellular matrix containing fibrous tissue proteins such as collagen, elastin, and proteoglycans. The vascular smooth muscle cells present in the media are responsible for many functions of the arterial wall, such as vasodilation or constriction.

2.3.3 Adventitia

The outer tissue layer, called the adventitia, is also a relatively loose tissue consisting mostly of bundles of collagen along with a few connective tissue cells. This layer also contain fibroblasts, nerves, lymph vessels, and blood capillaries known as vasa vasorum, which supply the adventitia and the outer part of the media with nutrients and oxygen

2.4 Risk factors

2.4.1 High blood cholesterol

Several lines of evidence have demonstrated that increased plasma cholesterol plays a primary role in the development of atherosclerosis. The formation of foam cells, the hallmark of atherosclerosis, is induced by modification of circulating LDL, especially oxidation of LDL. The oxidized LDL particles are recognized by macrophage scavenger receptors and taken up by the macrophages, forming lipid-laden foam cells (Lusis, 2000).

2.4.2 High blood pressure

Elevated blood pressure levels have been found to be highly predictive of cardiovascular events, including ischemic coronary disease, stroke, and peripheral arterial disease which resulting from atherosclerosis (Kannel, 2000). Pressure-induced stretch of the wall increases endothelial permeability to LDL and emphasizes accumulation of LDL in the intima (Meyer *et al.*, 1996). Hypertension also enhances the recruitment of monocyte by upregulating the expression of ICAM-1 and P-selectin. These favor the recruitment and the accumulation of monocytes and lymphocytes in the intima of vessels (Wang *et al.*, 2004).

2.4.3 Smoking

Cigarette smoking is also the risk factor associated with atherosclerosis. The risk is manifest both as an increased risk for thrombosis of narrowed vessels and as an increased degree of atherosclerosis in those vessels (Burns, 2003). Cigarette

smoking is promoted atherosclerosis possibly *via* mechanisms involving increased oxidative stress and nitric oxide (NO) inactivation in the vascular endothelium (Pittilo, 1990; Powell and Higman, 1994).

2.4.4 Diabetes

Diabetes mellitus is a major risk factor for atherosclerosis. Hyperglycemia-induced endothelial dysfunctions in the absence of insulin resistance by activates the hexosamine pathway in endothelial cells, activates nitric oxide production , elevates the production and activity of metalloproteinase 2 and 9. These accelerate the process of atherothrombotic complications (Federici and Lauro, 2005).

2.5 Cells of atherosclerotic plaque

2.5.1 Vascular smooth muscle cells

Vascular smooth muscle cells (VSMCs) proliferate in response to stimulation by various growth factors such as platelet derived growth factor (Murry *et al.*, 1997) and insulin like growth factors (Bayes-Genis *et al.*, 2000) due to injury to the arterial wall. The increasing in VSMCs migration has been reported to be the main cause of intimal hyperplasia (Miyata *et al.*, 2005). Also, the migration of VSMCs into and their accumulation within the intima have been recognized as hallmark events in atherosclerosis (Mutsusaka and Wakabayashi, 2005).

2.5.2 Endothelial cells

Endothelial cells forms a monolayer covering the inner surface of the blood vessels. Endothelial cell activation occurs due to the presence of inflammatory cytokines and oxidized LDL (Cines *et al.*, 1998). Endothelial cell expresses a range of adhesion molecules and chemotactic factors, such as vascular cell adhesion molecule-I and intracellular cell-adhesion molecule-I, responsible for the adhesion of leukocytes to the endothelium and their subsequent penetration into the intima (Jessup *et al.*, 2004).

2.5.3 Macrophages

Macrophages and the foam cells, which are lipid-laden macrophages, are one of the most important cell types present in the atherosclerotic plaque. The activated monocytes are attached to the endothelial cells and accumulate in the sub-endothelial layer of the artery. These macrophages proliferate in the atherosclerotic plaque under the influence of oxidized LDL, M-CSF (Hamilton *et al.*, 1999). Macrophages express scavenger receptors for oxidized LDL on their surface such as SR-A, CD36, and CD68 (Li and Glass, 2002). The macrophages uptake the oxidized LDL particles *via* these receptors and subsequently convert into foam cells (Suzuki *et al.*, 1997; Febbraio *et al.*, 2000).

2.5.4 Mast cells

Mast cells secrete certain proteases including chymase, tryptase and carboxypeptidase A. They also produce a variety of inflammatory cytokines such as TNF-α, TGF-β, IL-4, IL-5, IL-6 and IL-13 (Metcalfe *et al.*, 1997). The number of mast cells has been reported to be increased in the atherosclerotic plaques (Kaartinen *et al.*, 1996) and are involved in the recruitment of inflammatory cells, foam cell formation and destabilization of the atherosclerotic plaque (Kelley *et al.*, 2000).

2.6 Treatments

2.6.1 Statins

Statins, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, has dramatically reduced morbidity and mortality in patients with established cardiovascular disease by blocking the synthesis of cholesterol (Patel *et al.*, 2007). The efficacy of statins in the prevention of cardiovascular events mediated by reduction in LDL cholesterol has been well established (Shepherd *et al.*, 1995; Sacks *et al.*, 1996; Downs *et al.*, 1998)

2.6.2 Calcium blockers

Calcium blockers, which selectively inhibit transmembrane calcium influx into the cell, may exert antiatherogenic effects (Schmitz *et al.*, 1991; Cleland and Krikler, 1993). It has been clearly demonstrated that these drugs not only inhibit smooth muscle cell proliferation and migration but also diminish accumulation of cholesterol ester in macrophages, thereby reducing foam cell formation (Schmitz *et al.*, 1991).

2.6.3 Angioplasty

Angioplasty also named percutaneous transluminal angioplasty (PTA) or percutaneous coronary intervention (PCI) is a commonly used treatment option for atherosclerotic stenosis or even occlusions of the arteries (Dotter and Judkins, 1964). A balloon catheter is inserted into the arterial system, usually through the common femoral artery, and guided forward under fluoroscopic vision to the lesion in the artery (Fig 2). The balloon is inflated at high pressure and causes rupture and dissection of the plaque and overstretching of the vessel wall with an increased lumen as the result (Lyon *et al.*, 1987). Because of the tendency of early elastic recoil and the later negative remodeling of the vessel, balloon- or self-expandable stents are often used in combination with the PTA/PCI procedure (Fig.3). The implanted stent serves as a scaffold that keeps the artery open. Angioplasty and stenting techniques are widely used around the world and provide an alternative option to medical therapy and bypass surgery for improving blood flow to the heart muscle. However, one of the main drawbacks of PCI is the occurrence of restenosis (Dangas and Kuepper, 2002).

2.6.4 Bypass grafting

Bypass surgery is the most common type of heart surgery. According to the American Heart Association 427,000 coronary artery bypass grafting surgeries were performed in the United States in 2004 (AHA, 2004). Bypass surgery improves the blood flow to the heart with a new route, or "bypass," around a section of clogged or diseased artery. The surgery involves sewing a section of vein from the leg (saphenous vein) or artery from the chest or another part of the body to bypass a part

of the diseased coronary artery (Fig. 4). This creates a new route for blood to flow, so that the heart muscle will get the oxygen-rich blood which it needs to work properly.

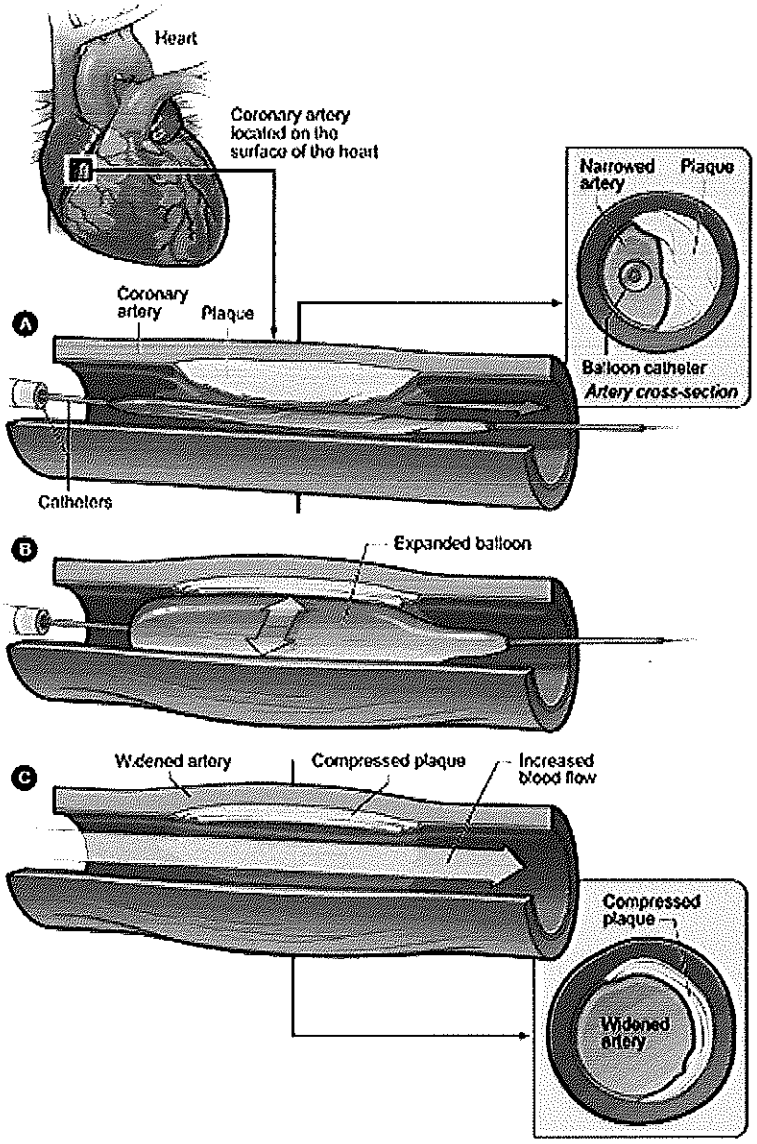


Figure 2. Coronary balloon angioplasty. **A**: the deflated balloon catheter inserted into the narrowed coronary artery. **B**: the balloon is inflated, compressing the plaque and restoring the size of the artery. **C**: the widened artery (available : www.nhlbi.nih.gov/, retrieved April 15, 2003).

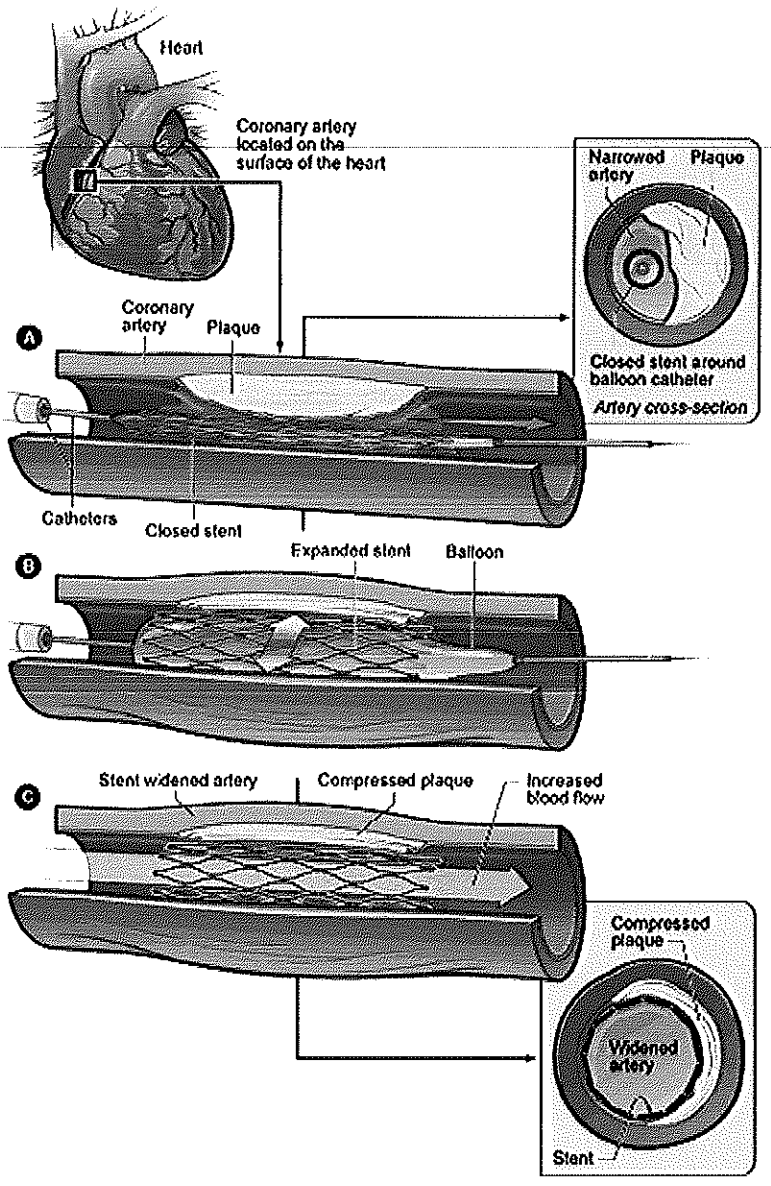


Figure 3. Stent placement. **A**: the deflated balloon catheter and closed stent inserted into the narrowed coronary artery. **B**: the balloon is inflated, expanding the stent and compressing the plaque to restore the size of the artery. **C**: the stent-widened artery (available : www.nhlbi.nih.gov/, retrieved April 15, 2003).

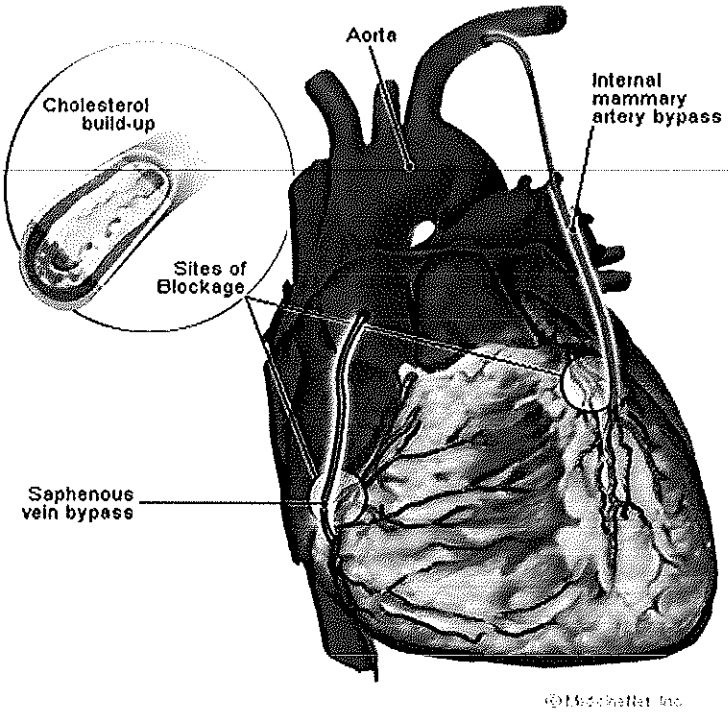


Figure 4. Coronary artery bypass grafting. The most commonly used vessel for the bypass is the saphenous vein from the leg. Bypass grafting involves sewing the graft vessels to the coronary arteries beyond the narrowing or blockage. The other end of this vein is attached to the aorta. Chest wall arteries, particularly the left internal mammary artery, have been increasingly used as bypass grafts. This artery is separated from the chest wall and usually connected to the left anterior descending artery and/or one of its major branches beyond the blockage (available : www.medicinenet.com/, retrieved April 15, 2003).

2.7 Restenosis

Restenosis is commonly defined as greater than 50% narrowing of vessel diameter compared with a reference artery after PCI usually within three to six months (Lee *et al.*, 2004). It is thought that the mechanism of this phenomenon is the response of the body's immune system to the injury induced by angioplasty (Fig. 5). At this point a repeat procedure may be needed. Compared to balloon angioplasty alone, having a chance of restenosis of 40%, stents reduce the chance of in-stent

restenosis to 10-25% (Pache *et al.*, 2003; Agenma *et al.*, 2004). Therefore, the majority of patients having angioplasty today are treated with stents. Two major processes are involved in the pathogenesis of restenosis are vessel recoil and neointimal formation (Zargham, 2008). Coronary stenting virtually eliminates vessel recoil, and in-stent restenosis is largely the result of VSMCs proliferation and migration, extracellular matrix formation, and neointimal hyperplasia (Lee *et al.*, 2004). When an artery is injured, activation of platelets, leukocyte infiltration, proliferation and migration of VSMCs, extracellular matrix formation occur. Growth factors and cytokines released by the blood cells involved stimulate the migration, growth, and multiplication of VSMCs (Chandrasekar, 2004). A few years ago, a breakthrough in the prevention of restenosis was introduced by a new generation of drug-eluting stents. These stents release a particular drug that prevents scar tissue growth in the artery in which the stent is placed, and therefore reduce the occurrence of restenosis. Recent data have demonstrated that patients treated with drug-eluting stents (DES) have decreased incidence of restenosis compared to those who received bare metal stents. Recently, however, these DES have been found associated with an increased incidence of stent thrombosis, presumably due to delayed tissue healing due to cytotoxic and cell cycle inhibitory effects of these agents (Iakovou *et al.*, 2005). Very recently, DES were reportedly associated with an increased rate of death, as compared with bare-metal stents, suggesting that DES may not be a perfect treatment of coronary artery disease (Lagerqvist *et al.*, 2007).

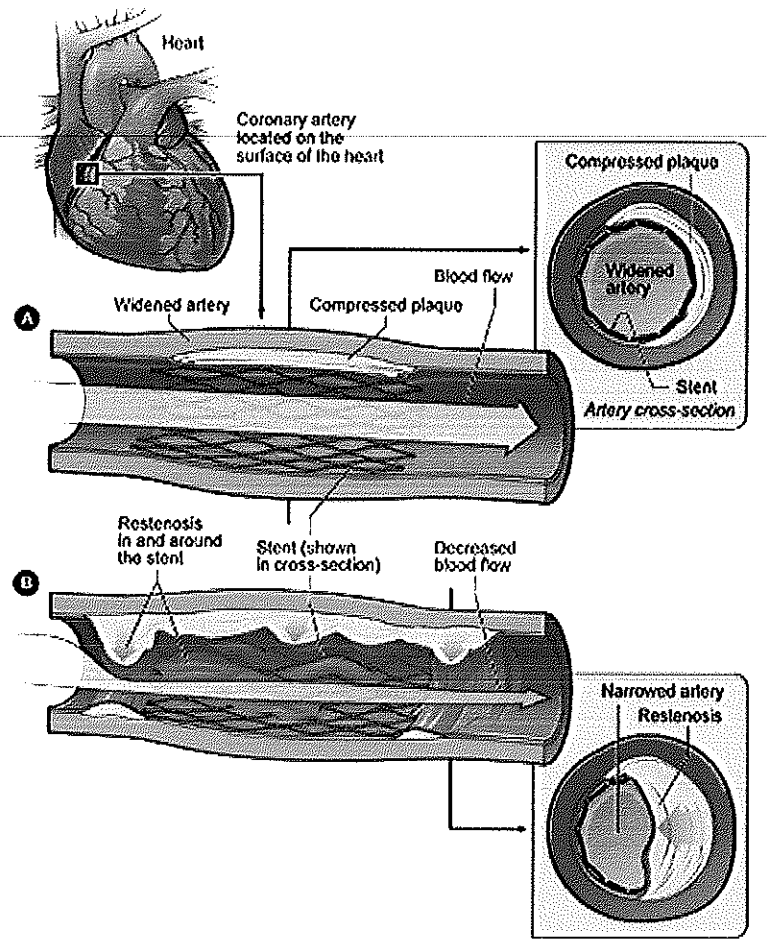


Figure 5. Restenosis of a stent-widened coronary artery. **A**: the expanded stent compresses plaque, allowing normal blood flow. **B**: over time, the plaque grows through and around the stent, causing a partial blockage and abnormal blood flow (available : www.nhlbi.nih.gov/, retrieved April 15, 2003).

2.8 Mouse models of atherosclerosis

A number of animal models have provided valuable insights into the progression of atherosclerosis, such as rabbit, pig and non-human primates (Faggiotto *et al.*, 1984a,1984b; Rosenfeld *et al.*, 1987; Gerrity, 1991a, 1991b; Lucas *et al*, 2003; Enkhmaa *et al.*, 2005; Juźwiak *et al.*, 2005). In recent years, atherogenic mechanisms

have been largely studied in mouse models, mainly due to the extensive genetic information available and the possibility to genetically engineer the mouse genome by insertion of new genes, insertions of mutants and removal or alteration of a specific gene (Table 2).

2.8.1 Inbred mouse as models of atherosclerosis

C57BL/6J inbred mice are firstly reported to be an atherosclerotic-induced mice by feeding them with enriched 50% fat-containing diet. However, the mortality rate is high by feeding this diet (Thompson, 1969). Sixteen years later Paigen and co-workers discovered that C57BL/6J inbred mice fed 15% fat-containing diet promote notable atherosclerosis development with lower mortality (Paigen *et al.*, 1985). However the lesions are small and restricted to the aortic root. While C57BL/6J16 mice are most susceptible for atherosclerosis, some strains such as the CH3 and BALB/c strain are more resistant to atherosclerosis and SV129 is an intermediate (Paigen *et al.*, 1985). Therefore, the use of this model gradually diminished as genetically engineered mice exhibiting larger lesions were induced.

2.8.2 Gene deletion and/or over expression as models of atherosclerosis

Apolipoprotein E (apoE) present on the surface of chylomicrons, very low density lipoprotein (VLDL) and high density lipoprotein (HDL) particles mediates lipoprotein uptake by several receptors. Deficiency of apoE elevates VLDL particles, leading to hyperlipidemia and eventually atherosclerosis even feeding with a normal diet (Piedrahita *et al.*, 1992; Plump *et al.*, 1992). The adhesion of monocyte to endothelium was observed in 2-month apoE-/- fed a normal diet. The lesions contained predominately macrophage foam cells at 10-30 weeks and lesions became more apparent at 15 weeks. The lesions of apoE-/- mice formed at aortic root and ascending aorta as well as branch points of several arteries which are the sites typically affected in human (Plump *et al.*, 1992; Reddick *et al.*, 1994; Rosenfeld *et al.*, 2000). However, most plasma cholesterol is carried in VLDL in these mice, rather than in LDL as in humans (Powell-Braxton *et al.*, 1998). When apoE-/- are fed diet

enriched in saturated fat, they were accelerated the progression of atherosclerosis (Nakashima *et al.*, 1994).

Another knock-out model which induced atherosclerosis is low LDL receptor null (LDLR-/-) mice which were generated by gene targeting of embryonic stem cells (Ishibashi *et al.*, 1993). LDLR-/- mice develop only modest hypercholesterolemia when maintained on regular diets. A lack of increased plasma cholesterol concentration is the presence of the synthesis of apolipoproteinB (apoB)-48 in the liver of mice, leading to a predominance of this apoB48 on circulating LDL. Lipoproteins containing apoB48 have high levels of apoE. This permit an enhanced binding of apoE and subsequent clearance with mechanisms other than the LDL receptor, such as LDL receptor-related protein (Powell-Braxton *et al.*, 1998). These mice showed large atherosclerotic lesions when fed a high cholesterol diet (Tangirala *et al.*, 1995). Although LDLR-/- mice fed on a regular diet do not develop atherosclerosis, this model can be distinguished from the diet-induced atherosclerotic model, because the lesion in LDLR-/- mice is more extensive than observed in C57BL/6 mice fed on a Western diet (Masucci-Magoulas *et al.* 1997). Furthermore, plasma lipid profiles of LDLR-/- mice are more similar to those in human hyperlipidemia than those of apoE-/- mice (Powell-Braxton *et al.*, 1998).

