

A STUDY OF THE PUTATIVE ANTI-ATHEROGENIC
MECHANISMS OF VITAMIN E AND VITAMIN C
IN SUBJECTS AT CORONARY RISK

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ABSTRACT

A Study Of The Putative Anti-Atherogenic Mechanisms Of Vitamin E And Vitamin C In Subjects At Coronary Risk

Julie C Williams

Epidemiological evidence suggests that populations with high intakes of antioxidant vitamins have a lower risk of cardiovascular disease, and animal studies have demonstrated that antioxidants can inhibit atherogenesis. In this thesis the effects of the antioxidant vitamins E and C on monocyte adhesion, platelet adhesion and platelet aggregation has been examined *in vitro* on cells isolated from healthy volunteers, and *ex vivo* using cells isolated from patients at coronary risk.

Preliminary *in vitro* investigations revealed that; pre-incubation of platelets with vitamin E inhibited platelet aggregation ($p<0.05$), platelet adhesion ($p<0.05$) and reduced platelet membrane microviscosity ($p<0.05$). Pre-incubation of platelets with vitamin C, however, failed to significantly affect platelet adhesion. Pre-incubation of monocytes with vitamin E inhibited their subsequent adhesion to plastic ($p<0.05$), whilst pre-incubation of the endothelial cells with vitamin E also significantly reduced subsequent monocyte adhesion ($p<0.05$).

Twenty eight patients, with a diagnosis of primary hypercholesterolaemia received placebo (soybean oil) for six weeks, followed by vitamin E at a dose of 400 IU per day. Following six weeks of vitamin E supplementation thrombin induced platelet aggregation was significantly inhibited ($p<0.01$), while monocyte adhesion remained unaffected.

In fifty six untreated elderly hypertensive and normotensive volunteers, monocyte adhesion to collagen coated microwells was significantly correlated with daytime pulse pressure ($r = 0.38$, $p<0.01$). Forty of these subjects were randomly allocated to a crossover trial of vitamin C 500 mg daily versus placebo each for 3 months. Vitamin C supplementation significantly reduced daytime systolic blood pressure ($p<0.05$) and mean arterial blood pressure ($p<0.05$) in the elderly hypertensive subjects. Platelet adhesion to collagen coated ($p<0.05$) and tissue culture plastic microwells ($p<0.01$) was also reduced in the elderly normotensive subjects. Monocyte adhesion was not significantly affected by vitamin C supplementation.

Eighty seven patients undergoing routine coronary angioplasty were randomly allocated to receive either placebo ($n = 42$) or vitamin E ($n = 45$) at a dose of 800 IU per day prior to the angioplasty procedure and for at least six months after. Neither placebo nor vitamin E supplementation had any significant effect on monocyte or platelet adhesion. However, vitamin E supplementation did prevent the rise in plasma levels of soluble P-selectin following angioplasty ($p<0.05$).

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ABBREVIATIONS

AA	arachidonic acid
ADP	adenosine diphosphate
apoB	apolipoprotein B
BHT	butylated hydroxytoluene
BMI	body mass index
BSA	bovine serum albumin
β-TG	β-thromboglobulin
CABG	coronary artery bypass graft
CD	cluster differentiation
CHD	coronary heart disease
CHOD-PAP	
CuSO ₄	copper sulphate
DBP	diastolic blood pressure
DG	diacylglycerol
DNA	deoxyribonucleic acid
dH ₂ O	deionised water
DPH	1,6-diphenyl-1,3,5-hexatriene
EDRF	endothelium derived growth factor
ELISA	enzyme linked immunosorbent assay
FCS	foetal calf serum
bFGF	basic fibroblastic growth factor
Gp	glycoprotein
GPO-PAP	
G proteins	guanosine triphosphate-binding regulatory proteins
HDL	high density lipoprotein
12-HETE	12-hydroxyeicosatetraenoic acid
HPLC	high pressure liquid chromatography
HOCl	hypochlorite
HRP	horse radish peroxidase
HT	hypertensive
ICAM	intercellular adhesion molecule
Ig	immunoglobulin
IGF-1	insulin-like growth factor-1
IL	interleukin
IP ₃	inositol 1,4,5-triphosphate
LDL	low density lipoprotein
LPS	lipopolysaccharide
MAP	mean arterial pressure
MCP-1	monocyte chemoattractant protein-1
MCSF	macrophage colony stimulating factor