The LDLR -/- has been crossed with other genetically modified mice to examine the role of genetic modifiers in atherosclerosis. A combination of apoE and LDLR knockout was generated (Ishibashi *et al.*, 1994). The lesion progression of these mice were increased when compared to the individual knockouts (Witting *et al.*, 1999). ApoBEC-1-/- LDLR-/- mice are the combination of apoB mRNA editing catalytic polypeptide-1 (apoBEC-1) deficiency and deletion of LDL receptors. These mice have markedly increased in plasma cholesterol concentrations, caused exclusively by increased LDL cholesterol and developed extensive lesions throughout the aorta, including most of the branch points (Powell-Braxton *et al.*, 1998). Unlike apoE-/- mice, the lipoprotein metabolism in these mice mirrors the pathophysiology of human familial hypercholesterolemia. Human with a atherosclerosis always have high plasma levels of apoB100-containing LDL, where as apoE-/- mice have a distinct accumulation of apoB48-containing lipoproteins and have normal low levels of apoB100-containing lipoproteins (Powell-Braxton *et al.*, 1998).

Table 2. Characteristics of selected mouse models of atherosclerosis (Daugherty, 2002)

Model	Characteristics
C57BL/6	Small lesions formed only in the aortic root when fed a modified diet
Apo E-/-	Mice are hyperlipidemic and also respond to modified diet. Lesions progress to an intermediate complexity of macrophage foam cells, necrotic cores, and fibrous caps. Lesions occur in several vascular beds.
LDL receptor-/-	Requires a modified diet to promote atherosclerosis. Lesions are representative of early lesions with a predominancy of macrophage foam cells
apoBEC-1-/- LDL receptor-/-	Mice are hyperlipidemic and form pronounced atherosclerotic lesions in many vascular regions.

2.9 Effects of flavonoids on atherosclerosis

Flavonoids are polyphenolic compounds that are abundant in fruits, vegetables, and a wide array of plants (Arts *et al.*, 2001; Henning *et al.*, 2004; Mulder *et al.*, 2005). They display a multitude of biological effects both *in vitro* and *in vivo* after consumption of flavonoid-containing foods. Epidemiologic studies show that increased consumption of flavonoids reduce the risk of cardiovascular disease and certain types of cancer (Koga and Meydani, 2001; Arts *et al.*, 2005). Flavonoids may exhibit antioxidant, antimutagenic, and free-radical scavenging activities (Moyers and Kumar, 2004). Moderate consumption of red wine, which contains a high content of polyphenols, is associated with a low risk of coronary heart disease (Bravo, 1998; Donovan *et al.*, 2002). Consumption of soy and soy products are related to biological

effects, including anticarcinogenic, antiatherosclerotic, and antihemolytic effects (Bravo, 1998; Moyers and Kumar, 2004).

2.9.1 Antioxidative property

Flavonoids have been shown to inhibit the oxidation of LDL *in vitro* (Fuhrman *et al.*, 2001; Kasaoka *et al.*, 2002; Naderi *et al.*, 2003). Furthermore, the addition of flavonoids, quercetin and catechin to the diet have been shown to reduce LDL oxidation ex vivo in rats (Fremont *et al.*, 1998) and was found to decrease atherosclerotic lesion area in apoE-deficient mice (Hayek *et al.*, 1997). The mechanisms whereby flavonoids inhibit LDL oxidation are unclear. They may reduce the formation or release of free radicals since they can react with superoxide anions (Afanas'ev *et al.*, 1989), hydroxyl radicals (Husain *et al.*, 1987), and lipid peroxy radicals (Torel *et al.*, 1986). These compounds may also act by chelating iron which is thought to catalyze processes leading to the appearance of free radicals (Afanas'ev *et al.*, 1989; Morel *et al.*, 1993).

2.9.2 Hypolipidemic activity

While the majority of research has focused on the antioxidant roles of flavonoids, a number of reports have suggested that these compounds may also influence atherogenesis through an effect on lipid and lipoprotein metabolism (Yotsumoto *et al.*, 1997; Borradaile *et al.*, 1999). Investigation of naturally occurring compounds as regulators of triglycerides and cholesterol metabolism has particular therapeutic importance, as evidenced by the discovery of the first HMG-CoA reductase inhibitors derived from fungal fermentation products, which are now widely used for the treatment of hyperlipidemia (Wilcox *et al.*, 1999). Flavonoids might represent another beneficial group of naturally occurring hypolipidemic compounds. Studies in rats have shown that the flavonoids, quercetin (Basarkar *et al.*, 1983), hesperidin (Monforte *et al.*, 1995), marsupin (Jahromi *et al.*, 1993), liquiritigenin (Jahromi *et al.*, 1993), and pratensein (Sharma *et al.*, 1979) cause significant reduction in serum total cholesterol (TC) and triglycerides (TG). In non-human primates, dietary genistein, the isoflavone analog of naringenin, significantly reduces plasma LDL and VLDL -cholesterol levels (Anthony *et al.*, 1997). Studies in

hyperlipidemic rats fed by high-fat diets showed that i.p. administration of a methanolic extract from *Prunus davidiana* and its flavonoid components (catechin, naringenin 7-O-glucoside (prunin), and hesperetin 5-O-glucoside) for 3 days resulted in a significant reduction in blood TG and TC (Choi *et al.*, 1991). Also, naringenin 7-O-glucoside or hesperetin 5-O-glucoside, when administered alone at doses of 20 mg/kg and 10 mg/kg, respectively, showed significant hypocholesterolemic effects (Choi *et al.*, 1991). Previous studies also suggested that hesperetin, citrus flavonoids, inhibits HMG-CoA reductase, the rate limiting enzyme in the cholesterol biosynthetic pathway, and lower plasma cholesterol level in animal models (Kim *et al.*, 2003; Choi *et al.*, 2004).

2.9.3 Enhance of endothelial nitric oxide

Nitric oxide (NO) produced by endothelial nitric-oxide synthase (eNOS) plays a protective physiological role in the vasculature (Li and Förstermann, 2000a). NO is a potent vasodilator and contributes to blood pressure control. Moreover, endothelial NO possesses multiple anti-atherosclerotic properties such as prevention of leukocyte adhesion to vascular endothelium and leukocyte migration into the vascular wall, decrease in endothelial permeability, reducing of lipoproteins into the vascular wall and inhibition of LDL oxidation (Li and Förstermann, 2000; Gewaltig and Kojda, 2002). Lack of NO synthesis causes significant peripheral vasoconstrction and elevation of blood pressure (Rees *et al.*, 1989). Similarly, mice deficiency in eNOS gene are hypertensive and lack endothelium-dependent, NO-mediated vasodilation (Huang *et al.*, 1995) and inhibition of eNOS caused accelerated atherosclerosis in rabbits (Cayatte *et al.*, 1994). Based on these antihypertensive and anti-atherosclerotic effects, the enhancement of endothelial NO production could be of prophylactic or therapeutic interest. Flavonoids have been shown to increase endothelial NO synthase activity. It was found that dioclein, a flavonoid from *Dioclea grandiflora*, increase vasorelaxation by enhancing synthesis of NO (Lemos *et al.*, 1999). The previous study also showed that epigallocatechin-3-gallate, a flavonoid from green tea, increased endothelial nitric oxide synthase activity in bovine aortic endothelial cells (Lorenz *et al.*, 2004). Recently, flavonoids from *Cynara scolymus* increased eNOS gene transcription in human endothelial cells (Li *et al.*, 2004).

2.9.4 Inhibition of matrix metalloproteinases 2

Matrix metalloproteinases (MMP-2), have been identified as major matrix metalloproteinases expressed in blood vessels and atherosclerotic plaques that contribute to the turnover of of collagens (Pasterkamp *et al.*, 2000; Galis and Khatri, 2002). Previous studies have demonstrated that both red wine polyphenols and green tea flavonoids strongly prevent the activation of MMP-2 induced by thrombin in vascular smooth muscle cells (Oak *et al.*, 2004; El Bedoui *et al.*, 2004). The ability of flavonoids from both red wine and green tea to inhibit degradation of matrix has also been demonstrated in a cell invasion assay which similar to that observed with the broad-spectrum MMP inhibitor, GM6001 (El Bedoui *et al.*, 2004; Oak *et al.*, 2004).

2.9.5 Prevention of vascular endothelial growth factor expression

Vascular endothelial growth factor (VEGF) is a major pro-angiogenic factor that triggers the proliferation and migration of endothelial cell and as well as the formation of new blood vessels (Ferrara and Davis-Smyth, 1997). Abundant VEGF expression is observed in human atherosclerotic plaques, predominantly by vascular smooth muscle cells and foamy macrophages (Chen *et al.*, 1999). Platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), and are the potential activators of VEGF expression in vascular smooth muscle cells (Bassus *et al.*, 2001). These activators enhance VEGF gene expression and the released VEGF stimulate angiogenesis by activating VEGF receptors located on endothelial cells. Recent study showed that polyphenols from red wine prevented VEGF expression and releasing by the prevention of the growth factor-induced redox-sensitive activation of the p38 MAPK pathway leading to VEGF gene expression (Oak *et al.*, 2003a). In addition, green tea flavonoids also prevented VEGF expression in several types of cancer cells by inhibiting epidermal growth factor receptor-related pathways of signal transduction (Masuda *et al.*, 2002).

2.9.6 Inhibition of migration of vascular cells

It has been shown that development of atherosclerosis is characterized by endothelial cell dysfunction, proliferation and migration of VSMCs, and increased extracellular matrix deposition (Ross, 1993). The proliferation and migration of

VSMCs as well as ECs are believed to play an important role in the pathogenesis of several cardiovascular diseases, including atherosclerosis and restenosis following balloon angioplasty (Taylor *et al.*, 2001). The recent studies have shown that epigallocatechin-3-O-gallate, green tea flavonoid, inhibited rat aortic smooth muscle cells migration (Han *et al.*, 2006) as well as inhibited rat thoracic aorta smooth muscle cells migration in a dose-dependent manner (Lo *et al.*, 2007). Also red wine polyphenols were demonstrated to blocked the smooth muscle cells migration through the inhibition of phosphoinositide-3 kinase (PI3K) activity and $p38^{MAPK}$ phosphorylation (Iijima *et al.*, 2002). Resveratrol, a polyphenol in red wine, suppressed the endothelial cells progression through S and G2 phases of the cell cycle by increasing the expression of the tumor suppressor gene protein p53 and the cyclin-dependent kinase inhibitor p21 (Hsieh *et al.*, 1999). Endothelial cell migration and proliferation are also inhibited by delphinidin, a polyphenol belonging to the class of anthocyanin, through the inhibition of cyclin D1- and A-dependent pathways (Favot *et al.*, 2003). Green tea flavonoids retained endothelial cells in the G1 phase of the cell cycle and epigallocatechin-3-gallate inhibited endothelial cell migration and proliferation by inducing apoptosis (Yoo *et al.*, 2002; Kojima-Yuasa *et al.*, 2003).

2.10 *Garcinia dulcis*

2.10.1 General description

Garcinia dulcis (Roxb.) Kurz is the sub-woody plant belonging to the Guttiferae family that grows mainly in Southeast Asia (Fig. 6). It is called Mundu in Indonesia and Malaysia, Baniti in the Philippines, and Maphuut in Thailand. *G. dulcis* is a medium sized tree. The tree has a short trunk and brown bark with white latex. The leaves are opposite, lanceolated shaped, 10-30 cm long and 3-5 cm wide. The leaves are pale green when young and become dark green and shiny on the upper surface at maturity. The lower leaves are often hairy. The midrib is prominent with numerous veinlets arranged in parallel. The thick petiole is short being only 2 cm long. Flowers are borne in the axil. They are yellowish white with a sour smell. Fruits are globose, 5-8 cm wide with slightly pointed ends, often rather compressed

and crowned by the persistent stigma. The fruit is soft with a thin skin and has a light yellow color, which turns to orange when ripe. The seeds are enveloped in an edible pulp of a darker color than the skin and have a pleasant taste (Subhadrabandhu, 2001).

2.10.2 Uses

The fruit can be eaten fresh, but they are sour and can be made into an excellent jam. Its leaves and seeds have been used in traditional medicine against lymphatitis, parotitis, struma in Indonesia (Kasahara and Henmi, 1986). Its stem bark has been used in Thai folk medicine as an antiseptic, the fruit juice as an expectorant and anti-scurvy, and the root extract for the relief of fever and to reduce poisoning and detoxification (Subhadrabandhu, 2001).

Accumulated data from phytochemical studies have revealed that various parts of this plant contain, in abundant, a broad range of polyphenols. Some possess potent biological activities, for example, the five xanthones isolated from the bark have shown inhibitory effects on *Plasmodium falciparum* (Likhitwitayawuid *et al.*, 1998), the three prenylated paraxanthonoids are cytotoxic against cancer cell lines (Soemiati *et al.*, 2004), and several phenolic compounds isolated from fruits and flowers also act as anti-oxidants and anti-bacterial agents (Deachathai *et al.*, 2005; Deachathai *et al.*, 2006; Hutadilok-Towatana *et al.*, 2007).

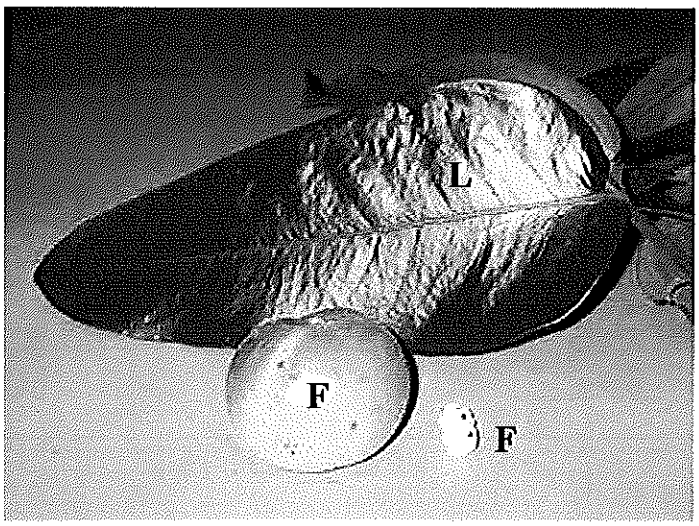


Figure 6. The leaf (L), fruit (F), and flower (FL) of *Garcinia dulcis*.

2.10.3 Morelloflavone

Morelloflavone (5, 7, 4', 5", 7", 3"', 4"'-heptahydroxy-[3, 8"]-flavonylflavanone), was firstly found in *Garcinia morella* (Karanjgaokar *et al.*, 1967). It represents a biflavonoid consisting of two flavones, one apigenin and another luteolin (Verbeek *et al.*, 2004), covalently linked to each other (Fig.7). It is composed of plants belonging to the Guttiferae family. It has been isolated from *G. spicata* (Konoshima *et al.*, 1969), *G. livingstonei* (Pelter *et al.*, 1971), *G. multiflora* (Chen *et al.*, 1975), *G. indica* (Cotterill *et al.*, 1977), *G. densivenia* (Waterman and Crichton, 1980), *G. spicata* (Gunatilaka *et al.*, 1984), *G. scortechinii* (Sukponddma *et al.*, 2005) and including local plants in southern of Thailand such as *G. dulcis* (Ansari *et al.*, 1976), *G. nervosa* (Babu *et al.*, 1988), and *G. atroviridis* (Permanna *et al.*, 2003). The biological activities of this compound have been investigated. Li and co-workers demonstrated that morelloflavone inhibited fatty acid synthase which is a potential antifungal target (Li *et al.*, 2002). It has shown that morelloflavone was an inhibitor against tyrosinase, enzyme that is responsible for melanization in animals, and stronger than the known potent inhibitor, kojic acid and arbutin (Masuda *et al.*, 2005). Morelloflavone was also showed to inhibit HIV-1 reverse transcriptase (HIV-1 RT) activity *in vitro* as well as in primary human lymphocytes tranfected with HIV-1 RT (Lin *et al.*, 1998). Moreover, Gil and co-workers demonstrated that morelloflavone is an inhibitor of secretory phospholipase A2 (PLA2) by inhibiting human synovial and bee venom PLA and exerted anti-inflammatory effects in mice, with a potent inhibition of 12-O-tetradecanoylphorbol 13-acetate (TPA)-induced edema (Gil *et al.*, 1997). Dechathai and co-workers also reported the antioxidant activity of morelloflavone which was able to trap the DPPH radical at the concentration of 10 μM (Dechathai *et al.*, 2005). Morelloflavone isolated from leaves of *Garcinia dulcis* was shown to increase the resistance of both iron-induced and AAPH-induced human-LDL oxidation *in vitro* and thus may act as an anti-atherosclerotic agent (Hutadilok-Towatana *et al.*, 2007). Recently, Decha-Dier and co-workers discovered that morelloflavone reduced the plasma triglycerides and cholesterol levels and decreased the atherosclerotic plaque in hypercholesterolemic rabbits (Decha-Dier *et al.*, 2008).

Since morelloflavone has not been demonstrated to reduce the progression of atherosclerosis in genetically manipulated animal models such as

apoBEC-1/LDLR double knockout mice which is mimic to hypercholesterolemia and atherosclerosis that are observed in human and the mechanism underlying the protective property of this compound is still unclear. Therefore, it is worth to study the effect of morelloflavone on cardiovascular disease, atherosclerosis, using transgenic mice.

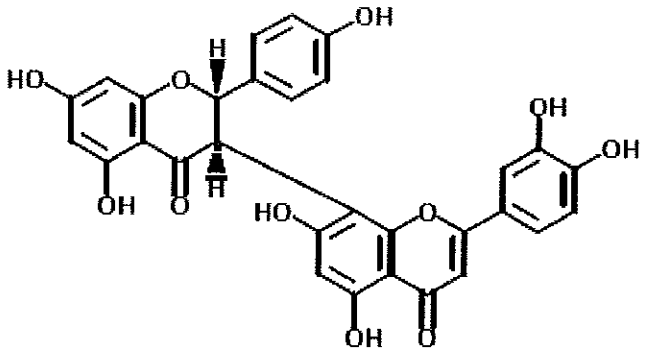


Figure 7. Structure of morelloflavone. Morelloflavone (MW = 556), a major component of the leaf and fruit of *Garcinia dulcis*, represents a biflavonoid consisting of two flavones (apigenin and luteolin) covalently linked to each other.

Aims of the thesis and benefit gain

The general aims of the thesis are to investigate whether morelloflavone could prevent the progression of atherosclerosis in apoBEC-1/LDLR-DKO mice and inhibit restenosis in apoE-KO mice.

The research works were then divided in three systems:

1. Vascular smooth muscle cells (VSMCs): to test the effects of morelloflavone on proliferation, death, and migration of VSMCs as well as the mechanisms underlying those effects.
2. ApoE knockout mice (apoE-KO): to examine whether morelloflavone could block carotid artery injury-induced neointimal formation using apoE-KO mice.
3. ApoBEC-1/LDLR double knockout mice (apoBEC-1/LDLR-DKO): to evaluate the effects of morelloflavone on lipid profiles and the progression of atherosclerosis in apoBEC-1/LDLR-DKO mice.

Benefit Gain

It is speculated that the results from this study will lead to a discovery of a natural substance, potentially useful as pharmacological agent in the treatment or prevention of atherosclerosis as well as restenosis.

CHAPTER 3

EFFECT OF MORELLOFLAVONE ON VASCULAR SMOOTH MUSCLE CELL AND INJURY-INDUCED NEOINTIMAL FORMATION IN APOE KNOCKOUT MICE

3.1 Introduction

Atherosclerosis in coronary arteries, or coronary artery disease (CAD), is the leading cause of death in adults in the developed countries. In the U.S., CAD is the single most frequent cause of death, accounting for more than 1 in 5 of deaths, with the economic burden to the society of $133.2 billion annually (AHA, 2004). Although patients with CAD can be treated by medication or coronary artery bypass grafting (CABG) surgery, an ever increasing number of patients now undergo percutaneous coronary intervention (PCI) where the atherosclerotic narrowings of coronary arteries are opened by balloons and stents. PCI was performed in more than 900,000 patients in 2003 in the U.S (Zipes and Braunwald, 2005). Unlike CABG, PCI does not require thoracotomy and are much less invasive and less frequently associated with cerebrovascular complications than CABG (Newman *et al.*, 2001). An Achilles' tendon of PCI is restenosis—re-narrowing of dilated or stented arteries—which is a process due chiefly to the migration and proliferation of α-actin-immunoreactive vascular smooth muscle cells (VSMCs) (Morimoto *et al.*, 1990; Nobuyoshi *et al.*, 1991; Karsch *et al.*, 1991). In recent years, drug-eluting stents (DES)—stents coated with cytotoxic agents such as sirolimus (Moses *et al.*, 2003) and paclitaxel (Park *et al.*, 2003) —have been used to circumvent VSMCs growth with great clinical success. Recently, however, these DES have been found associated with an increased incidence of stent thrombosis, presumably due to delayed tissue healing due to cytotoxic and cell cycle inhibitory effects of these agents (Iakovou *et al.*, 2005). Very recently, DES were reportedly associated with an increased rate of

death, as compared with bare-metal stents (Lagerqvist *et al.*, 2007), suggesting that DES may not be a perfect treatment of CAD. Although an ideal strategy would be to use an anti-restenosis agent without cytotoxic or cell cycle inhibitory effects, there have been no such agents on the immediate horizon.

Despite the previous studies and the fact that flavonoids in general are cardioprotective, the exact role of morelloflavone in cardiovascular system remains unknown. In order to start to evaluate the effect of morelloflavone on arterial injury and repair, the effects of morelloflavone on vascular smooth muscle cells (VSMCs) and in a mouse model of restenosis were studies. Here, morelloflavone was reported to blocks the migration of VSMCs without causing apoptosis or cell cycle arrest and decreases neointimal hyperplasia in a mouse model of restenosis. Based on these data, morelloflavone was proposed to be a viable anti-restenotic agent and a potential alternative to DES-based strategies.

3.2 Materials

3.2.1 Plant

The leaves of *Garcinia dulcis* were collected from Songkhla province in the southern part of Thailand. The voucher specimen (Coll. No. 02, Herbarium No. 0012652) has been deposited at Prince of Songkla University Herbarium, Biology Department, Faculty of Science, Prince of Songkla University, Thailand.

3.2.2 Mouse vascular smooth muscle cells

Mouse vascular smooth muscle cells (VSMCs) were a gift from Prof. Dr. Kenichi Fujise from The University of Texas Medical Branch, Texas, USA.

3.2.3 Animals

Male apoE knockout mice, aged 10-12 weeks, were obtained from the Jackson Laboratory (Maine, USA).

3.2.4 Diets

5001®rodent diets were purchased from LabDiet (Indiana, USA). Morelloflavone incorporated diets were prepared by mixing 1.5 g of morelloflavone with 1 kg of 5001 rodent diets and manufacturer by Research Diets, Inc. (New Jersy, USA).

3.3 Methods

3.3.1 Preparation of morelloflavone

Dried *Garcinia dulcis* leaves were finely powdered and extracted with acetone. After removal of the insoluble matter by filtration, the filtrate was concentrated in vacuo. A second extraction was achieved with hexane and the hexane-insoluble fraction was subsequently extracted with dichloromethane. The obtained greenish-yellow residue from dichloromethane-insoluble fraction was subjected to quick-column chromatography on silica 60H and eluted with dichloromethane-acetone in a polarity gradient manner. The eluted fractions were combined on the basis of their thin-layer chromatography (TLC) behavior. Finally, the purified compound was concentrated in vacuo, dried and ground. TLC was used to confirm desirable fraction every steps of extraction and purification. The purity of this compound was determined by using a high-performance liquid chromatography (HPLC) system (Agilent 1100 Series, Germany), equipped with a solvent delivery pump (BinPump G1312A), an autosampler (ALS G1313A), photodiode-array detector (DAD G1315B) and data output (LC Chemstation, Rev. A.10.02). An ODS-2 column (5 mm particle size, 4.6 x 2 50 mm i.d.; InertsilTM, Shimadzu, Japan) was used. The mobile phase consisting of 45% (v/v) acetonitrile and 55% (v/v) of 1% acetic acid was pumped at a flow rate of 1 ml/min. The effluent was monitored at 289 nm. Morelloflavone in the sample was identified by comparing its spectral data with that of a standard, previously purified from *G. dulcis* flowers (Dechathai *et al.*, 2006).

3.3.2 Cell culture

Mouse vascular smooth muscle cells (VSMCs) were used between passages 4 and 9. The cells were routinely maintained in 231 media (Cascade Biologics, USA) supplemented with smooth muscle cell growth supplement (Cascade Biologics, USA) in a humidified incubator at 37°C with 5% CO_2. All experiments were performed in subconfluent, unsynchronized cells growing in smooth muscle growth supplement (SMGS) except for the lamellipodium formation assay.

3.3.3 Cell cycle analyses

One million each of VSMCs were seeded on 10-cm dishes and incubated overnight. Next day the cells were subjected to various concentrations of morelloflavone and incubated for 24 h. After 24 h of incubation, the cells were harvested, washed twice with PBS, fixed with 70% ethanol and incubated at -20°C overnight. After centrifugation, the pellets were stained with 25 μg/mL of propidium iodide (Sigma, USA) and 20 μg/mL of RNase in PBS, incubated at 37°C for 15 min, and then subjected to flow cytometric DNA content analysis using Epics XL (Beckman-Coulter, USA). Percentages of G1, S, and G2/M phases were determined using Multi-cycle system software (Phoenix Flow System, USA).

3.3.4 BrdU incorporation assay

VSMCs were seeded at 2 x 10^4 cells per well in 96-well culture plates and incubated overnight. Cell cycle assay was achieved by using BrdU Cell Proliferation Assay kit (Calbiochem, USA). After exposure to morelloflavone at final concentrations of 0 to 100 μM for 2 h, 1 μM of BrdU was added into the medium and incubated for 8 h in a humidified incubator at 37°C with 5% CO_2. Then the cells were fixed with the Fixative/Denaturing Solution for 30 min and incubated with anti-BrdU monoclonal antibody for 1 h at room temperature, respectively. After unbound antibody was washed away, horseradish peroxidase-conjugated goat anti-mouse was added and incubated for 30 min at room temperature. Finally, tetra-methylbenzidine substrate was added, incubated in the dark at room temperature for 15 min, and Stop Solution was added, respectively. The colored reaction product was quantified using

a spectrophotometric plate reader at dual wavelengths of 450 and 540 nm. Protein content was analyzed using the Bradford assay (Bio-Rad, USA).

3.3.5 MTT cell death assay

VSMCs were plated at 1 x 10^4 cells per well in 96-well culture plates and incubated overnight. The cells were treated with various concentrations of morelloflavone and incubated for 48 h in a humidified atmosphere. The MTT labeling reagent was then added to each well and the plate was incubated for 4 h. After the incubation period, the solubilization solution (10% SDS in 0.01 M HCl) was added and the plate was allowed to stand overnight. Finally, the formed formazan was measured using a microplate reader at 600 nm with the reference wavelength of 700 nm.

3.3.6 DNA fragmentation assay

VSMCs were seeded at 1 x 10^5 cells per well in 24-well culture plates and incubated overnight. DNA fragmentation assay was achieved by using Cell Death Detection ELISA^{PLUS} (Roche, USA). Briefly, the cells were incubated in the presence of increasing concentrations (0-100 μM) of morelloflavone. After 24 h of incubation, the cells were harvested and counted. Then 1 x 10^5 cells of each morelloflavone treated concentration were lysed and incubated for 30 min at room temperature. The lysate was subsequently centrifuged at 200 g for 10 min. The supernatants were then carefully transferred into the streptavidin coated microplate. The immunoreagent, anti-histone antibody conjugated to biotin and anti-nucleosomal-DNA-antibody-conjugated to horse radish peroxidase, was added to each well and incubated under gently shaking for 2 h at room temperature. After washing 3 times, the 2,2'-azino-bis-[3-ethylbenzthiazoline-6-sulfonic acid] (ABTS) substrate solution was added and the plate was incubated on a plate shaker at 250 rpm. When the color development is sufficient for a photometric analysis, the ABTS Stop Solution was added. Finally, the plate was measured the spectrophotometrical absorbance at 405 nm against ABTS solution with the reference wavelength of 490 nm.

3.3.7 Migration assay

VSMCs that had been grown to confluence in 6-well culture plates were scratched with a sterile 1000-µl pipette tip and exposed to morelloflavone at various concentrations. The cells were allowed to migrate into the plastic surface for 18 h and photographed. A migration index was determined at the number of cells migrated into 1 mm^2 of free plastic surface, using the Image J software (NIH, USA).

3.3.8 Invasion assay

The lower chambers of ChemoTx® Disposable Chemotaxis System (NeuroProbe, USA) were filled with 29 µl of smooth muscle cell growth supplement (SMGS) diluted in 231 medium at the appropriate concentrations (0-10%). A filter plate (5 µm pore size) was positioned over the lower wells, and 1 x 10^4 VSMCs suspended in 25 µL of 231 medium were placed on the top of the filter plate. Cells were allowed to attach for 4 h. After 4 h of incubation, the droplets on the upper surface were removed, replaced with 25 µl of 231 media containing 1 µM of morelloflavone or vehicle (0.1% DMSO) and incubated for additional 8 h to allow cells to migrate through the membrane. After 8 h of incubation, the cells that had not migrated were scraped off from the upper surface by Q-tips. Cells that had migrated onto the opposite side were fixed with 4% paraformaldehyde for 30 min, permeabilized with 70% methanol for 20 sec, and stained with hematoxylin solution for 15 min, followed by three washes with tap water. Stained cells were counted and migration indices were calculated at the number of cells migrated per 8.0 (mm^2) test site surface area.

3.3.9 Attachment assay

The lower chambers of ChemoTx® Disposable Chemotaxis System (NeuroProbe, USA) were filled with 29 µl of smooth muscle cell growth supplement (SMGS) diluted in 231 medium at concentration of 5%. A filter plate (5 µm pore size) was positioned over the lower wells, and 1 x 10^4 VSMCs suspended in 25 µL of 231 medium containing 1 µM of morelloflavone or vehicle (0.1% DMSO) were placed on the top of the filter plate. Cells were allowed to attach for 4 h. Cells that

had attached on the upper surface were fixed with 4% paraformaldehyde for 30 min, permeabilized with 70% methanol for 20 sec, and stained with hematoxylin solution for 15 min, followed by three washes with tap water. Stained cells were counted and attachment indices were calculated at the number of cells migrated per 8.0 (mm^2) test site surface area.

3.3.10 Lamillipodium formation assay

Lamellipodium formation assay was performed as described previously (Liu *et al.*, 2006). In brief, mouse VSMCs were serum starved for 24 h and then seeded onto fibronectin-coated wells of chamber slides (CultureSlide, BD BioCoat Fibronectin, USA) and incubated with various concentrations of morelloflavone (0, 1, and 10 μM) in the presence or absence of serum for 3 h at 37°C. After incubation, cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, stained with Alexa Fluor 568-Phalloidin (Invitrogen-Molecular Probes, USA) and DRAQ5 (Biostatus, Ltd, Leicestershire, UK) before they were observed by Leica DM6000 confocal microscope (Leica, USA).

3.3.11 Western blot analysis

For the evaluation of phosphorylated FAK and c-Src, western blot analyses were performed as described previously (Guo *et al.*, 2003). Briefly, VSMCs were seeded on 10-cm dishes, treated with various concentrations of morelloflavone for 5 min and then harvested into RIPA buffer with protease inhibitor cocktails. Cleared cell lysates (500 μg of protein) were incubated with anti-FAK or anti-c-Src antibodies at 4°C for 1 h in a final volume of 1 mL RIPA buffer, and incubated for another 1 h with protein A/G agarose beads (Santa Cruz ; SCBT sc-2003). Immunoprecipitated proteins were eluted into SDS-loading buffer and subjected to 12% SDS-PAGE and immunoblotted using anti-FAK (Santa Cruz; A-17; sc-557) and anti-c-Src (Santa Cruz; SRC-2; sc-18) antibodies for total FAK and c-Src and anti-phosphotyrosine antibody (Santa Cruz; PY-20; sc-508) for phosphorylated FAK and c-Src. Densitometric analyses were performed using Adobe Photoshop (Adobe Systems Incorporated, USA).

To assess the phosphorlyation status of ERK, VSMCs were seeded on 10-cm dishes, treated with various concentrations of morelloflavone for 5 min, and harvested into RIPA buffer with protease inhibitor cocktails. The whole cell lysates (30 μg of protein) were loaded on SDS-PAGE and then performed the western blot using anti-phosphorylated ERK (Santa Cruz; E-4; sc-7383), and anti-ERK (Santa Cruz; K-23; sc-94). Densitometric analyses were performed using Adobe Photoshop (Adobe Systems Incorporated, USA).

To detect active RhoA, Rac1 and Cdc42, GST-tagged RhoA binding domain of Rhotekin protein (GST-Rhotekin-RBD) and GST-tagged p21-binding domain of p21-activated kinase 1 (PAK1)(GST-PAK1-PBD) were generated. Briefly, *E. Coli* BL21 cells transformed with pGEX4T-PAK1-PBD or pGEX4T-Rhotekin-RBD were grown at 37°C. The expression of recombinant protein was induced by addition of 0.1 mM IPTG for 3 h. Cells were resuspended in lysis buffer (50mM Tris-HCl, pH8.0, 10% glycerol, 20% sucrose, 2 mM DTT, 1 ug/ml leupeptin, 1 ug/ml pepstatin, and 1 ug/ml aprotinin), sonicated, and centrifuged at 4°C for 30 min at 45,000 g. The supernatant was incubated with glutathione sepharose 4B beads (GE-Amersham, Piscataway, NJ) for 1 h at 4°C, and then washed three times in lysis buffer. GTPase activation assays was performed by either GST-Rhotekin-RBD- or GST-PAK1-PBD- pull-down assays as described previously (Sander *et al.*, 1998). Briefly, VSMCs were seeded on 10-cm dishes, treated with various concentrations of morelloflavone for 5 min and then harvested in resuspended in lysis buffer (50 mM Tris, pH8.0, 500 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 10% glycerol, 10 mM $MgCl_2$, 10 ug/ml leupeptin and aprotinin, and 1 mM phenylmethylsulfornyl fluride) were cleared, and then the supernatants containing approximately 500 μg of protein were incubated with 5 μg of recombinant GST-Rhotekin-RBD or GST-PAK1-PBD, both conjugated to agarose beads, for 1 h at 4°C, washed with lysis buffer, and eluted into SDS-loading buffer. Eluents were size-fractionated by SDS-PAGE, transferred to nitrocellulose membranes, and probed by anti-RhoA, anti-Rac1, and anti-Cdc42 antibodies. Densitometric analyses were performed using Adobe Photoshop (Adobe Systems Incorporated, USA). These experiments were performed three times with the same results.

3.3.12 Mouse carotid artery injury assay

Mouse carotid artery injury assay was performed as described previously by Kuhel and others (Kuhel *et al.*, 2002). Male apoE-/- mice were obtained from the Jackson Laboratory (Maine, USA) and were maintained on a 12-h light/dark cycle. Each mice were fed with either normal rodent chow diet (5001, LabDiet, USA) (N=10) or normal chow diet containing 0.15% morelloflavone (w/w) (N=9). This corresponded to 200 mg/kg morelloflavone if 30 gram animals consume 4 gram of chow. All animal experimentation protocols were performed under the institutional guidelines of animal welfare, in accordance with National Institutes of Health guidelines. The animals were placed on these diets for 7 days before they underwent carotid artery denudation by the insertion of an epoxy resin (Epon) probe as described previously (Kuhel *et al.*, 2002). Briefly, the entire length of the left carotid artery was exposed and the distal bifurcation of the carotid artery was looped proximally and ligated distally with 7-0 suture. A transverse arteriotomy was made between the 7-0 silk sutures and the resin probe was inserted, advanced toward the aortic arch, and withdrawn 5 times. The probe was then removed, the proximal 7-0 suture was ligated, a 6-0 suture was secured, and the incision was closed with 5-0 sterile surgical gut. After surgery, mice were maintained on these diets for 14 days before they were euthanized. After blood was sampled, the arteries of these mice were perfusion-fixed with 10% buffered formalin (pH 7.0) solution at a constant pressure of 100 mmHg. The entire neck from each mouse was dissected, fixed further in 10% buffered formalin, decalcified, and then embedded in paraffin. Identical whole neck cross sections of 5 μm were made from the distal side of the neck beginning at the point of the distal 7-0 ligature. Whole neck sections were used to evaluate both the injured and the uninjured control vessels on the same section. For each mouse, the 4 levels of serial sections were taken at 500-μm intervals. These sections were stained by Verhoeff-van Gieson (VVG) staining and subjected to morphometric analyses. Images were digitized and captured using a Sony video connected to a personal computer. Measurements were performed at a magnification of 200 using a Scion Image analysis computer program (Frederick, MD). Data were obtained from the first 2 levels where endothelial denudation occurred. For each artery, the luminal

area, area inside the internal elastic lamina (IELA), and the area encircled by external elastic lamina-IELA were measured. The intimal area (IA) was calculated as the IELA minus luminal area.

For the determination of serum morelloflavone levels, animal sera were first digested by proteinase K in the presence of 0.01% SDS. Morelloflavone was then extracted into ethyl acetate and lyophilized. The quantification of morelloflavone was performed by HPLC as described in the "preparation of morelloflavone" sub-section of Materials and Methods with following modifications. The pure morelloflavone was diluted to vary concentrations (0 -100 μM), aliquoted into multiple tubes, and lyophilized. The samples were dissolved in 200 μL of 25% acetonitrile/0.55% acetic acid of which 100 μL were injected into a Vydac C-18 reversed phase HPLC column (Grace Davison Discovery Sciences, Deerfield, IL) in an Agilent 1100 HPLC system (San Jose, CA). Morelloflavone was eluted from the column by a 25-55% acetonitrile/0.55% linear gradient with a flow rate of 1 ml/min with UV monitoring at 289 nm. The peaks were integrated and the signal to noise value was obtained from the HPLC software, using the baseline appearing after the morelloflavone peak to calculate noise. The limit of detection (LOD) is defined as the concentration of morelloflavone that yielded a signal to noise of 2:1, and was calculated at 1.06 μM, from the 20 μM standard peak (signal to noise = 37.6:1). None of samples from control animals contained morelloflavone concentrations higher than the limit of detection.

For the analyses of intimal cell proliferation and apoptosis, Ki-67 and Terminal deoxynucleotidyl transferase (TdT)-deoxyuridine nick-end labeling (TUNEL) staining, respectively, were performed. Ki67 was detected using a monoclonal rabbit antibody (Clone TEC-3, DAKO North America, Inc., Carpinteria, CA) as described previously (Ihling *et al.*, 1997; Ihling *et al.*, 1998). Briefly, after the quenching of endogenous peroxidase with 1% H_2O_2 for 30 min, serial sections were incubated with 0.5% normal bovine serum to reduce nonspecific background staining. Thereafter, the slides were incubated with monoclonal antibodies directed against Ki-67. Antigens were either unmasked by pressure cooking in 10 μmol citric acid, pH 6, for 3 min. All slides were then incubated with biotinylated secondary antibody at

room temperature, followed by incubation with avidin and biotinylated horseradish peroxidase complex (ABC method, Vector Labs). Peroxidase activity was visualized by Diaminobenzidine (DAB) to yield a brown reaction product. The nuclei were slightly counterstained with hematoxylin. Terminal deoxynucleotidyl transferase (TdT) deoxyuridine nick-end labeling (TUNEL) staining (Surh and Sprent, 1994) was performed using a FragEL™ DNA fragmentation detection kit (Oncogene Research Products, Boston, MA) according to the manufacturer's instructions. In this assay terminal deoxynucleotidyl transferase (TdT) binds to exposed 3'-OH ends of DNA fragments generated in response to apoptotic signals and catalyzes the addition of biotin-labeled and unlabeled deoxynucleotides. Biotinylated nucleotides are detected using a streptavidin-horseradish peroxidase (HRP) conjugate. Diaminobenzidine (DAB) reacts with the labeled sample to generate an insoluble colored substrate at the site of DNA fragmentation. Counterstaining with hematoxylin aids in the morphological evaluation and characterization of normal and apoptotic cells. The Ki-67 and apoptotic indices, defined as the number of cells with DAB positive nuclei divided by the total number of cells counted and expressed as a percentage, were then calculated. All cells within the intima were counted.

3.3.13 Statistical analysis

Values are expressed as means ± SD. Comparisons of parameters between two groups were made with Student's t test. When appropriate, ANOVA was performed to compare multiple groups. A value of $P < 0.05$ was considered statistically significant.

3.4 Results

3.4.1 Isolation of morelloflavone

Morelloflavone was purified from the leaves of *Garcinia dulcis* as described in Materials and Methods, and subjected to HPLC analysis. As shown in Fig. 8, HPLC analyses revealed the morelloflavone preparation to be 94% pure.

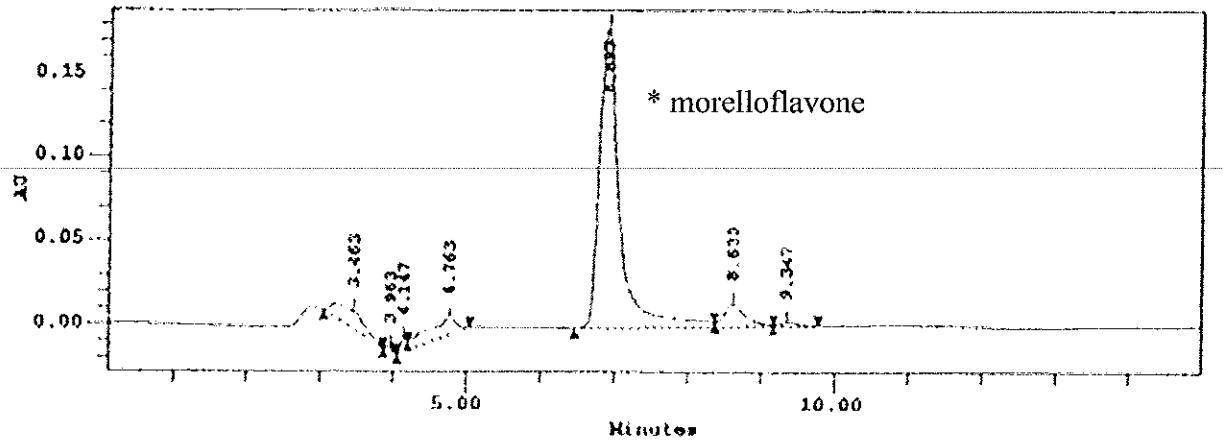


Figure 8. Purification of morelloflavone from the leaves of *Garcinia dulcis*. High pressure liquid chromatography (HPLC) of morelloflavone demonstrates that the purity of the current preparation is 93.4%. Morelloflavone was purified from leaves of *Garcinia dulcis*, using established chemical extraction and column-based methods.

3.4.2 Effect of morelloflavone on cell cycle progression of VSMCs.

In order to test whether morelloflavone affects VSMCs cell cycle progression, VSMCs were treated with 0-100 μM morelloflavone and subjected them to flow cytometric analysis. As shown in Fig. 9-10, morelloflavone did not significantly change the cell cycle progression up to 10 μM, although it blocked G2/M→G1 progression at 100 μM. Consistently, BrdU incorporation assay showed that morelloflavone did not affect DNA synthesis at concentrations up to 10 μM, while it significantly reduced DNA synthesis at 100 μM ($P < 0.001$) (Fig. 11). These data suggest that morelloflavone does not affect cell cycle progression or DNA synthesis of VSMCs, except at very high concentration.

3.4.3 Effect of morelloflavone on proliferation and death in VSMCs.

In order to test if morelloflavone affects the viability of VSMCs, cells were treated with various concentrations of morelloflavone and subjected them first to MTT assay. As shown in Fig. 12, morelloflavone failed to show any cytotoxicity at

concentration between 0 and 10 μM. In order to determine whether morelloflavone would induce apoptosis in VSMCs, we performed a standard DNA fragmentation assay on VSMCs challenged by 0, 1, 10, and 100 μM morelloflavone. Intriguingly, morelloflavone did not induce DNA fragmentation in VSMCs but rather reduced it in a dose dependent fashion (P = 0.032) (Fig. 13), suggesting that morelloflavone does not cause VSMC apoptosis and that the reduced survival seen at 100 μM (Fig. 12) was due to necrosis, not apoptosis.

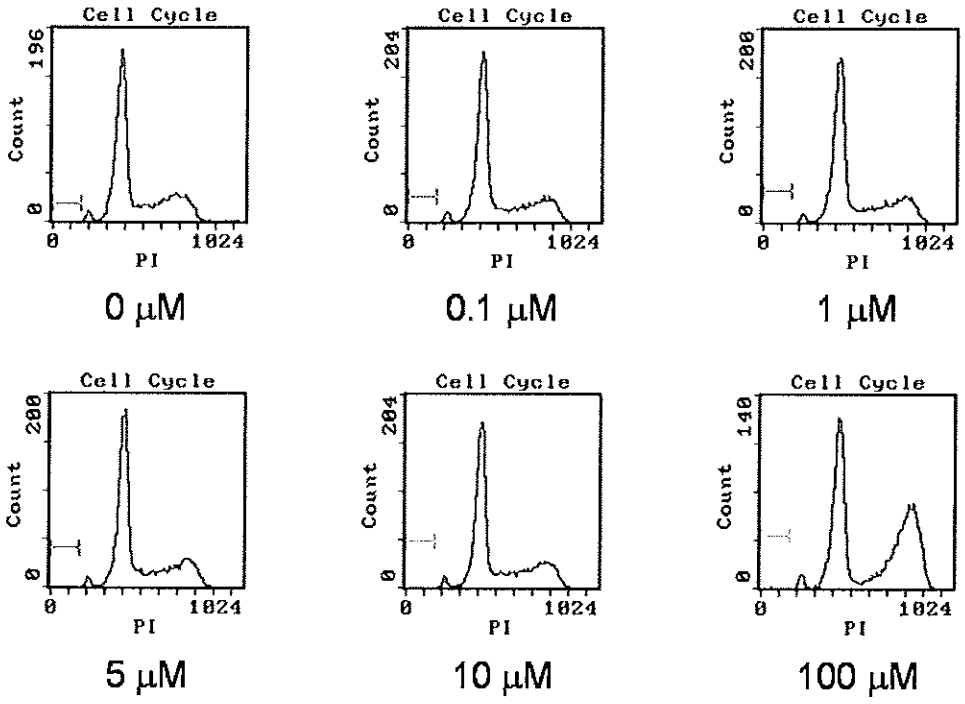


Figure 9. Histogram of flow cytometry. Flow cytometric analysis of VSMCs treated with various concentrations (0-100 μM) morelloflavone. Subconfluent VSMCs were treated with 0 – 100 μM morelloflavone for 24 h and subjected to flow cytometric determination of DNA contents..

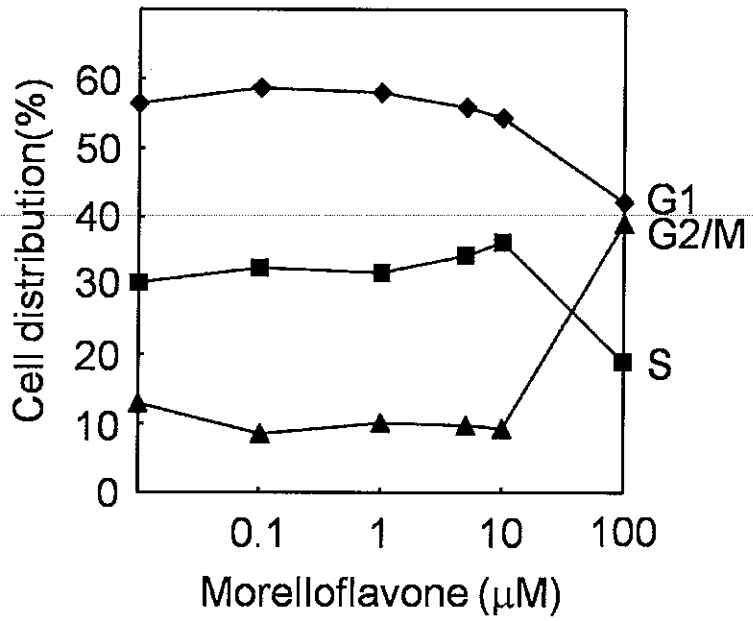


Figure 10. Percentages of G1, S, and G2/M cells. Flow cytometric analysis of VSMCs treated with various concentrations (0-100 μM) morelloflavone. Subconfluent VSMCs were treated with 0 – 100 μM morelloflavone for 24 h and subjected to flow cytometric determination of DNA contents.