MI	myocardial infarction
MIP-1 β	macrophage inflammatory protein-1 β
mmLDL	minimally modified low density lipoprotein
MNC	mononuclear cell
Na ₂ CO ₃	sodium bicarbonate
NaOH	sodium hydroxide
NO	nitric oxide
NT	normotensive
O ₂ [•]	superoxide
Ox LDL	oxidised low density lipoprotein
PBS	phosphate buffered saline
PDGF	platelet derived growth factor
PF4	platelet factor 4
PGI ₂	prostacyclin
PIP ₂	phosphatidyl-inositol-4,5-biphosphate
PKC	protein kinase C
PLA ₂	phospholipase A ₂
PLC	phospholipase C
PP	pulse pressure
PPP	platelet-poor plasma
PRP	platelet-rich plasma
PTCA	percutaneous transluminal coronary angioplasty
PUFA	polyunsaturated fatty acids
RNA	ribonucleic acid
ROS	reactive oxygen species
SBP	systolic blood pressure
sE-selectin	soluble E-selectin
sICAM-1	soluble ICAM-1
sL-selectin	soluble L-selectin
SOD	superoxide dismutase
sP-selectin	soluble P-selectin
TGF- β	transforming growth factor β
TMB	tetramethyl benzidine
TNF	tumour necrosis factor
TXA ₂	thromboxane A ₂
TXB ₂	thromboxane B ₂
vWF	von Willebrand factor

α	alpha
β	beta
λ	wavelength of light (nm)
η	apparent microviscosity
%	percentage

ANOVA	analysis of variance
°C	degrees Celsius
CI	confidence interval
conc	concentration
Da	dalton
EC ₅₀	Effective concentration required to produce a response which is half of the maximal response
g	acceleration due to gravity
G	grating factor
h	hour
IU	international unit
kg	kilogram
mg	milligram
l	litre
ml	millilitre
µl	microlitre
log	logarithm to the base 10
LTU	light transmission unit
m	metre
mm	millimetre
µm	micrometre
nm	nanometre
mm Hg	unit of pressure equal to one millimetre of mercury
mol	amount of substance containing Avogadro's number of particles
n	number
OD	optical density
p	probability
P	fluorescence polarisation
QC	quality control
r	correlation coefficient
<i>r</i>	fluorescence anisotropy
r ²	coefficient of determination
rpm	revolutions per minute
s	seconds
SD	standard deviation
SEM	standard error of the mean
t	time
Tmax	maximum light transmission
U	units
UV	ultraviolet
vs	versus
v/v	volume to volume ratio
w/v	weight to volume ratio

CHAPTER 1

GENERAL INTRODUCTION

1.1 ATHEROSCLEROSIS, AN INTRODUCTION.

Atherosclerosis, a term taken from Greek meaning '*athero*' (gruel) and '*sclerosis*' (hardening), is a focal intimal disease of arteries, involving the participation of multiple cell types including smooth muscle cells, endothelial cells, inflammatory cells and platelets. The atherosclerotic process, is proposed to be a response to insults of the endothelium and smooth muscle cells of the wall of the artery (reviewed by Ross 1986), and begins in early life. By the third decade the characteristic fibrous or fibrolipid plaques are almost ubiquitous in western populations. Distribution of the disease is far from uniform; some large arteries such as the internal mammary are mainly spared while others such as the coronary arteries are at high risk of being affected.

1.1.1 Epidemiology.

Despite accumulating knowledge about the pathogenesis of atherosclerosis, it continues to be responsible for nearly half of all deaths of the adult population in western society. Complications of atherosclerosis, which arise because of a lack of blood supply to tissues supplied by the occluded artery, include coronary heart disease (CHD), stroke, aneurysm, peripheral vascular disease or gangrene. In the United Kingdom coronary heart disease is the single most common cause of death in both men and women.

1.1.2 Risk Factors.

Atherosclerosis is a disease of multifactorial causation and the combination of risk factors can be used to predict an individual's probability of developing this disease. A risk factor may be defined as 'a habit or trait or abnormality associated with a sizeable increase in susceptibility to disease' (Starnler *et al.* 1972). Risk factors for atherosclerosis include plasma cholesterol concentration, cigarette smoking, elevated blood pressure, diabetes and obesity (Table 1.1).