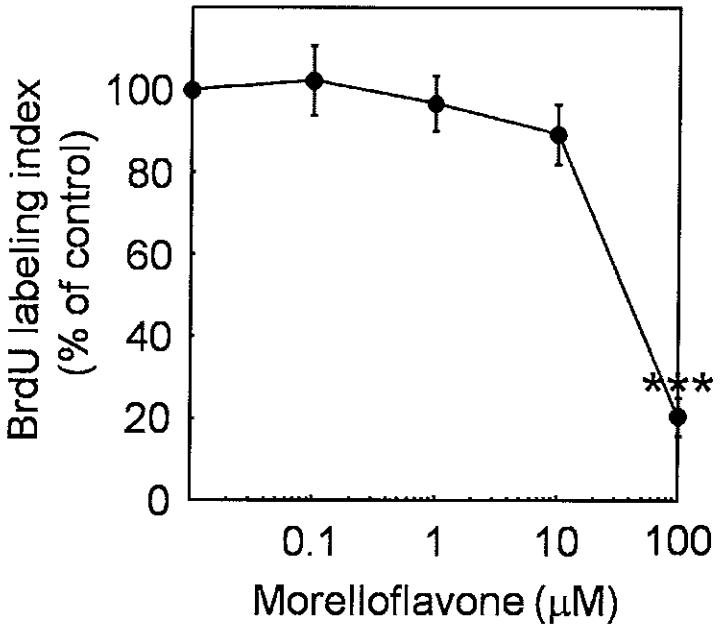


Figure 11. BrdU assay. BrdU assay of VSMCs treated with various concentrations (0-100 μM) of morelloflavone. ***, $P < 0.001$ by ANOVA ($N = 4$).

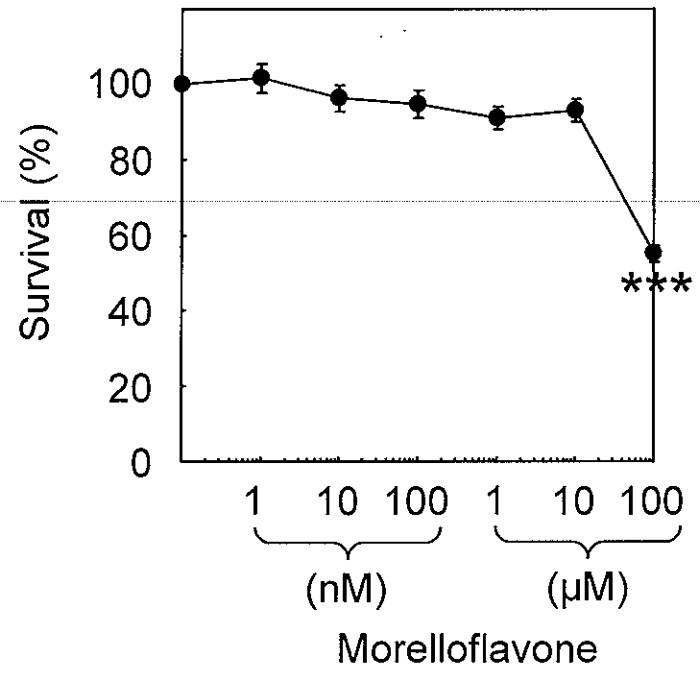


Figure 12. MTT assay. VSMCs were treated with various concentrations (0 – 100 μM) of morelloflavone and incubated for 48 h. Viability of cells was determined using MTT as described in Methods. ***, $P < 0.001$ by ANOVA ($N = 4$).

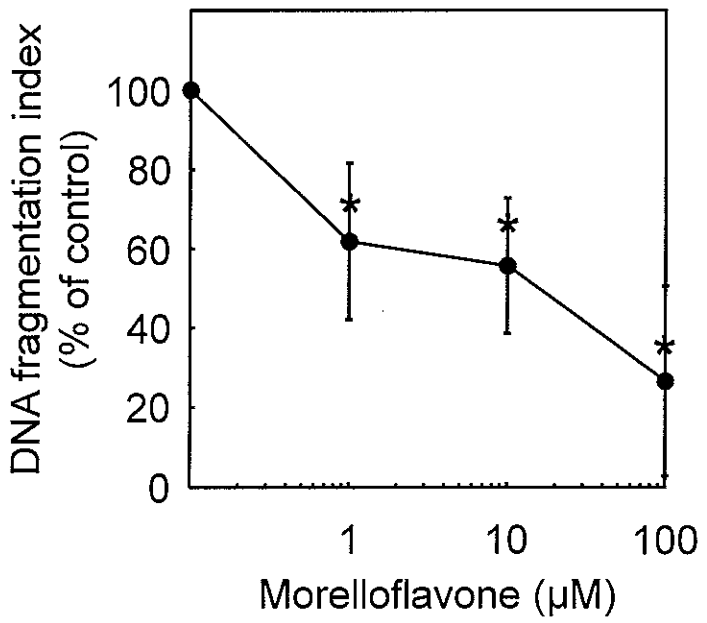


Figure 13. DNA fragmentation assay. VSMCs were exposed for 24 h at 37°C to different concentrations of morelloflavone. After lysis, cells were centrifuged and supernatant was analyzed in ELISA as described in Methods. *, $P < 0.032$ ($n = 2$).

3.4.4 Effect of morelloflavone on migration of VSMCs

In order to determine whether morelloflavone plays a role in the regulation of VSMC migration, a standard scratch assay was performed. VSMCs were grown to confluency, scratched, and allowed to migrate into the scratched area in the presence of 0, 1, 10, and 100 μM morelloflavone for the next 18 h. As shown in Fig. 14-15, the migration of VSMCs was inhibited in a dose dependent fashion (migration indices at 0, 1, 10 and 100 μM morelloflavone = 568.7 ± 33.5, 487.6 ± 36.9, 411.3 ± 39.5, and 191.86 ± 32.8 [cells/mm^2], respectively; $P < 0.001$ by one-way ANOVA). In order to evaluate the effect of morelloflavone on VSMCs invasion, a modified Boyden chamber assay was performed. VSMCs were placed on upper surface of the porous membrane which was placed on the media containing various concentrations of VSMCs growth supplement (SMGS) to allow them to attach for 4 h. After 4 h of incubation, medium containing 1 μM morelloflavone or vehicle were replaced and incubated for additional 8 h. Cells were allowed to migrate through 5μm pores for 8 h. As shown in Fig. 16-17, 0% of SMGS did not cause VSMCs to migrate in the presence or absence of morelloflavone. 1 μM morelloflavone significantly blocked VSMCs migration at 2.5 and 5.0% SMGS (morelloflavone vs. control = 252.7 ± 31.7 vs. 372.0 ± 50.9 [cells] at 2.5%; 273.0 ± 7.1 vs. 444.5 ± 50.2 [cells] at 5.0%, both $P < 0.05$), while the inhibitory effect of morelloflavone on migration at 10% was not significantly different from control (morelloflavone vs. control = 390.0 ± 82.0 vs. 450.5 ± 24.8 [cells], NS). Taken together with the data presented in Fig. 3.3A-D, these data suggest that morelloflavone is a potent inhibitor of VSMCs migration and invasion.

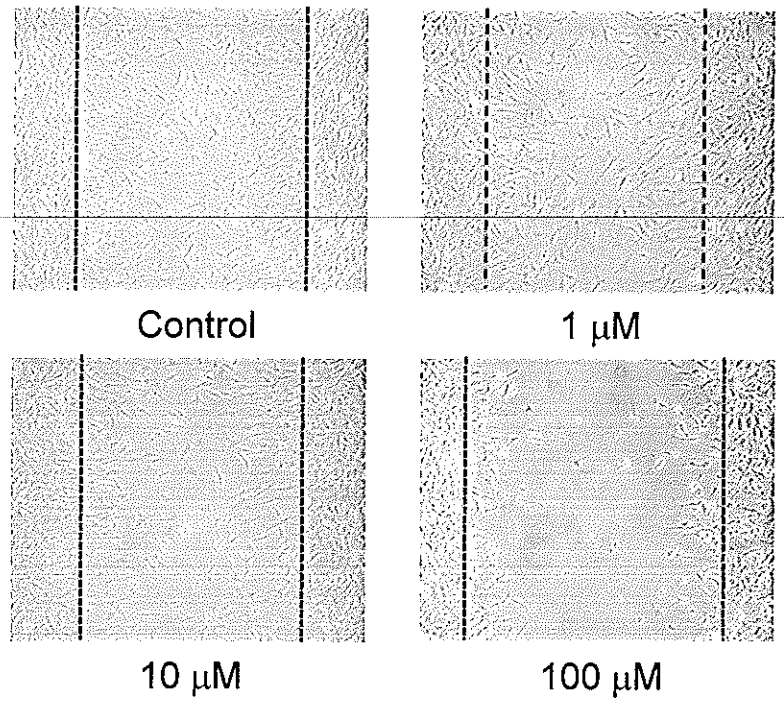


Figure 14. Photomicrograms of migration patterns of VSMCs. Migration patterns of VSMCs in the presence of various concentrations (0-100 μM) of morelloflavone.

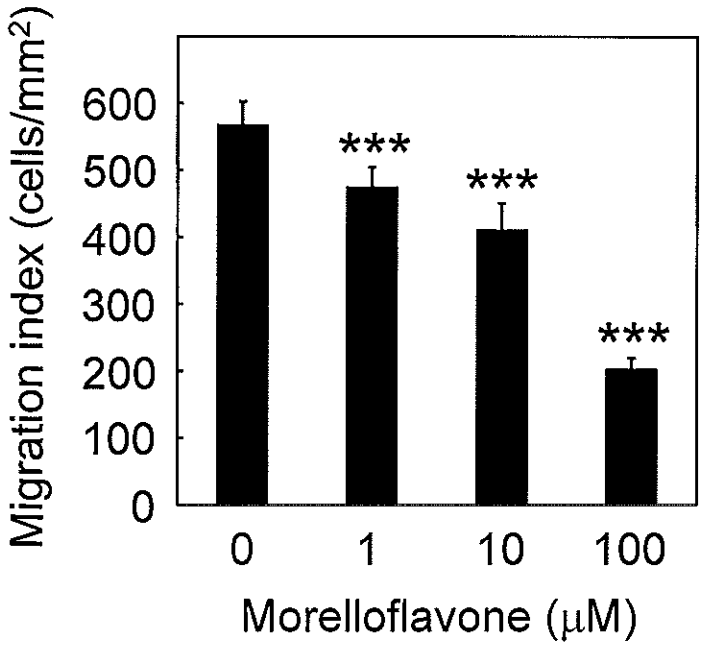


Figure 15. Migration index in scratch assay. Migration index calculated at migrated cells per unit area (cells/mm^2). ***, $P < 0.001$ by ANOVA ($N = 5$).

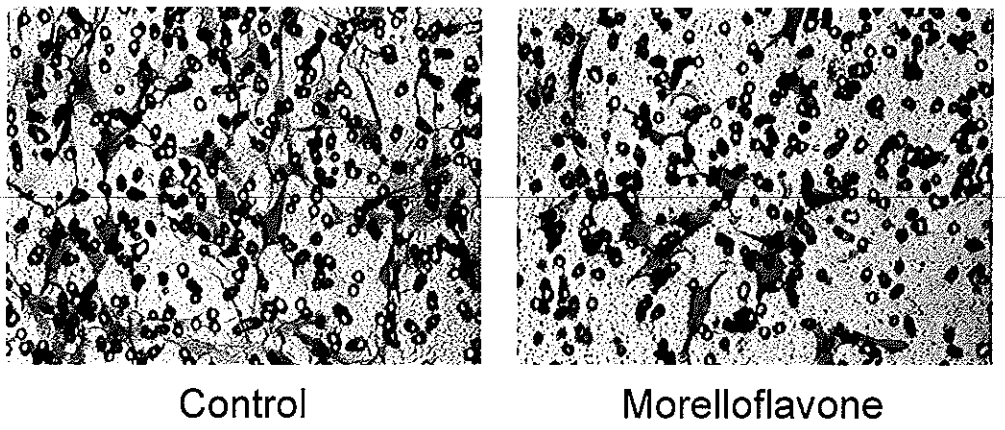


Figure 16. Photomicrograms of invasion patterns of VSMCs. Patterns of VSMCs in the presence and absence of morelloflavone (1 μM) using a modified Boyden chamber system (ChemoTx® Disposable Chemotaxis System, pore size = 5 μm, NeuroProbe, Gaithersburn, MD).

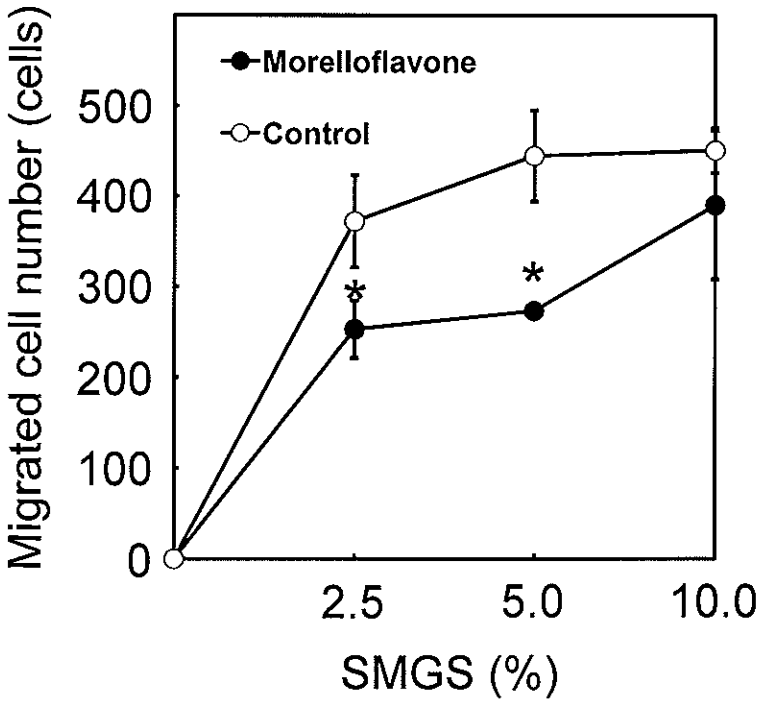


Figure 17. Migrated cell number in modified Boyden chamber. Migrated cell number representing the total cell numbers on each test sites (8.0 [mm^2]). *, $P < 0.05$ by ANOVA (N = 3).

3.4.5 Effect of morelloflavone on attachment of VSMCs

In order to evaluate the effect of morelloflavone on VSMCs attachment, a modified Boyden chamber assay was performed. VSMCs suspended in media containing either 1 μM morelloflavone or vehicle were seeded on the upper surface of the porous membrane which was placed on the media containing 5% SMGS. Cells were allowed to attach on the membrane for 4 h. As shown in Fig. 18-19, morelloflavone at any concentrations did not inhibit VSMCs attachment at 5.0% SMGS (attachment cells at 0, 1, 2, 4 and 8 μM morelloflavone = 81 ± 6, 86 ± 9, 76 ± 14, 78 ± 17, and 82 ± 13 [cells], NS). These data suggest that morelloflavone does not inhibit the attachment of VSMCs.

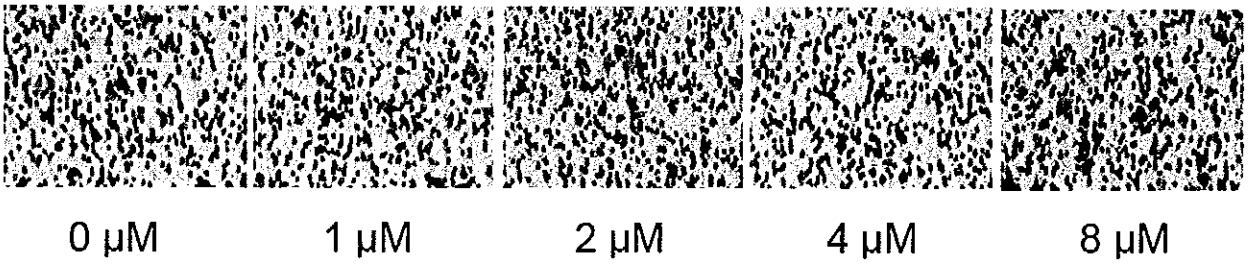


Figure 18. Photomicrograms of attachment patterns of VSMCs. Patterns of VSMCs various concentrations of morelloflavone using a modified Boyden chamber system (NeuroProbe, Gaithersburn, MD).

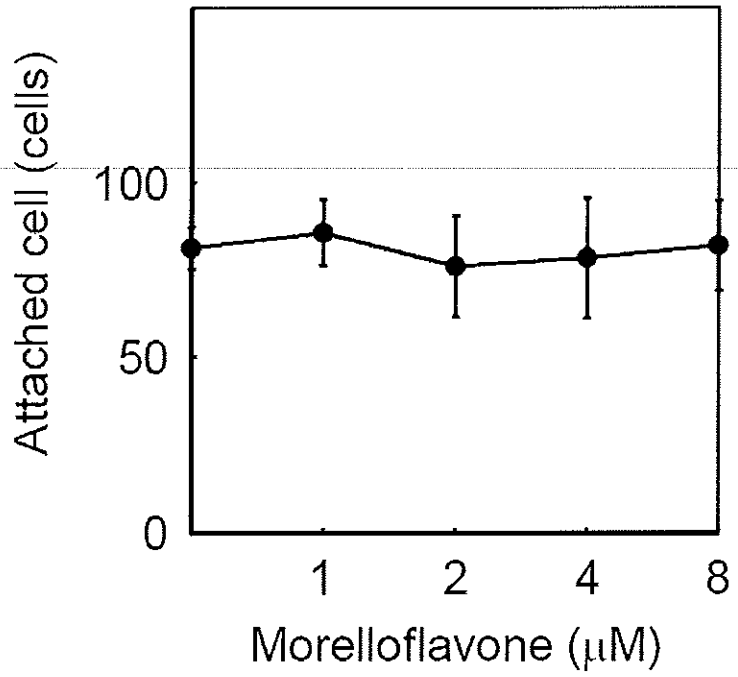


Figure 19. Attached cell in a modified Boyden chamber. Attached cell number representing the total cell numbers on each test sites (8.0 [mm^2]). NS, (N = 3).

3.4.6 Effect of morelloflavone on the formation of lamellipodia in VSMCs.

In order to determine whether morelloflavone affects the formation of the VSMCs migratory apparatus—lamellipodia (Small *et al.*, 2002; Liu *et al.*, 2006), VSMCs were subjected to serum starvation for 24 h. VSMCs were then seeded onto fibronectin-coated wells of chamber slides and incubated these cells in the presence and absence of sera with various concentrations (0, 1, and 10 μM) of morelloflavone. After 3 h of incubation, cells were stained with Alexa Fluor 568-Phalloidin and DRAQ5 and examined by a confocal microscope. In the absence of sera, morelloflavone did not change the morphology of VSMCs regardless of its concentrations (Fig. 20). In the absence of morelloflavone, the number of lamelliopodia statistically significantly increased upon serum stimulation (Fig. 20 Serum[-] and [+] at morelloflavone 0 μM, Fig. 21 columns 1 and 2; serum (-) vs. (+) = 0.105 ± 0.006 vs. 0.05 ± 0.01, $P < 0.005$). In this system, morelloflavone

significantly decreased lamellipodium indices in a dose-dependent fashion (Fig. 21, 0, 1, and 10 μM = 0.105 ± 0.006, 0.05 ± 0.001, 0.033 ± 0.006, $P < 0.001$ by ANOVA).

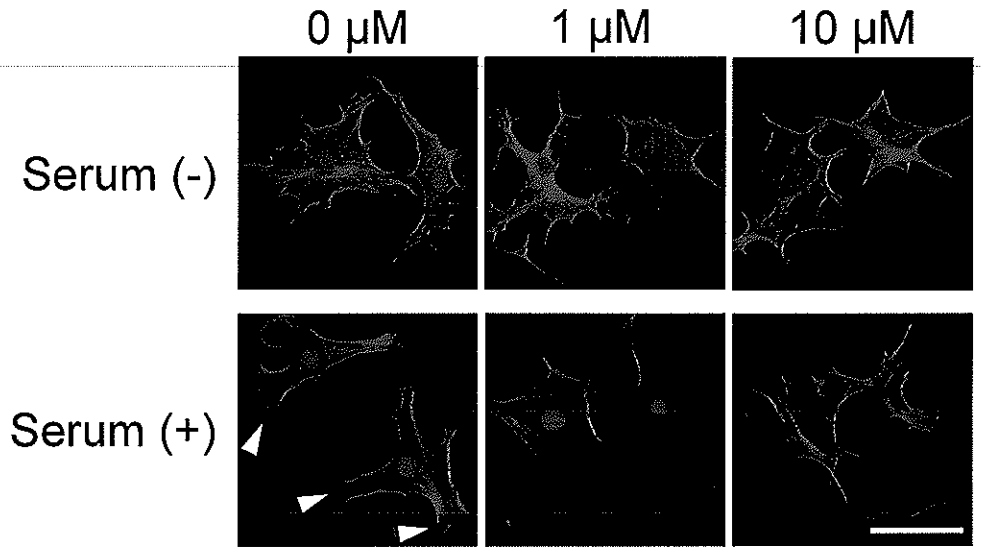


Figure 20. Confocal microscope of VSMCs in lamellipodia assay. VSMCs were stimulated by sera in the presence of various concentration of morelloflavone (0-10 μM). Arrow, lamellipodia; size bar, 50 μm.

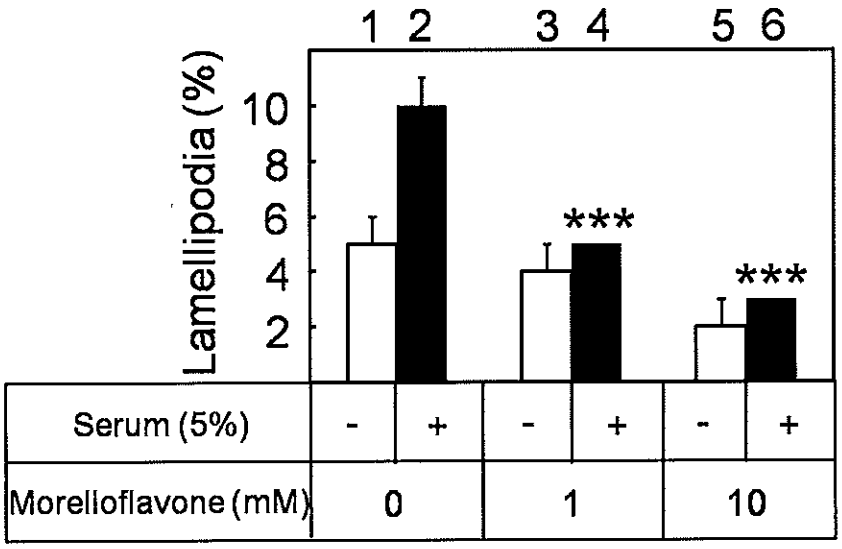


Figure 21. Lamellipodia index. Lamellipodium index is calculated from the number of lamellipodia divided by the total number of cells counted. Open bar, no serum; closed bar, 5% serum. ***, $P < 0.001$ by ANOVA ($n = 3$).

3.4.7 Effect of morelloflavone on the migration-related kinases.

In order to evaluate the pathways involved in morelloflavone-induced inhibition of VSMCs migration, the role of morelloflavone on migration related pathways was systematically evaluated. In order to evaluate the phosphorylation status of focal adhesion kinase (FAK) and c-Src, FAK and c-Src were immunoprecipitated, then performed western blot analyses on the precipitated proteins using anti-FAK, anti-c-Src, and anti-phosphotyrosine antibodies. As shown in Fig. 22-23, morelloflavone inhibited the phosphorylation of FAK and c-Src. Then western blot analysis was performed on lysates from VSMCs treated with various concentrations (0-10 μM) of morelloflavone using anti-ERK and anti-phosphorylated ERK antibodies. As shown in Fig. 24, morelloflavone robustly inhibited the phosphorylation of ERK. Finally, the effects of morelloflavone on the activation status of small GTPases—RhoA, Rac1, and Cdc42 were evaluated. As shown in Fig. 25-27, morelloflavone blocked the activation of RhoA at low concentrations (0.1 and 1μM), and blocked the activation of Cdc42 at higher concentration (10 μM), but it had no significant effects on Rac1. In summary, morelloflavone blocks the activation of FAK, c-Src, ERK, and RhoA (and Cdc42 at a higher concentration) which are key migration-related kinases. These data explaining why morelloflavone can exert such a powerful inhibitory effect on migration.

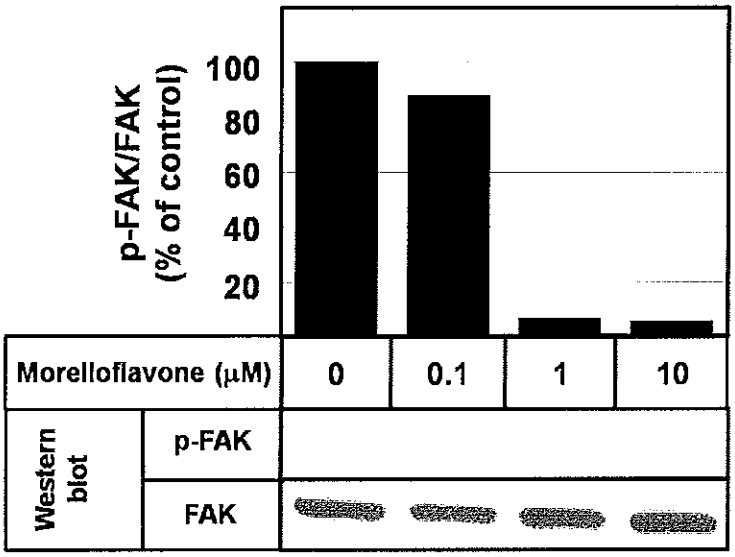


Figure 22. Morelloflavone inhibits phosphorylation of FAK.

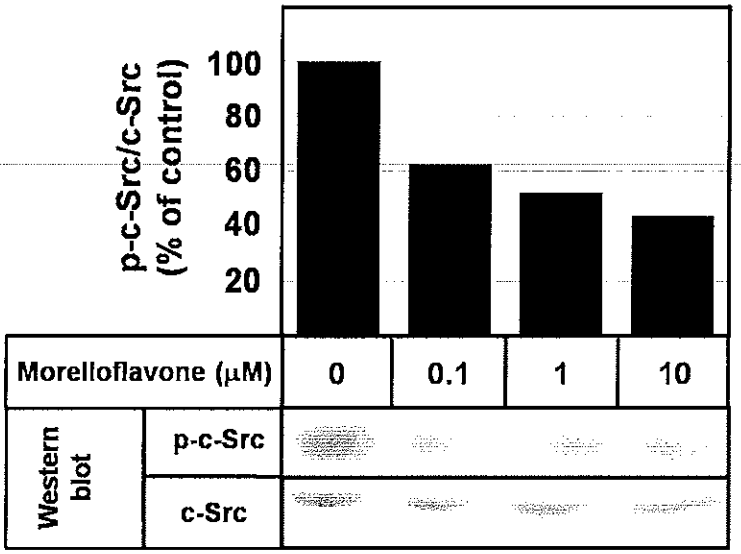


Figure 23. Morelloflavone inhibits phosphorylation of c-Src.

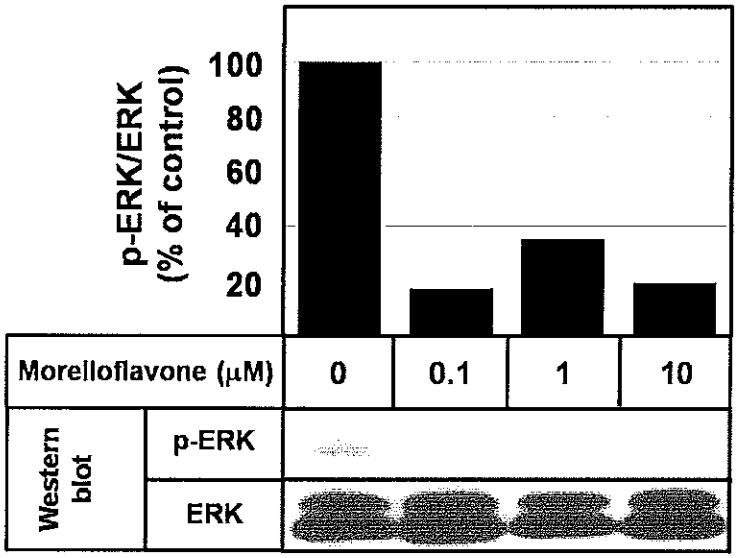


Figure 24. Morelloflavone inhibits phosphorylation of ERK.

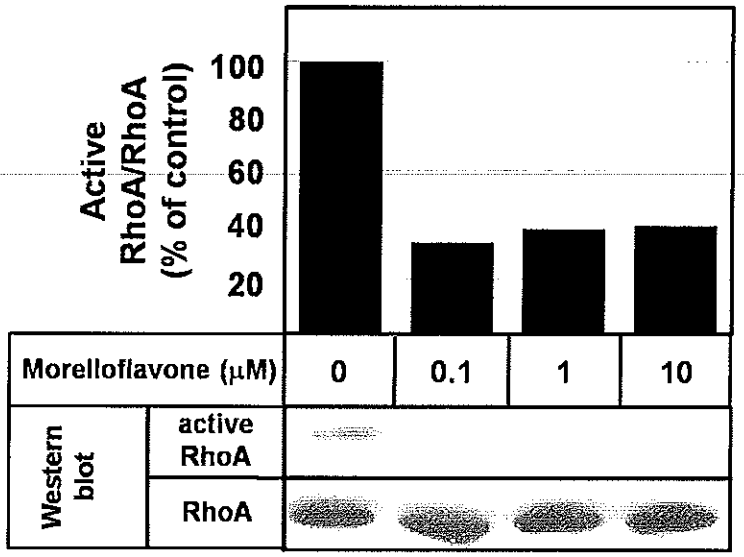


Figure 25. Morelloflavone inhibits activation of RhoA.

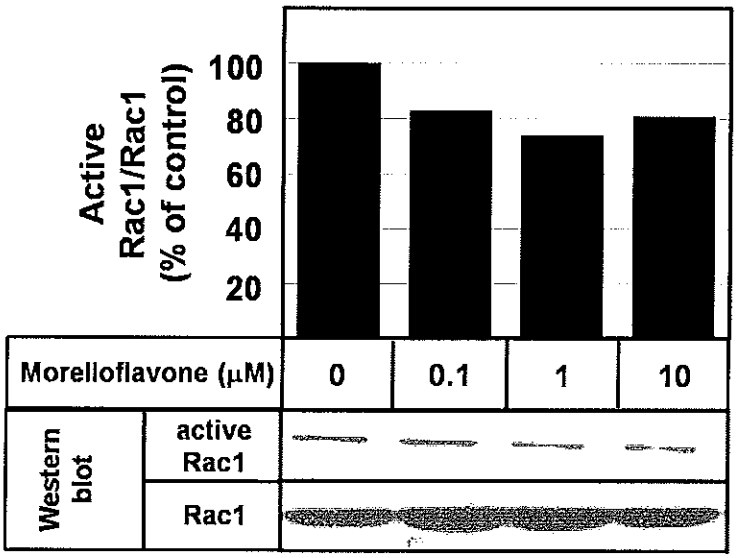


Figure 26. Morelloflavone inhibits activation of Rac1.

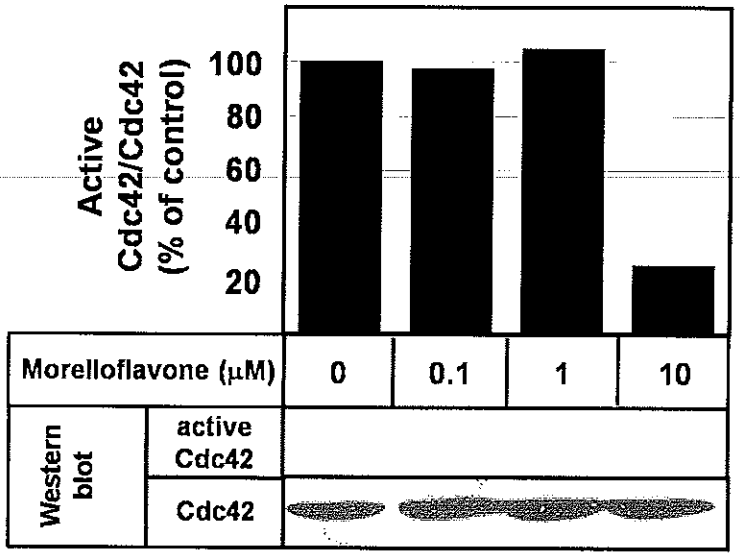


Figure 27. Morelloflavone inhibits activation of Cdc42.

3.4.8 Effect of morelloflavone on neointimal formation in a mouse carotid artery injury model

In order to test whether morelloflavone's inhibitory effects on VSMC migration *in vitro* could be translated *in vivo* to the reduction of injury-induced neointimal formation, Male apoE-/- mice were placed on normal chow or chow containing morelloflavone (0.15% w/w) for 1 week, induced endothelial denudation by the insertion of an epoxy resin (Epon) probe as described previously (Kuhel *et al.*, 2002), and incubated the animals on the same diets for 2 weeks. Animals were then sacrificed to harvest right (uninjured) and left (injured) carotid arteries. Weights of animals were almost the same between just prior to both injury and sacrifice (weights at injury, control vs. morelloflavone = 23.8 ± 2.0 vs. 24.0 ± 2.0 [g], NS; weight at sacrifice, 23.0 ± 1.0 vs. 23.3 ± 1.5 [g], NS). The mean serum concentration of morelloflavone of treated animals was 1.37 ± 0.78 μM. As shown in Fig. 28-29, injured carotid arteries from morelloflavone-treated mice exhibited significantly reduced neointimal formation compared to those from control mice (control vs. morelloflavone = 17,333 ± 13,465 vs. 4,804 ± 5,019 [μm^2], P = 0.0178). Ki67 staining failed to show any difference in Ki-67 indices between control and

morelloflavone groups (control vs. morelloflavone = 0.19 ± 0.34 vs. 0.17 ± 0.41 %, NS, Fig. 30, 32). TUNEL staining showed that there is no difference in TUNEL indices between control and morelloflavone groups (control vs. morelloflavone = 19.9 ± 6.1 vs. 16.0 ± 4.6, NS, Fig. 31, 33). These data, taken together with the data presented in Figs 14-17, suggest that morelloflavone reduced injury-induced neointimal formation by inhibiting VSMCs migration from the media to intima in apoE-/- mice but not by either increasing apoptosis or inhibiting cell proliferation in the neointima.

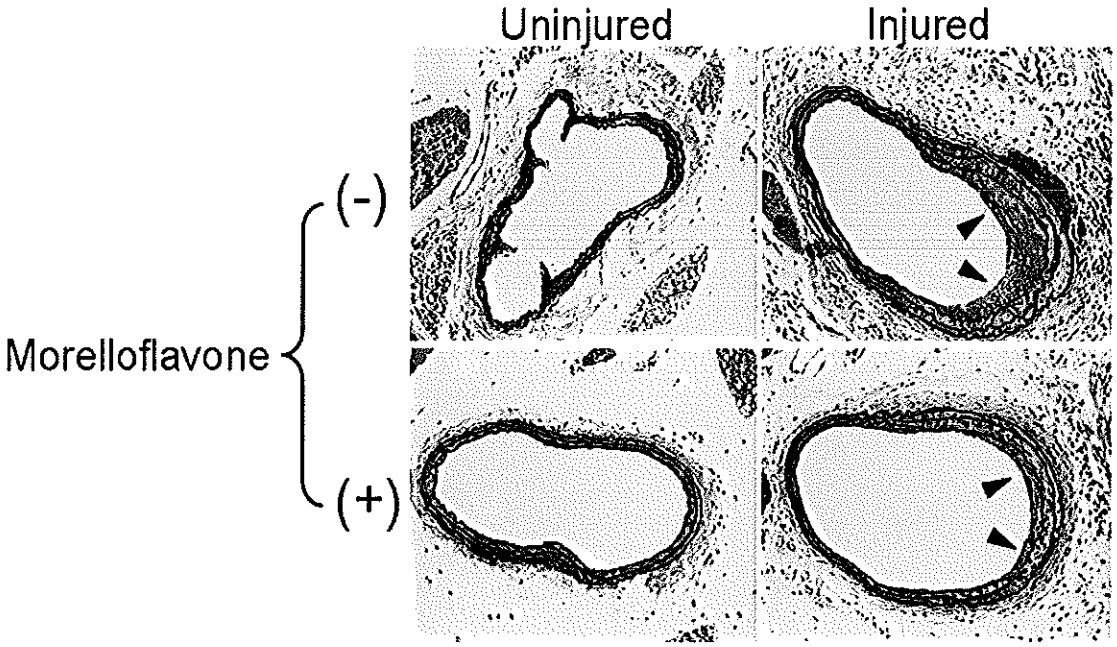


Figure 28. Verhoeff–van Gieson (VVG) staining of mouse carotid arteries. Uninjured, right carotid arteries that are sham operated; Injured, left carotid arteries where endothelial cells were denudated by the insertion of an epoxy resin probe. Arrows, neointimal formation. Male apoE-/- mice were fed with normal chow diet or chow containing 0.15% morelloflavone (w/w) (N = 9) for 1 week before subjected to left carotid artery denudation by the insertion of an epoxy resin probe. Animals were fed on the same diets for the next 14 days. Right (uninjured, control) and left carotid arteries were fixed, embedded, sectioned and VVG-stained before they were subjected to morphometric analyses.

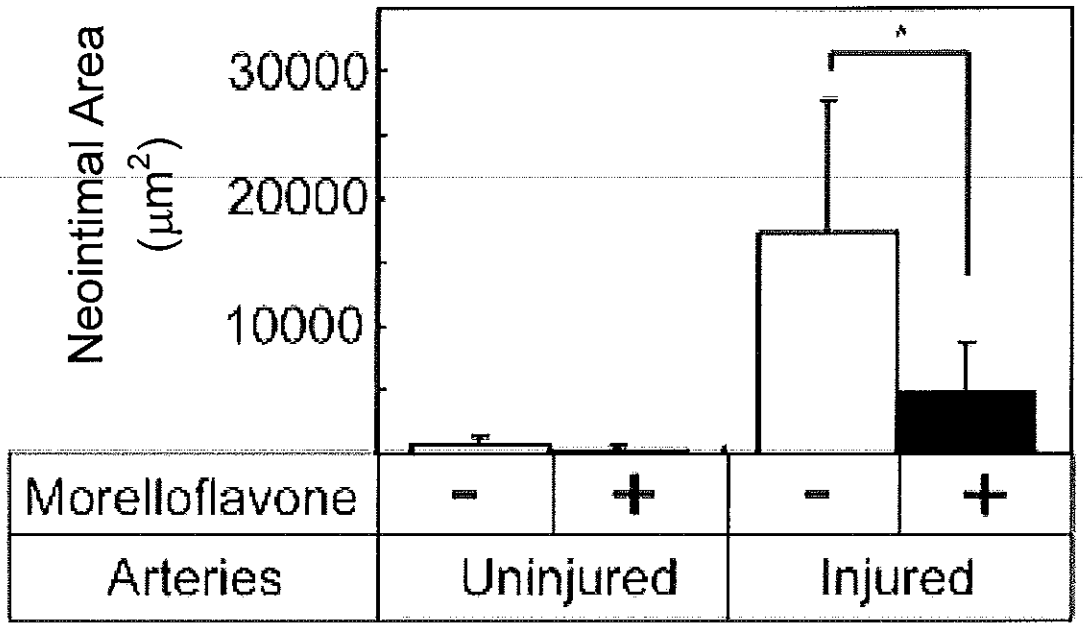


Figure 29. Morphometric analyses of injured and uninjured mouse carotid arteries. *, $P < 0.05$. Mice were placed either on normal chow (N = 10) or chow containing morelloflavone (N = 9) (0.15% w/w).

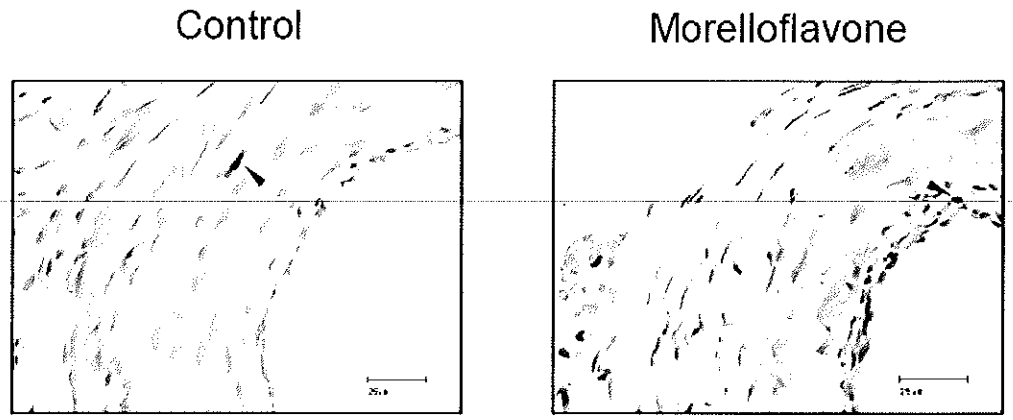


Figure 30. Ki67 staining of sections from mouse injured carotid arteries. Arrows, positively stained cell. Sections were blocked in BSA overnight and incubated with primary antibody Ki67. Peroxidase activity was visualised with DAB. Hematoxylin was used as a counterstain.

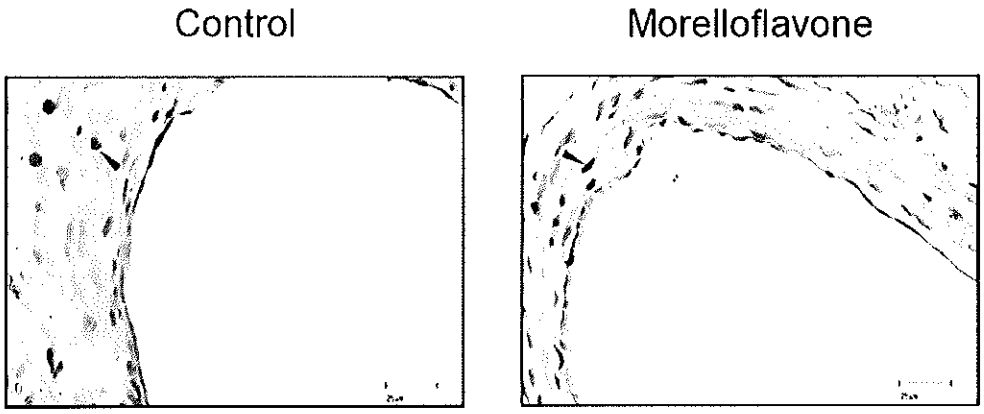


Figure 31. TUNEL staining of sections from mouse injured carotid arteries. Arrows, positively stained cell. Sections were detected as described in Methods section.

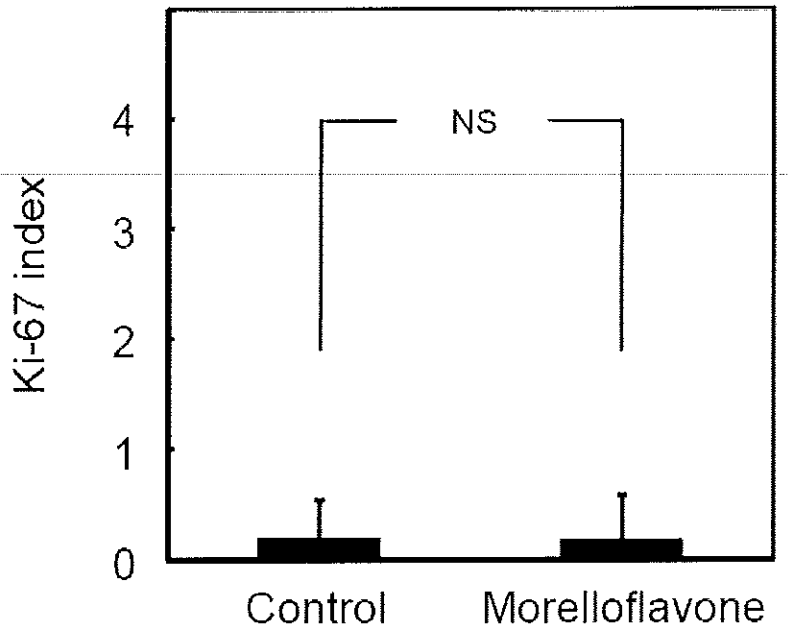


Figure 32. Ki-67 index. Ki-67 index representing the percentage of positive cells / total number of cells in the neointima on each mouse (N = 6), NS.

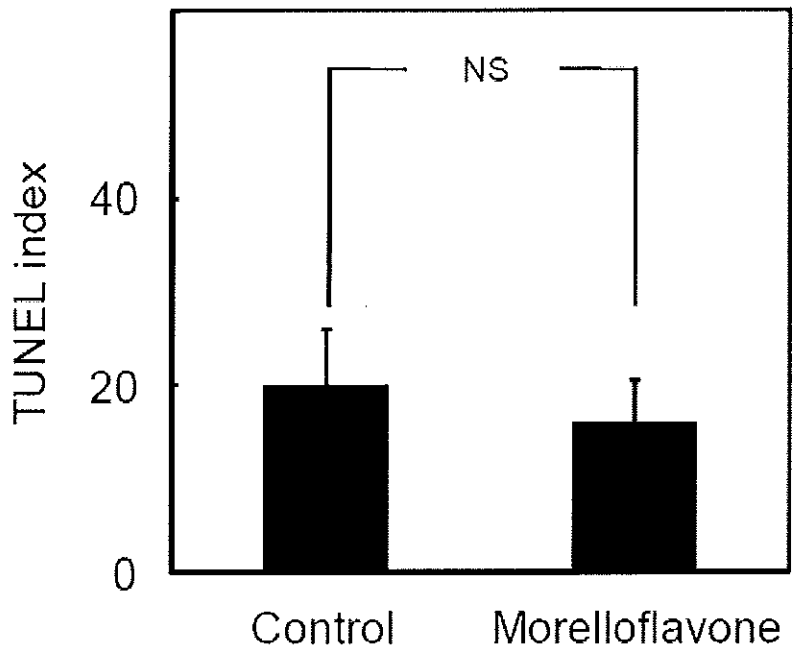


Figure 33. TUNEL index. TUNEL index representing the percentage of positive cells / total number of cells in the neointima on each mouse (N = 6), NS.

3.5 Discussion

Morelloflavone has been a poorly-studied flavonoid with a few reports on its anti-inflammatory and anti-oxidative properties (Gil *et al.*, 2007; Hutadilok-Towatana *et al.*, 2007; Sanz *et al.*, 1994). Its effects on vascular cells or arteries have not been studied. Here, the biological effects of morelloflavone in both VSMCs in tissue culture system and injured arteries of whole animals were characterized. Morelloflavone was found to possess highly unique biological properties. It does not have any effects on cell cycle progression (Fig. 9-11) or cell survival (Fig. 12-13) at concentrations up to 10 μM, while it has profound effects on migration at very low concentrations (0.1-1 μM) (Fig. 14-17). Morelloflavone's negative regulatory effects on migration was through various migration-related molecules—FAK, c-src, Erk and RhoA (Fig. 22-27). This suggests that the most significant biological activity of morelloflavone is the inhibition of VSMC migration (Fig. 14-17). Strikingly, oral administration of morelloflavone resulted in the reduction of neointimal formation in injured mouse carotid arteries (Fig. 28-29) without affecting the degree of apoptosis or proliferation of neointimal cells (Fig. 30-33). It has not been demonstrated before that morelloflavone has such profound effects on VSMCs migration and that it blocks neointimal formation in a mouse model of post-angioplasty restenosis. The current data are highly and clinically relevant since they suggest that morelloflavone could be orally administered to patients undergoing bare-stent implantation to reduce the risk of in-stent restenosis and that stents coated with morelloflavone might not have as deleterious effects as sirolimus- and paclitaxel-coated stents.

Sirolimus, also known as rapamycin, is a potent inhibitor of VSMCs migration although its mechanism of action is unknown (Poon *et al.*, 1996). Unlike morelloflavone, sirolimus exhibits profound inhibitory effects on cell cycle progression of VSMCs (Marx *et al.*, 1995). The inhibitory effects are mediated by a reduction in the phosphorylation of retinoblastoma protein and manifest themselves in cell cycle arrest at G1/S transition (Marx *et al.*, 1995). Sirolimus also blocks TNF-α-NFκB-pathway and facilitates VSMCs apoptosis (Giordano *et al.*, 2006). Paclitaxel is a potent anti-microtubule agent, inducing tubulin depolymerization and the formation

of abnormally stable and nonfunctional microtubules (Schiff *et al.*, 1979), leading to the disruption of normal cell cycle progression in VSMCs (Axel *et al.*, 1997; Wiskirchen *et al.*, 2004) and other cell types (Liebmann *et al.*, 1994). Paclitaxel's cell cycle arrest occurs—at a very low concentration around 10 nM—at G2/M phase (Wiskirchen *et al.*, 2004, Liebmann *et al.*, 1994), instead of at G1 phase in the case of sirolimus (Marx *et al.*, 1995). It has been shown paclitaxel-induced cell cycle arrest is often accompanied by apoptosis (Wang *et al.*, 2000). In addition, paclitaxel reportedly decreased VSMCs migration in a modified Boyden chamber assay (Wiskirchen *et al.*, 2004). Therefore, morelloflavone distinguishes itself from sirolimus and paclitaxel, both of which have profound cytotoxic and cell cycle inhibitory effects that morelloflavone peculiarly lacks.

The mechanism by which morelloflavone inhibits VSMCs migration involves multiple pathways—FAK, c-Src, ERK, and RhoA pathways. FAK and c-Src play key roles in cell migration. Fibroblasts from mice lacking FAK have a reduced migration rate (Ilic *et al.*, 1995). Similarly, fibroblasts from mice lacking c-Src also show a decreased rate of spreading (Klinghoffer *et al.*, 1999). Thus, it is not surprising that morelloflavone, which inhibits the phosphorylation (and activation) of both FAK and c-Src (Fig. 22-23), robustly blocks VSMCs migration (Figs. 14-17). Morelloflavone severely inhibited the phosphorylation of ERK at 0.1 – 10 μM (Fig. 24). ERK is implicated in both cell cycle progression (Cambard *et al.*, 2007) and migration (Mutsusaka *et al.*, 2005). Intriguingly, morelloflavone at the concentrations that reduced ERK phosphorylation decreased cell migration (Fig. 14-17) but had no effects on cell cycle progression (Fig. 9-11). One explanation may be that the degree of the inhibition by morelloflavone of the phosphorylation of ERK was sufficient to negatively affect cell migration but insufficient to compromise normal cell cycle progression. Further studies are called for to evaluate these possible differential effects by morelloflavone on cell migration and cell cycle progression.

The small G proteins, including Cdc42, Rac1, and RhoA, are very early elements of signaling pathways, all driving cell migration (Gerthoffer, 2007). It has been shown that Cdc42, Rac1, and RhoA act in a hierarchical cascade where Cdc42 activates Rac, which in turn activates RhoA (Hall, 1998). It is not clear why

morelloflavone at 10 μM inhibited Cdc42 activation without affecting Rac1 activation. It is possible that in the complex RhoGTPase network, morelloflavone negatively regulates Cac42 and RhoA through certain guanine nucleotide exchange factors (GEFs) specific to them (Bar-Sagi and Hall, 2000). Again further studies are needed to solve this puzzle. In the current work, morelloflavone drastically decreased activated RhoA (Fig. 25). Brock and others showed inhibition of RhoA completely inhibited lamellipodium formation, suggesting that RhoA function is required for lamellipodium formation (Brock and Ingber, 2005). In this study, morelloflavone completely inhibited lamellipodium formation in VSMCs at 1 μM. It is likely that morelloflavone blocked lamellipodium formation by preventing RhoA from being activated and activated RhoA from exerting its biological effect, i.e., lamellipodium formation (Fig. 20-21). Although morelloflavone substantively decreased the phosphorylation of FAK, c-Src, and ERK and the activity of RhoA (Fig. 22-25) and these molecules have been implicated in cell migration, further experiments are needed in order to clearly establish the cause-effect relationship between the inhibition by morelloflavone of these molecules and the inhibition of VSMC migration by morelloflavone.

In the current study, morelloflavone was shown to block injury-induced neointimal formation in mouse carotid arteries (Fig. 28-29) without affecting the degree of apopotosis or proliferation of neointimal cells (Fig. 30-33), suggesting that upon further studies, morelloflavone or its derivatives are promising anti-restenotic agents. For the prevention of in-stent restenosis, morelloflavone can be administered orally after the implantation of bare-metal stents. Alternatively, stents coated with morelloflavone (DES) can be implanted in coronary arteries. Due to its peculiar lack of cytotoxic and cell cycle inhibitory effects, it is hoped that morelloflavone-based strategies are not associated with increased subacute thrombosis rate unlike sirolimus- and paclitaxel-coated stents (Iakovou *et al.*, 2005).

CHAPTER 4

EFFECT OF MORELLOFLAVONE ON ATHEROSCLEOROSIS IN APOBEC-1/LDLR-DKO MICE

4.1 Introduction

Flavonoids are one of the largest groups of natural products known. These phenolic compounds are ubiquitous in plants and have a great variety of chemical nature. Their remarkable array of biological activities beneficial to human health has long been recognized (Havsteen, 2002). In the area of activities that may act in the prevention of cardiovascular disease, evidences from both *in vitro* and *in vivo* studies have emerged that flavonoids possess several anti-atherosclerotic actions (Middleton *et al.*, 2000; Calixto *et al.*, 2003; Lucas *et al*, 2003; Calixto *et al.*, 2004; Enkhmaa *et al.*, 2005; Juźwiak *et al*, 2005).

In 1997, Gil and co-workers have demonstrated that morelloflavone, a biflavonoid isolated from *Garcinia spicata* not only exerts potent anti-inflammatory effects in animal models but also scavenges reactive oxygen species generated by human neutrophils (Gil *et al.*, 1997). Later, anti-oxidation activities of this compound have been well established *in vitro* (Sanz *et al.*, 1994; Deachathai *et al.*, 2006; Hutadilok-Towatana *et al.*, 2007), and have led to the evaluation of its anti-atherosclerotic activities in experimental animals. Morelloflavone-rich fraction prepared from *Garcinia dulcis* leaves, has been demonstrated to reduce plasma lipids as well as aortic fatty streak formation in cholesterol-fed rabbits (Decha-Dier *et al.*, 2008). However, this animal model cannot be regard as the optimal model for human atherosclerosis due to many limitations. Although the early lesions developed in rabbits are similar to human fatty streaks, they do not develop as tissue plaques, which are the trademarks of atherosclerosis in humans (Kolodgie *et al.*, 1996). Also, VLDL rather than LDL is the dominant circulatory lipoprotein (Björkhem *et al.*, 1990) and

their plasma cholesterol levels are extraordinarily high which are very dissimilar to humans (Jawien *et al.*, 2004). Yet, there is no data pertaining to anti-atherogenic potential of morelloflavone in the genetic deficient animals, as far as more relevant models of atherosclerosis are considered.

The new animal models became available for experimental atherosclerosis research in 1990s. At that time, the gene targeted animal models, namely apolipoprotein E gene knockout (apoE-/-) mice (Piedrahita *et al.*, 1992) as well as low density lipoprotein receptor gene knockout (LDLR-/-) mice were developed (Ishibashi *et al.*, 1993). Deficiency of apoE elevates VLDL particles, leading to hyperlipidemia and eventually atherosclerosis even feeding with a normal diet (Piedrahita *et al.*, 1992). However, most plasma cholesterol is carried in VLDL in these mice, rather than in LDL as in humans (Powell-Braxton *et al.*, 1998). LDLR-/- mice develop only modest hypercholesterolemia when maintained on regular diets. Although LDLR-/- mice on a regular diet do not get atherosclerosis, their plasma lipid profiles are similar to those in human hyperlipidemia (Masucci-Magoulas *et al.* 1997). More recently, apoBEC-1 and LDLR double knockout (apoBEC-1/LDLR-DKO) mice have been created (Powell-Braxton *et al.*, 1998). These mice have markedly increased in plasma cholesterol concentrations, caused exclusively by increased LDL-cholesterol and developed extensive lesions throughout the aorta, including most of the branch points (Powell-Braxton *et al.*, 1998). They have more severe hyperlipidemia and atherosclerosis than mice deficient for LDLR alone (Powell-Braxton *et al.*, 1998). Unlike apoE-/- mice, the lipoprotein metabolism in apoBEC-1/LDLR-DKO mice mirrors the pathophysiology of human familial hypercholesterolemia. Humans always have high plasma levels of apoB100-containing LDL during atherosclerosis, whereas apoE-/- mice have a distinct accumulation of apoB48-containing lipoproteins and have normal low levels of apoB100-containing lipoproteins (Powell-Braxton *et al.*, 1998). Thus, apoBEC-1/LDLR DKO mouse is nowadays considered as a one of the most relevant models to study the anti-atherogenic potential of drugs.

In the present study, an effort has been made to evaluate the effects of morelloflavone against development of aortic atherosclerosis in apoBEC-1/LDLR-DKO mouse, a well-established animal model for atherosclerosis.

4.2 Materials

4.2.1 Morelloflavone

Morelloflavone was prepared from *Garcinia dulcis* leaves as previously described in Chapter 3.

4.2.2 Animals

ApoBEC-1 and LDLR genes knockout (apoBEC-1/LDLR-DKO) mice were generous gifts from Assoc. Prof. Dr. Ba-Bie Teng of Institute of Molecular Medicine for Prevention of Human Diseases, The University of Texas Health Science Center at Houston, Texas, USA.

4.2.3 Diets

Normal rodent diets (5001®) were purchased from Lab Diet (Indiana, USA). Diets containing 0.003% w/w morelloflavone were manufactured by Research Diets Inc. (New Jersey, USA).

4.3 Methods

4.3.1 Animal treatments

In order to confirm the progression of atherosclerosis in apoBEC-1/LDLR-DKO mice, an animal model used in this study, forty male apoBEC-1/LDLR-DKO mice were randomly divided into 5 groups. Each group (eight mice) was maintained on a normal diet for 10, 20, 30, 40, and 50 weeks, respectively. Four animals were housed in the same cage in an air-conditioned room with 12-h light/dark cycle and accessed to diet and water *ad libitum*. At the end of given time, mice were sacrificed under isoflurane anesthesia. A blood sample was then obtained *via* a heart puncture and collected into microfuge tubes containing EDTA, and the heart was excised with a small portion of the ascending aorta remaining and embedded in Tissue-Tek® O.C.T. Compound (Sakura, USA). All animal experimentation

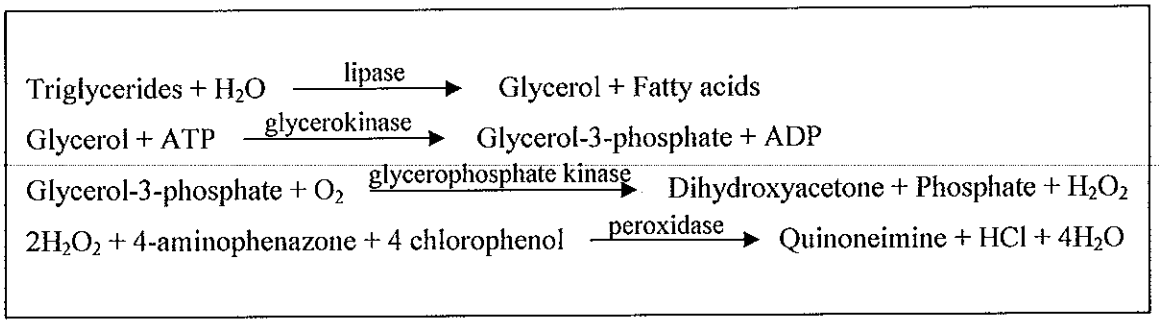
protocols were performed under The University of Texas Health Science Center at Houston's guidelines of animal welfare, in accordance with the USA National Institutes of Health guidelines.

In order to examine whether morelloflavone could attenuate atherosclerosis in apoBEC-1/LDLR-DKO mice. Male apoBEC-1/LDLR-DKO mice aged two months were randomly divided into two groups of twelve animals. The control group was on normal diet and the test group was fed normal diet containing 0.003% morelloflavone (w/w) daily, for 8 months. This corresponds to 4 mg/kg morelloflavone if 30 gram animals consume 4 grams of chow. Four animals were housed in the same cage in an air-conditioned room with 12-h light/dark cycle and accessed to diet and water *ad libitum*. After eight months of feeding, the mice were anesthetized with isofurane. A blood sample was then obtained *via* a heart puncture and collected into microfuge tubes containing EDTA and a whole aorta was removed. All animal experimentation protocols were performed under The University of Texas Health Science Center at Houston's guidelines of animal welfare, in accordance with the USA National Institutes of Health guidelines.

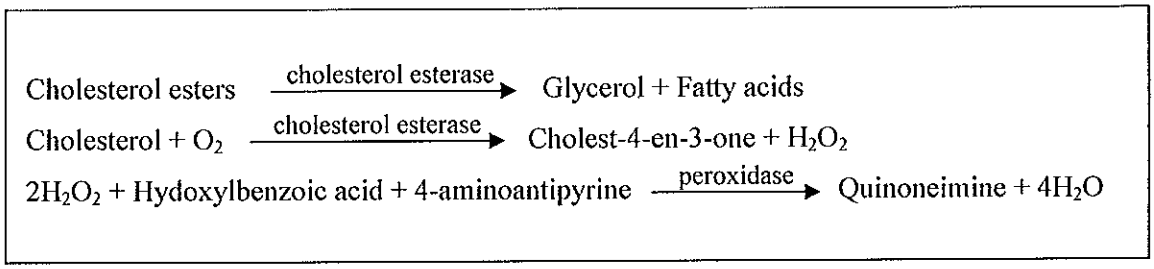
4.3.2 Lipids determination

After a given time, the mice were anesthetized with isofurane. A blood sample was then obtained via a heart puncture and collected into microfuge tubes containing EDTA. Samples were immediately centrifuged at 5,000 g for 10 min at room temperature, and the plasma was stored at 80°C. The plasma triglycerides (TG), total cholesterol (TC), phospholipids (PL), and non-esterified fatty acids (NEFA) were measured by using commercially available assay kits.

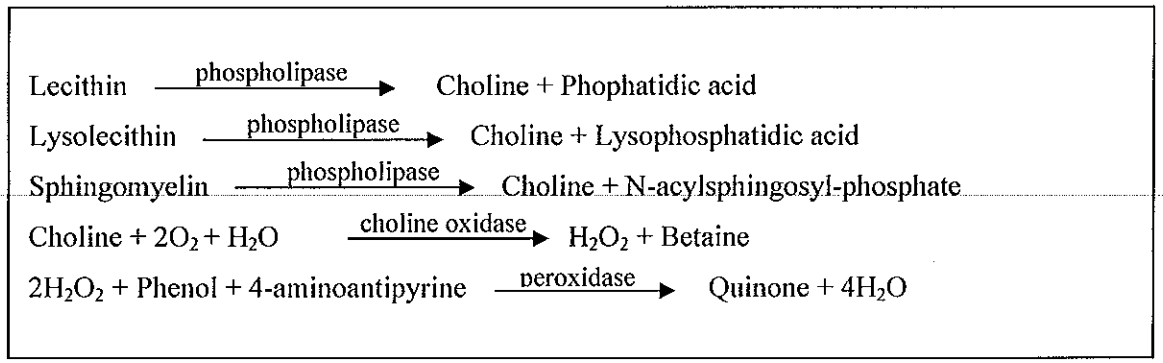
For TG determination, TGs were hydrolyzed with lipases to form glycerol and fatty acids. Glycerol was then changed to glycerol-3-phosphate which subsequently changed to dihydroacetone phosphate, and hydrogen peroxide by glycerol kinase and glycerol-3-phosphate oxidase, respectively. The quinoneimine, formed from hydrogen-peroxide, 4-aminophenazone and 4-chlorophenol under the catalytic influence of peroxidase, was measured at 500 nm (Randox, UK).



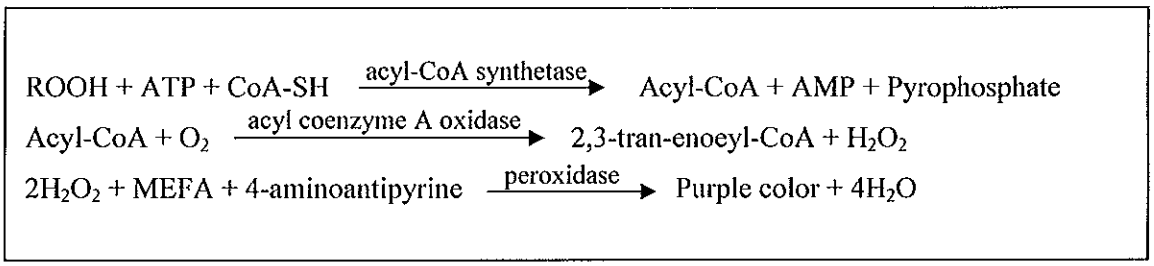
For TC determination, cholesterol esters were hydrolyzed by cholesterol esterase to cholesterol and free fatty acids. Free cholesterol was then oxidized by cholesterol oxidase to cholest-4-en-3-one and hydrogen peroxide. The quinoneimine formed by hydrogen peroxide combines with hydroxybenzoic acid and 4-aminoantipyrine to form which can be quantitated at 500 nm (Thermo Electron, USA).



For PL analysis, phospholipids (lecithin, lysolecithin and sphingomyelin) are hydrolyzed to free choline by phospholipase. The liberated choline is subsequently oxidized to betaine by choline oxidase with the simultaneous production of hydrogen peroxide. The hydrogen peroxide, which is produced quantitatively, oxidatively couples 4-aminoantipyrine and phenol to yield a chromogen with a maximum absorption at 600 nm (Wako USA).



For NEFA measurement, the enzymatic method relies upon the acylation of coenzyme A (CoA) by the fatty acids in the presence of added acyl-CoA synthetase. The acyl-CoA thus produced is oxidized by added acyl-CoA oxidase with generation of hydrogen peroxide, in the presence of peroxidase POD permits the oxidative condensation of 3-methy-N-ethyl-N(β-hydroxyethyl)-aniline (MEFA) with 4-aminoantipyrine to form a purple colored adduct which can be measured colorimetrically at 550 nm (Wako, USA).



4.3.3 Assessment of aortic atherosclerotic lesions

The mice were sacrificed under isoflurane anesthesia. The whole aorta was removed and stripped off any external fatty deposits. It was cut longitudinally from the aortic arch down to the iliac juncture, pinned on a waxed-paperboard. After fixing with 10% formalin overnight, the aorta was rinsed with water and then 78% methanol. The aorta was stained by oil red O for 1 h and rinsed twice with 78% methanol for 2 min to remove excess dye. The aorta was subsequently mounted on the glass slide and dried at RT for 24 h. The entire aortic surface was photographed and scanned digitally, and planimetry of oil red O–positive stained lesions was

performed on the digitized images using SigmaScan Pro software. Quantification of atherosclerotic lesion area was expressed as the total aortic surface area covered by oil red O-positive lesions in square millimeters.

To measure the atherosclerotic lesion in histological sections, the heart was excised with a small portion of the ascending aorta remaining and embedded in Tissue-Tek® O.C.T. Compound (Sakura, USA). Sectioning started within the heart and worked in the direction of the aorta. Once the aortic valves were identified, 5-μm cryostat sections were taken and mounted on slides. One slide was stained with hematoxylin and the rest were stained for immunohistochemistry. The images of sections were captured digitally under identical lighting and stored in TIFF format using ScanScope slide scanning systems (Nikon, USA). The extent of lesion was assessed by using Image J software (NIH, USA).

4.3.4 Immunohistochemistry

For the analyses of smooth muscle cells, macrophages, cell proliferation and apopotosis in atherosclerotic lesion, smooth muscle cell alpha-actin (SMA), surface glycoproteins of macrophages (F4/80), Ki67 and terminal deoxynucleotidyl transferase (TdT)-deoxyuridine nick-end labeling (TUNEL) staining, respectively, were performed. SMA, F4/80, and Ki67 were detected using rabbit polyclonal (#5694, Abcam, USA), rabbit monoclonal (#6640, Abcam, USA), and rabbit monoclonal (Clone TEC-3, DAKO, USA) antibodies, respectively as described previously (Ihling *et al.*, 1997; Ihling *et al.*, 1998). Briefly, cryo-sections were fixed in cold acetone, dried and hydrated by dipping in 95% ethanol, 70% ethanol, and PBS. After the quenching of endogenous peroxidase with 3% H_2O_2 for 10 min, sections were incubated with Biocare Blocking Reagent (Biocare, USA) to reduce nonspecific background staining. Thereafter, the slides were incubated with antibodies directed against SMA, F4/80, and Ki67. For immunostaining of Ki67, antigens were unmasked by pressure cooking in 10 mM citric acid, pH 6, for 3 min. All slides were then incubated with biotinylated secondary antibody (Vector, USA) at room temperature, followed by incubation with horseradish peroxidase complex (Biogenex, USA). Peroxidase activity was visualized by diaminobenzidine (DAB, Dako, USA) to yield a brown reaction product. The nuclei were slightly

counterstained with hematoxylin. Terminal deoxynucleotidyl transferase (TdT) deoxyuridine nick-end labeling (TUNEL) staining (Surh and Sprent, 1994) was performed using a FragEL™ DNA fragmentation detection kit (Oncogene Research Products, USA) according to the manufacturer's instructions. In this assay, terminal deoxynucleotidyl transferase (TdT) binds to exposed 3'-OH ends of DNA fragments generated in response to apoptotic signals and catalyzes the addition of biotin-labeled and unlabeled deoxynucleotides. Biotinylated nucleotides are detected using a streptavidin-horseradish peroxidase (HRP) conjugate. Diaminobenzidine (DAB) reacts with the labeled sample to generate an insoluble colored substrate at the site of DNA fragmentation. Counterstaining with hematoxylin aids in the morphological evaluation and characterization of normal and apoptotic cells. The SMA, and F4/80 indices defined as the number of cells with DAB positive nuclei per section or divided by the total atherosclerotic lesion area. The Ki67 and apoptotic indices, defined as the number of cells with DAB positive nuclei divided by the total number of cells counted and expressed as a percentage, were then calculated. All cells within the intima were counted.

4.3.5 Statistical analysis

Values are expressed as means ± SD. Comparisons of parameters between two groups were made with Student's t test. When appropriate, ANOVA was performed to compare multiple groups. A value of $P < 0.05$ was considered statistically significant.

4.4 Results

4.4.1 Lipids profile and atherosclerotic lesion in mouse model

After being maintained on regular diet for 10, 20, 30, 40 or 50 weeks, apoBEC-1/LDLR-DKO mice were sacrificed and their blood were collected and examined for plasma lipids profile. As shown in Table 3, levels of plasma cholesterol (TC), triglycerides (TG), the non-esterified fatty acids (NEFA) and phospholipids (PL) in mice deficient in apoBEC-1 and LDLR did not increase with time but

remained unaltered between each time-point (Table 3). However, the significantly different TC, TG, and PL values only detected in 30-and 40-week groups, respectively, were rather biological variation among the animals. The plasma lipids values of apoBEC-1/LDLR-DKO mice obtained from this study, however, were comparable to those of Powell-Braxton and co-workers, who have also reported that the plasma cholesterol level in apoBEC-1/LDLR-DKO mice fed low-fat chow diet is about two-fold higher than that in wild-type mice, while the triglycerides level is mildly elevated (Powell-Braxton *et al.*, 1998).

After apoBEC-1/LDLR-DKO mice were maintained on normal rodent diet for 10, 20, 30, 40, or 50 weeks, mice were then sacrified and their hearts were dissected, embedded in Tissue-Tek® O.C.T. Compound, sectioned, stained with hematoxylin, and analyzed. In contrast to their stable lipid levels, mice deficient in apoBEC-1 and LDLR developed more severe atherosclerosis from 10 to 50 weeks of normal diet (Fig. 34), as demonstrated by significantly increased atherosclerotic lesions (lesions area, 10 weeks *vs.* 20, 30, 40, or 50 weeks = 0.18 ± 0.07 *vs.* 0.96 ± 0.32, 3.28 ± 0.75, 4.48 ± 1.26, 7.36 ± 1.87 [$x10^3$ μm^2], $P < 0.05$). These data, taken together with lipids profile, suggests that apoBEC-1/LDLR-DKO mouse is a suitable model to study the anti-atherogenic potential of morelloflavone.

Table 3. Plasma lipids level in apoBEC-1/LDLR-DKO mice.

	Time during study (weeks)				
	10	20	30	40	50
TG (mg/dL)	161.87±29.67	182.69±21.36	196.83±17.23*	177.63±11.93	153.86±15.06
TC (mg/dL)	403.25±77.92	418.79±105.65	498.10±103.07	375.31±55.92	405.42±70.91
PL(mg/dL)	295.05±19.95	304.18±16.07	312.73±46.74	261.58±29.21*	294.57±12.76
NEFA (mEq/L)	0.60±0.11	0.59±0.09	0.67±0.10	0.68±0.18	0.51±0.09

Values are expressed as mean ± SD (N=8 for 10, 20, 30, and 50-weeks mice, N=5 for 40-weeks mice)* $P < 0.05$ compared with 10 weeks.

Table 4. Plasma lipids level in apoBEC-1/LDLR-DKO fed normal rodent or morelloflavone-containing diet.

	Control	Morelloflavone	P-value
TG (mg/dL)	158.47±32.87	179.89±28.00	0.10
TC (mg/dL)	512.63±102.01	507.23±95.79	0.89
PL (mg/dL)	321.99±52.65	320.66±65.50	0.96
NEFA (mEq/L)	0.81±0.08	0.89±0.13	0.10

Values are expressed as mean ± SD (N=12)

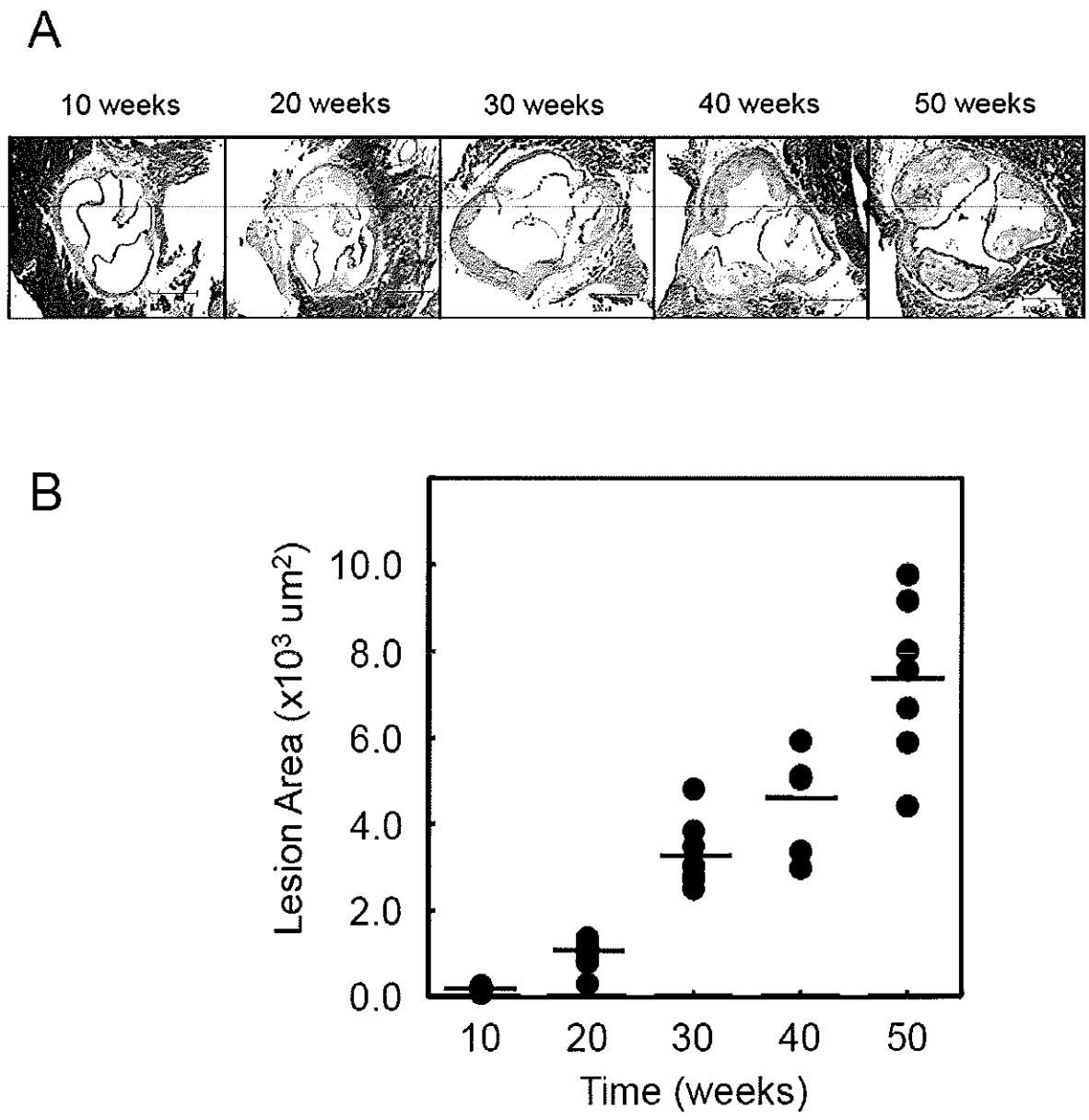


Figure 34. Aortic atherosclerosis progression in apoBEC-1/LDLR-DKO mice. Representative HE-stained sections from the aortic valve area of mice (A). Quantitative estimation of aortic atherosclerotic lesion involvement (N=8). Each data point represents total area of atherosclerotic lesion involvement in aortic valve area (B).

4.4.2 Effect of morelloflavone on body weight and food consumption

In order to investigate whether morelloflavone affected the body weights and food consumption, two-month male apoBEC-1/LDLR-DKO mice were fed normal rodent diets either alone or containing 0.003% morelloflavone for 8 months. As shown in Fig. 35, changes in body weight and food consumption were similar between the control and morelloflavone-treated groups (body weights at 8-month time point, control vs. morelloflavone = 27.8 ± 1.0 *vs.* 28.4 ± 2.3 [g], NS; food consumption, 4.0 ± 0.1 *vs.* 3.9 ± 0.1 [g], NS). The calculated dose of morelloflavone consumed over the 8-month study was 4 mg/kg per day. These results suggest that morelloflavone does not affect body weights and food consumption.

4.4.3 Effect of morelloflavone on plasma lipids profile

In order to test if morelloflavone affected plasma lipids profile, the apoBEC-1/LDLR-DKO mice were fed for 8 months with normal rodent diet or morelloflavone-containing diet (0.003% w/w). Then mice were sacrified and their blood were collected, centrifuged, and measured for plasma lipids level. As shown in Table 4, after 8 month-period, both control and morelloflavone-treated mice developed severe hyperlipidemia. There was no substantial difference in either TC, TG, PL, or NEFA between the two groups. These data importantly suggest that treatment with morelloflavone does not influence the concentrations of cholesterol, triglycerides, phospholipids as well as non-esterified fatty acid in blood.

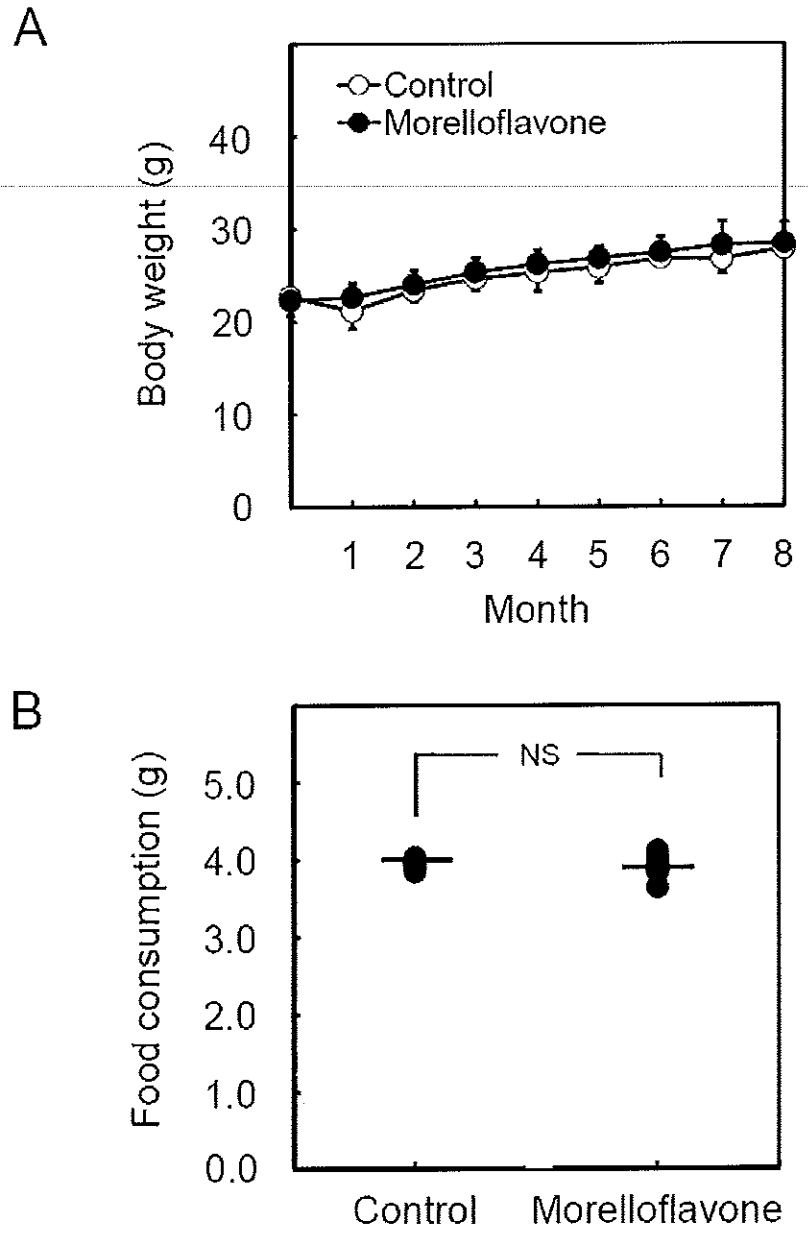


Figure 35. Body weight and food consumption of fed mice. The apoBEC-1/LDLR-DKO mice (N=12) were fed with normal rodent diet or rodent diet supplemented with 0.003% morelloflavone for 8 months. Food consumption (B) and body weight (A) were measured daily and monthly, respectively. NS, not significant.

4.4.4 Effect of morelloflavone on the progression of atherosclerosis in apoBEC-1/LADK-DKO mice

In order to test whether morelloflavone reduced atherosclerosis in apoBEC-1/LDLR-DKO mice, they were fed with normal rodent diet or morelloflavone-containing diet (0.003% w/w) for 8 months. Mice were then sacrificed and the whole aorta was removed, cut longitudinally from the aortic arch down to the iliac juncture, pinned on a waxed-paperboard, fixed with formalin, stained with Oil Red O, and measured the atherosclerotic lesions area. Also, the heart was excised, embedded in Tissue-Tek® O.C.T. Compound, sectioned, stained with hematoxylin, and the extent of lesion was measured. As shown in Fig. 36-37, the aortic lesion areas were significantly lower in morelloflavone-treated mice than in control mice after 8 months of feeding. (atherosclerosis, control *vs* morelloflavone = 33.76 ± 5.88 *vs* 24.86 ± 6.87 [% of total aortic area], P = 0.0025). The decreased atherosclerotic lesion area in morelloflavone-treated mice was 26% compared to control mice. Also as shown in Fig. 38-39, cross-sectioning of aortic roots revealed the difference in lesions area. The atherosclerotic lesion from morelloflavone-treated mice was quantitatively lower than that from control mice (lesion area, control *vs.* morelloflavone = 7.64 ± 1.30 *vs.* 5.65 ± 1.05 [x10^3 μm^2], P < 0.0025). In both groups, raised atherosclerotic lesions were visible grossly and microscopically in the aortic intima. Mean lesion area in morelloflavone-treated mice was significantly 26% lower than that in control mice. The data obtained from both *en face* and cross-section method thus suggest that morelloflavone treatment significantly reduces atherosclerosis in apoBEC-1/LDLR-DKO mice.

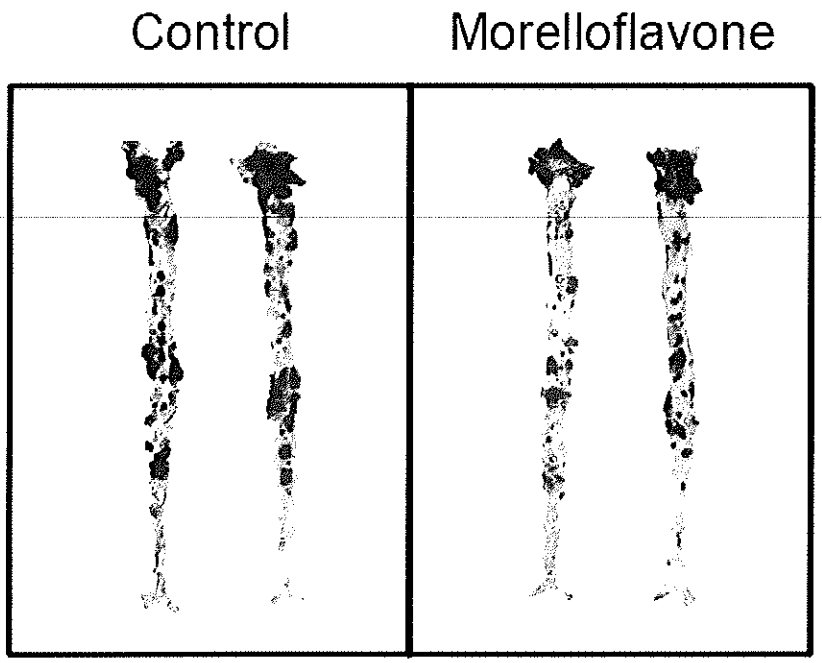


Figure 36. Atherosclerotic lesions in aortic of apoBEC-1/LDLR-DKO mice. Mice were fed a normal rodent diet or 0.003% morelloflavone-containing diet with for 8 months. Gross view of aortas stained with Oil Red O; red indicates the presence of atherosclerotic lesions

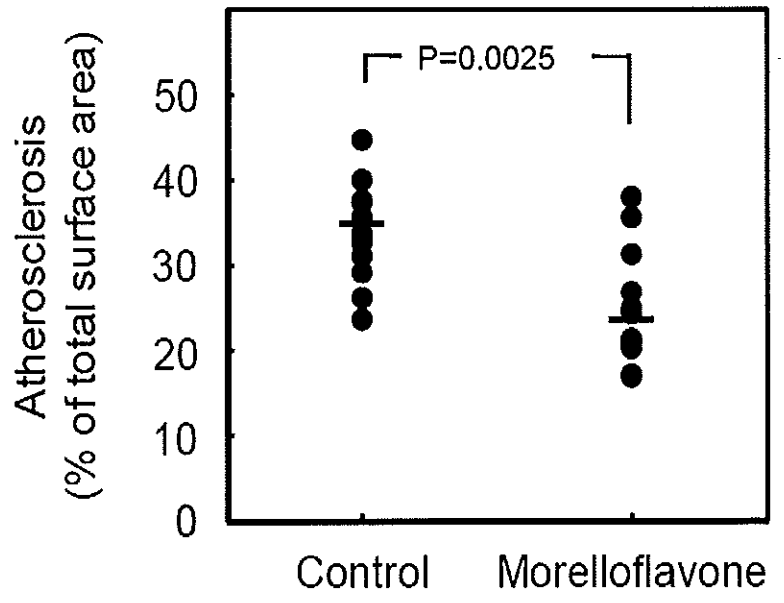


Figure 37. Degree of atherosclerosis by *en face* method. The total area affected by atherosclerosis is substantially lower in morelloflavone-treated mice than in control mice (N=12), P = 0.0025.

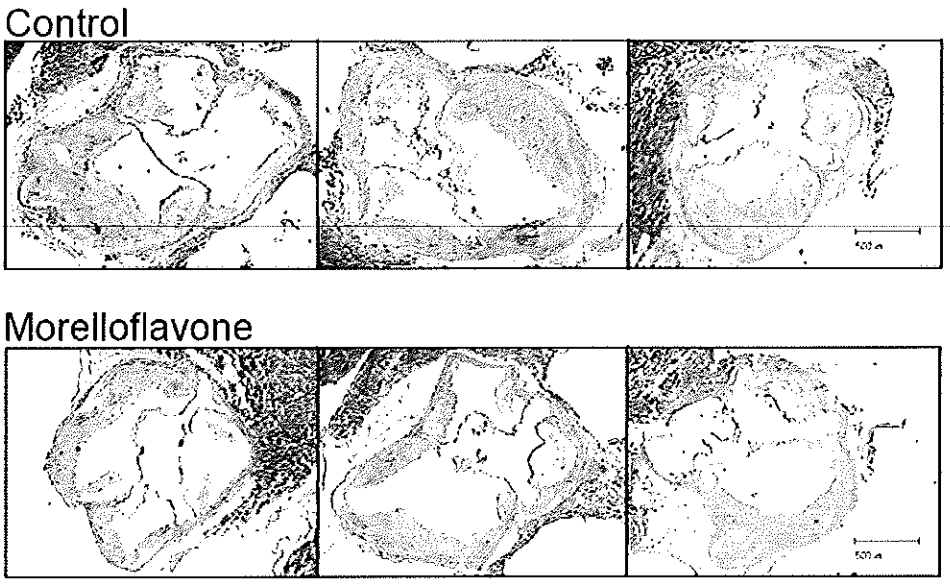


Figure 38. Cross-sectional aortic atherosclerosis in apoBEC-1/LDLR-DKO mice. Mice were fed a normal chow diet or morelloflavone–containing diet for 8 months. Representative HE-stained sections from the aortic valve area of apoBEC-1/LDLR-DKO mice fed with normal diet or morelloflavone–containing diet (N=6).

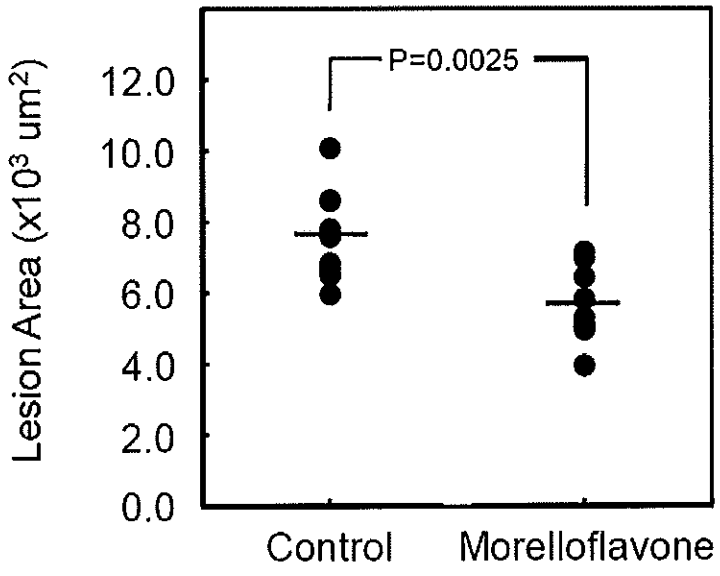


Figure 39. Atherosclerotic lesion area by cross-sectioned methods. Quantitative estimation of aortic atherosclerotic lesion involvement. Each data point represents total area of atherosclerotic lesion involvement in aortic valve area (N=6).

4.4.5 Effect of morelloflavone on smooth muscle cells in atherosclerotic lesions

In order to determine whether morelloflavone reduced the size of atherosclerotic lesions by inhibited VSMCs migration, the sections was examined by staining with SMA antibody. As shown Fig. 40, morelloflavone-treated mice had a number of smooth muscle cells less than that in control mice. The mice fed with morelloflavone showed a significant 32% decrease in smooth muscle cells in atherosclerotic lesion, parallel with the decrease in plaque area. (SMA-positive cells, control *vs.* morelloflavone = 27.00 ± 10.69 *vs.* 15.44 ± 3.50 [cells/section], P = 0.0077; SMA index, control *vs.* morelloflavone = 0.0038 ± 0.0014 *vs.* 0.0026 ± 0.0007 [cells/μm^2], P = 0.0491). These data, together with the previous ones, suggest that decreased smooth muscle cell numbers may contribute to the pathogenic prevention mechanism leading to the decrease in atherosclerotic lesions in apoBEC-1/LDLR-DKO.

4.4.6 Effect of morelloflavone on macrophages in atherosclerotic lesions

In order to determine whether morelloflavone affects macrophages in lesions, the positive cells were quantified for the anti-rabbit monoclonal antibody, F4/80. As shown Fig. 41, there was no significant change in macrophages in lesions as indicated by number of macrophages per section or lesion area (F4/80-positive cells, control *vs.* morelloflavone = 33.63 ± 3.78 *vs.* 32.25 ± 8.03 [cells/section], NS; F4/80 index, control *vs.* morelloflavone = 0.0045 ± 0.0009 vs. 0.0042 ± 0.0018 [cells/μm^2], NS). This data suggest that morelloflavone does not affect macrophages infiltration in atherosclerotic lesions.

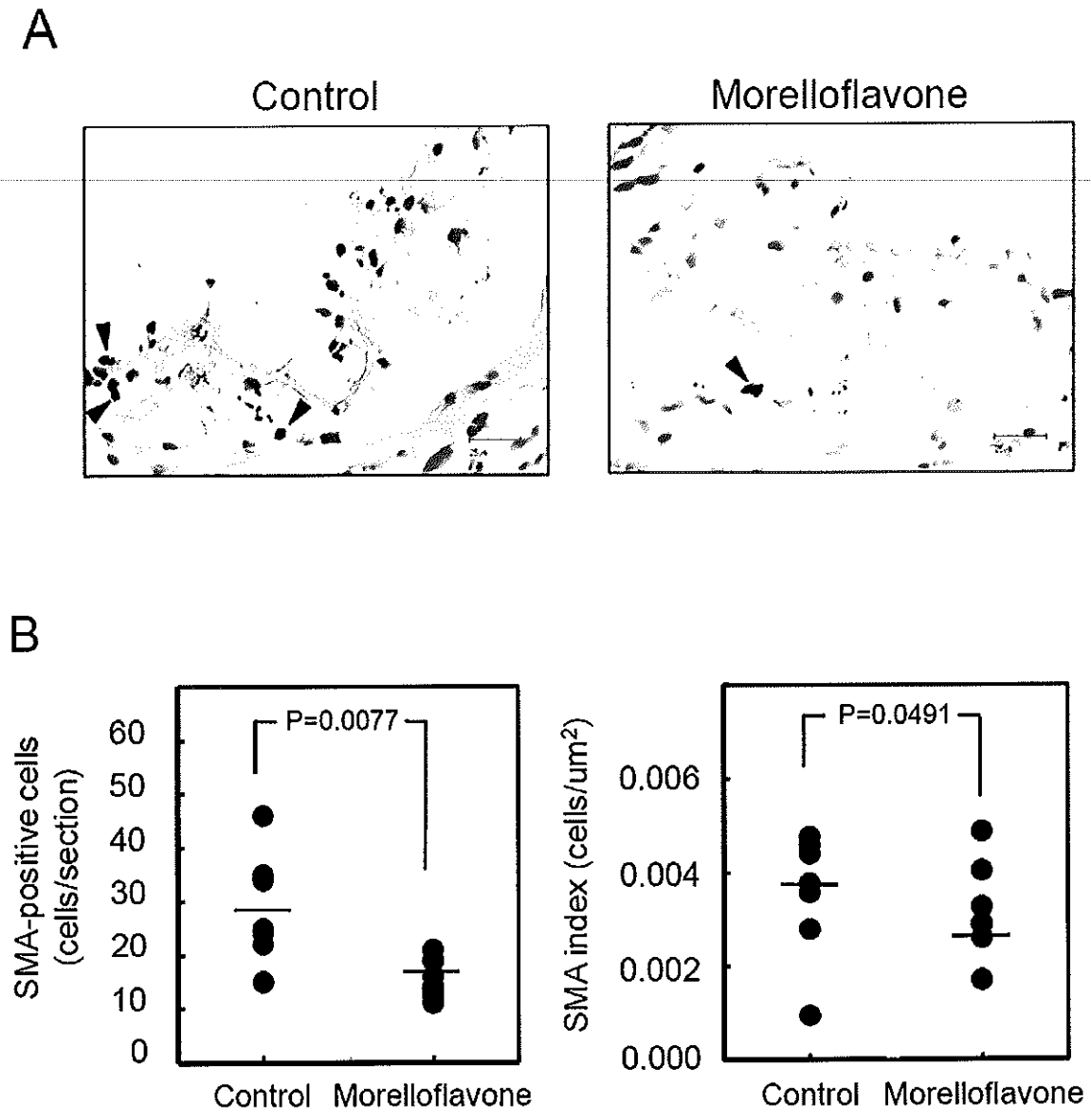


Figure 40. SMA-positive cells and SMA index. The sections (N=7) were stained for SMA and couterstained with hematoxylin (A). Quantitative analysis of SMA-positive cells in atherosclerotic lesion (B).

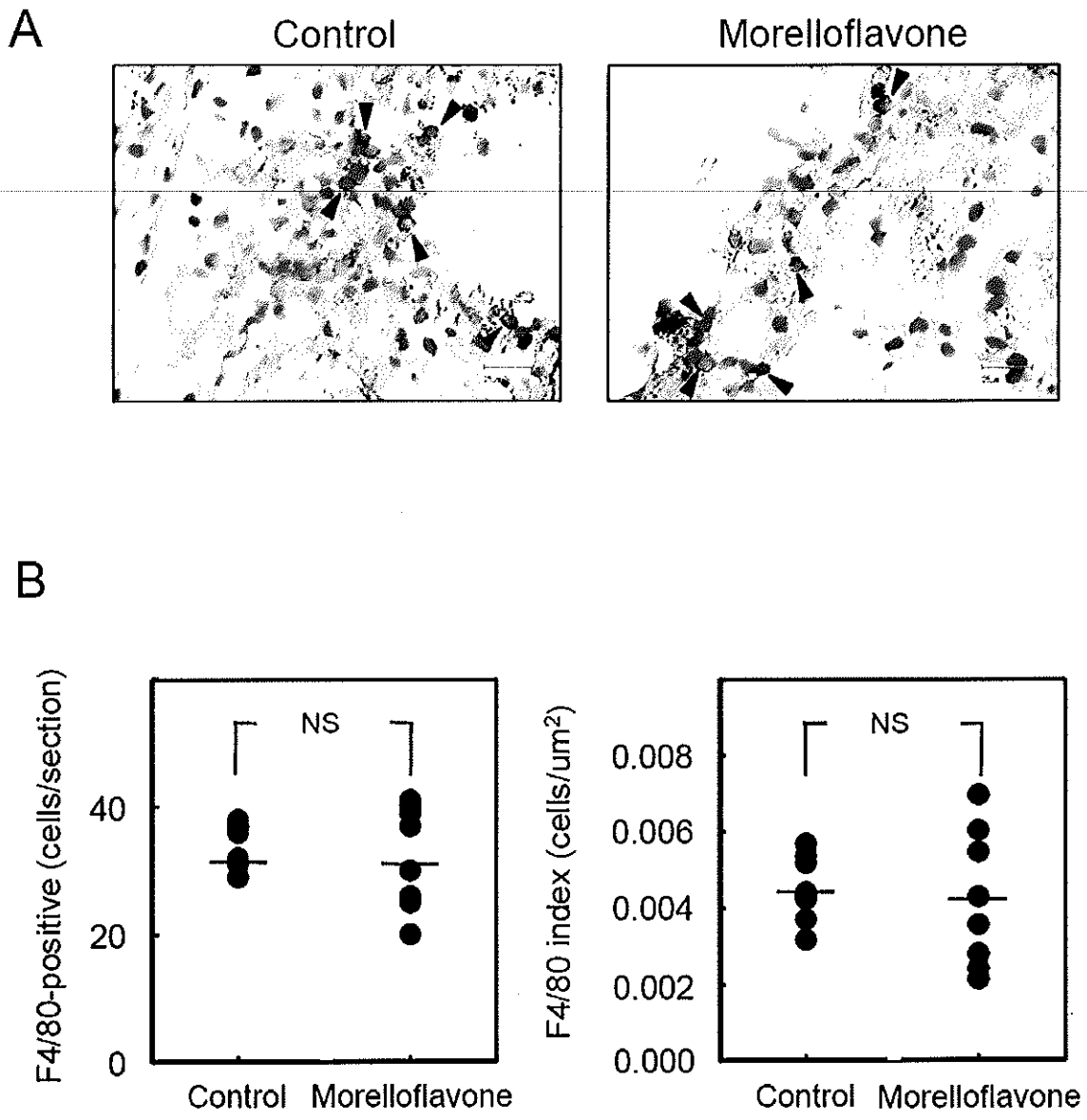


Figure 41. F4/80-positive cells and F4/80 index. The sections (N=7) were stained for F4/80 and couterstained with hematoxylin (A). Quantitative analysis of F4/80-positive cells in atherosclerotic lesion (B).

4.4.7 Effect of morelloflavone on proliferation and apoptotis of cells in atherosclerotic lesions

In order to investigate whether morelloflavone decreased the size of atherosclerotic lesions by inhibited proliferative activity, proliferation was examined by Ki67 staining. The sections were blocked in blocking reagent and incubated with Ki67 antibody. Peroxidase activity was visualized with DAB. Hematoxylin was used as a counterstain and subjected to morphometric analyses to count positively stained cells. As shown in Fig 42, lesions from morelloflavone-treated mice had a frequency of Ki67-positive cells and Ki67 index, which were not different from control mice (Ki67-positive cells, control *vs.* morelloflavone = 0.0013 ± 0.0003 *vs.* 0.0011 ± 0.0003 [cells/um2], NS; Ki67 index, control *vs.* morelloflavone = 1.8 ± 0.7 *vs.* 1.6 ± 0.5 [%], NS). In order to investigate whether morelloflavone decreased the size of atherosclerotic lesions by inhibited apoptotic activity, apoptosis was determined by the TUNEL technique and subjected to morphometric analyses to count positively stained cells. As shown in Fig. 43, the average frequency of apoptotic cells in the lesions of morelloflavone-treated mice was not significantly different from those of control mice as indicated by TUNEL-positive cells and TUNEL index (TUNEL-positive cells, control *vs.* morelloflavone = 0.00021 ± 0.00010 *vs.* 0.00025 ± 0.00008 [cells/um2], NS; TUNEL index, control *vs.* morelloflavone = 0.4 ± 0.2 *vs.* 0.4 ± 0.2 [%], NS). These data suggest that morelloflavone does not cause a decrease in apoptotic cells or inhibit proliferating cells.

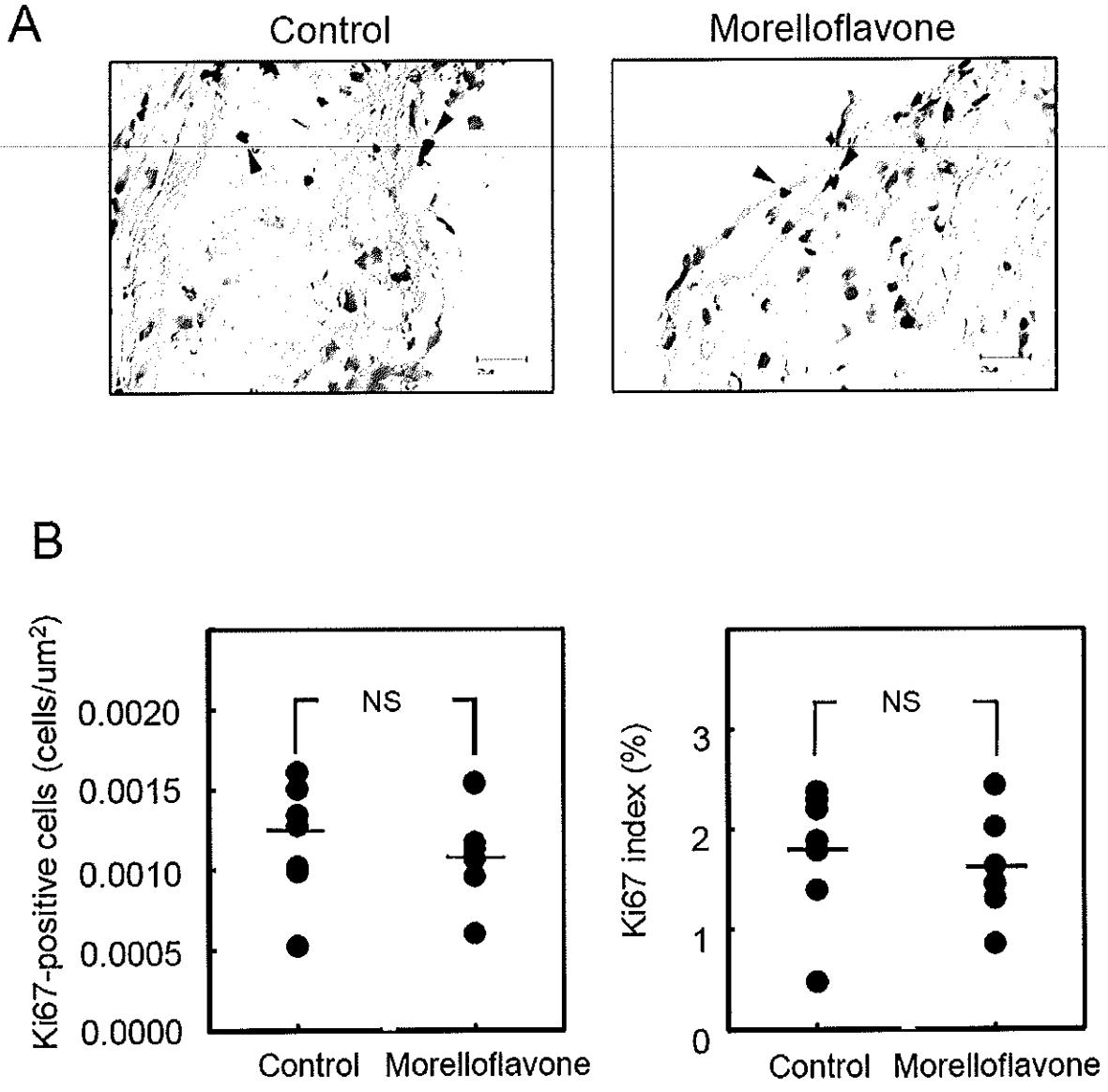


Figure 42. Ki67-positive cells and Ki67 index. The sections (N=7) were stained for Ki67 and couterstained with hematoxylin (A). Quantitative analysis of Ki67-positive cells in atherosclerotic lesion (B).

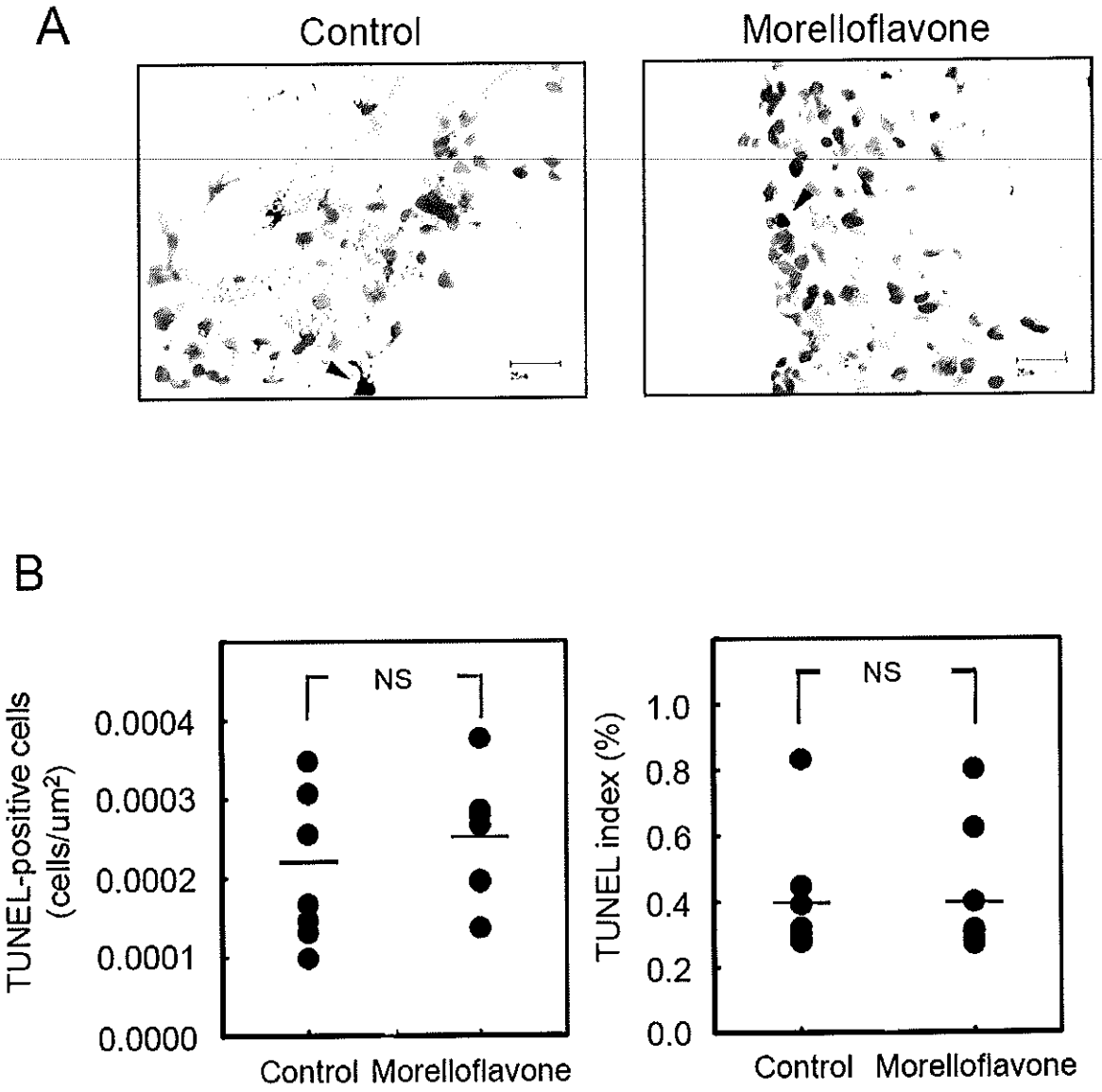


Figure 43. TUNEL-positive cells and TUNEL index. The sections (N=7) were stained for TUNEL staining and couterstained with hematoxylin (A). Quantitative analysis of TUNEL-positive cells in atherosclerotic lesion (B).

4.5 Discussion

Despite anti-inflammatory and anti-oxidative properties of morelloflavone, the biflavonoid from *Garcinia dulcis*, is well recognized (Gil *et al.*, 1997;. Sanz *et al.*, 1994; Deachathai *et al.*, 2006; Hutadilok-Towatana *et al.*, 2007), its effects on aorta have not yet been investigated. In the present study, the cardioprotective activities of morelloflavone were demonstrated for the first time in the genetic deficient animal model of atherosclerotic disease. ApoBEC-1/LDLR-DKO mice are the combination of apoB mRNA editing catalytic polypeptide-1 (apoBEC-1) deficiency and deletion of LDL receptors. Deletion of LDLR in mice leads to modest hypercholesterolemia and do not develop considerable atherosclerotic lesions when maintained on normal diets (Powell-Braxton *et al.*, 1998). This comes from the fact that the mouse liver produces lipoprotein containing a truncated form of apolipoprotein B48 due to the action of apoBEC-1 (Davison *et al.*, 1995a; Davison *et al.*, 1995b). Unlike LDLR-/- mice, these double genetically manipulated mice, deficient for both apoBEC-1 and LDLR, have markedly increased plasma cholesterol concentrations and develop extensive lesions throughout the aorta including most of the branch points that mirror pathophysiology of human familial hypercholesterolemia (Powell-Braxton *et al.*, 1998). These unique features, however, were also observed in the present study and thus confirms that apoBEC-1/LDLR-DKO mice can be used as an experimental tool to address how morelloflavone affects atherosclerosis.

Obviously, morelloflavone, given orally at a relatively low dose, was able to decrease formation of atherosclerotic lesions in aortas of apoBEC-1/LDLR-DKO mice fed with normal diet (Fig. 36-39) without altering their lipids metabolism (Table 4). This is not so surprising as cholesterol-lowering effect of flavonoids appears minimal in general (Gross, 2004). Unlike, probucol, a well-known phenolic antioxidant that attenuates atherogenesis and also protects coronary arteries from restenosis after angioplasty in humans and animals (Choy *et al.*, 2005), morelloflavone attenuated atherosclerosis without affecting the cellular composition and proliferation in atherosclerotic cells (Fig. 41-43). Collectively, such findings and that morelloflavone significantly decreased VSMCs numbers in atherosclerotic

lesions (Fig. 40) as well as its inhibitory activity on VSMC migration (chapter 3) suggest that morelloflavone may exerts anti-atherosclerotic activity through the inhibition of VSMC migration from media to intima, independent of inhibition of lipid oxidation which is commonly considered a cause of atherosclerosis (Stocker and Keaney, 2004). Lesions of atherosclerosis are only found in the intima, resulting from the comprising of smooth muscle cells in these lesions (Schwartz *et al.*, 1995). This simple fact implies that formation of the intima, presumably by migration of cells from the media, is a key event in the early stages of atherosclerosis. In previous study (chapter 3), morelloflavone was demonstrated to potently block VSMC migration by inhibiting the phosphorylation of FAK, c-Src, ERK, and activating RhoA. Therefore, with this inhibitory ability of morelloflavone, the vessels would be protected.

Cell proliferation and cell death are key processes in the progression of atherosclerosis (Ross, 1993). Proliferation of smooth muscle cells and endothelial cells and infiltration of macrophages contribute to lesion development (Ross, 1993). Cell death including apoptosis has been postulated to play an important role in the development and homeostasis of complicated lesions. Apoptotic cell death occurs in plaques, both in humans and in animal models (Kochx, 1998; Geng *et al.*, 1995; Isner *et al.*, 1995). In this work, morelloflavone did not decrease both apoptosis (TUNEL-positive cells) and proliferating cells (Ki67-positive cells). Therefore, neither decreased cell proliferation nor decreased apoptosis was accounted for the reduction in atherosclerosis by this compound when given orally to apoBEC-1/LDLR-DKO mice.

Over the past decade, inflammation has been considered as a primary process of atherosclerosis (Libby, 2002). In this regard, morelloflavone which inhibits inflammation *via* secretory phospholipase A2 and myeloperoxidase inhibition (Gil *et al.*, 1997) may play a protection role against atherosclerosis. Also, its anti-migratory activity may affect migration of blood-borne inflammatory cells into local tissue (Luster *et al.*, 2005).

In conclusion, morelloflavone was shown to prevent the progression of atherosclerosis in apoBEC-1/LDLR-DKO mice without affecting macrophages, cellular proliferation and apoptosis, as well as plasma lipids profile. Therefore, a decrease in smooth muscle cells in atherosclerotic lesions is likely to be the

underlying prevention mechanism of morelloflavone from atherosclerosis in this model. These suggest that upon further studies, morelloflavone or its derivatives are promising agents against atherosclerosis that may be important contributors in the prevention of cardiovascular disease.

CHAPTER 5

CONCLUSIONS AND FUTURE STUDIES

5.1 Conclusions

Morelloflavone inhibited VSMC migration without inducing apoptosis or cell cycle arrest in cell culture model and could prevent the development of intimal hyperplasia in induced-injury apoE-KO mice. Its anti-VSMC activity was found to be mediated through inhibition of migration-related kinases including FAK, c-Src, ERK, and RhoA. The potential of morelloflavone to attenuate the progression of atherosclerosis was also demonstrated in apoBEC-1/LDLR-DKO mice. In this animal model, morelloflavone could attenuate the progression of atherosclerosis without affecting plasma lipids profile or decreasing accumulation of macrophages in lesions. Rather, it exerted the anti-atherosclerotic activity by blocking VSMC migration.

5.2 Future studies

1. In atherosclerosis, blood-borne monocytes transmigrate across the endothelial monolayer and differentiate into tissue macrophages that progressively accumulate in the atherosclerotic plaque and take up modified LDL to form foam cells and, eventually, large lipid cores (Ludewig and Laman, 2004). Therefore, if monocyte transmigration is blocked, the progression of atherosclerosis could be retarded. It is interesting to evaluate such ability of morelloflavone on atherogenesis in both cellular systems and experimental animals.

2. Cancer metastasis requires the movement of cancerous cells from one site to another. It has been shown that small GTPases-such as RhoA, Cdc42, and Rac1, integrin-containing focal adhesion assembly and disassembly, secreted and

plasma membrane-tethered proteases, and the actomyosin contractile machinery, all play critical regulatory roles in cancer cell migration (Gupta and Massague, 2006). If morelloflavone negatively regulates the migration and motility of cancerous cells, and thus reducing cancer metastasis. Again, animal studies are called for in order to elucidate the effect of morelloflavone in the complex mechanisms of cancer metastasis (Condeelis and Segall, 2003).

3. Further investigation on morelloflavone absorption and its metabolism *in vivo* is another research area that may allow us to better understand the role of this compound in the prevention and also to develop a unique strategy against these chronic diseases.

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List of Publications and Proceedings

Publications:

1. Fujita, T., Felix, K., **Pinkaew, D.,** Hutadilok-Towatana, N., Liu, Z., and Fujise, K. 2008. Human fortilin is a molecular target of dihydroartemisinin. FEBS. Lett. 582, 1055-1060.
2. Decha-Dier, U., Hutadilok-Towatana, N., Sawangjaroen, K., Mahabusarakam, W., and **Pinkaew, D.** 2008. Anti-atherogenic effects of morelloflavone from *Garcinia dulcis* leaves in hypercholesterolemic rabbits. J. Nat. Rem. 8(2), xxx-xxx.
3. **Pinkaew, D.**, Cho, S.G., Hui, D.Y., Wiktorowicz, J.E., Hutadilok-Towatana, N., Mahabusarakam, W., Tonganunt, M., Stafford, J., Phongdara, A., Liu, M. and Fujise, K. 2008. Morelloflavone blocks injury-induced neointimal formation by inhibiting vascular smooth muscle cell migration. J. Mol. Cell. Cardiol. (accepted).

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