Table 1.1 Principal risk factors for atherosclerosis.

Risk factors	References
Unalterable risk factors	
Age	Stary 1989
Gender	Lerner & Kannel 1986
Race	Tejada <i>et al</i> 1968
Family history	Colditz <i>et al</i> 1986
Genetic	Brown & Goldstein 1986
(i.e. Familial hypercholesterolaemia)	
Hyperhomocysteinaemia	Alfthan <i>et al</i> 1997
Partially alterable risk factors	
Diabetes	Kannel <i>et al</i> 1991
Hypertension	Kannel <i>et al</i> 1969
Obesity	Hubert <i>et al</i> 1983
Blood fibrinogen levels	Ernst 1993
Alterable risk factors	
Raised plasma total cholesterol	Keys 1980
Raised plasma triglycerides	Austin 1991
Decreased plasma HDL cholesterol	Miller <i>et al</i> 1977
Cigarette smoking	Doll & Peto 1976
Diet	Ulbricht & Southgate 1991
Physical inactivity	Paffenbarger <i>et al</i> 1986

1.1.3 Treatment.

1.1.3.1 Lifestyle changes.

The objective of treatment is to relieve symptoms and slow down the progression of plaque development. The progression of atherosclerotic plaques can be slowed down by alteration of risk factors such as diet and exercise and by the cessation of smoking. Alterations in diet, especially the reduction of dietary fat, can result in reductions in total cholesterol of up to 20% in men, though the effects in women are not so great (Keys & Parlin 1966). Other influential dietary factors include the availability of antioxidant vitamins and minerals. Increased physical activity will improve general physical fitness, with benefits to the heart in lowering blood pressure and pulse rate. Exercise can also have a positive effect on triglyceride levels and HDL concentrations (Manson *et al.* 1992). The risk also improves on the cessation of smoking, so that after 3-5 years risk is similar to those who never smoked (Doll & Peto 1976)

1.1.3.2 Drug therapy.

One of the main aims is to lower lipid levels and if necessary can be achieved by the use of drugs such as bile acid binding resins, fibrates, nicotinic acid and statins. Angina can be controlled by drug therapy such as nitrates, beta-adrenoreceptor blockers, calcium channel blockers and potassium channel openers.

1.1.3.3 Surgical intervention.

In advanced cases, however, surgical intervention may be required and could comprise coronary artery angioplasty or coronary artery bypass grafting (CABG).

1.1.3.3.1 Percutaneous transluminal coronary angioplasty.

Percutaneous transluminal coronary angioplasty (PTCA) is an alternative to coronary bypass surgery for some patients with angina. The first PTCA was performed in September 1977 by Grüntzig following his modification of a dilatation catheter for use in coronary arteries (Grüntzig 1978). This technique involves a catheter system being introduced via the femoral artery under local anaesthesia. The guiding catheter carries a narrow bore (0.5 - 1.25 mm diameter) dilatational catheter with a sausage-shaped distensible segment. This segment can be inflated with fluid to an outer diameter of around 4 mm, thereby dilating the lumen of the artery and compressing the atherosclerotic material in the wall. Although the immediate success rate is greater than 90% (Grüntzig 1987), acute closure and longer-term recurrence of the stenosis are major problems and occur in a substantial proportion of the patients, reaching clinical significance in about 30% of cases within the first 6 months (Holmes *et al.* 1984).

1.1.3.3.2 Coronary bypass surgery.

Coronary artery bypass surgery involves bypassing the occluded segment of artery by engrafting a section of the patient's saphenous vein or internal mammary artery. Reactive oxygen species (ROS) mediated injury during reperfusion is a problem following bypass surgery (Granger *et al* 1981; Barsacchi *et al* 1992). While long-term limitation to the outcome is the susceptibility of the new graft to atherosclerosis, which may also become partially or totally occluded. Evidence suggests that internal mammary

arteries are more resistant to the development of atheroma and as such are increasingly becoming the vessel of choice (Grondin *et al.* 1984).

Several studies have compared PTCA with coronary bypass surgery and have yielded consistent results (RITA Trial Participants 1993; King *et al.* 1994; Pocock *et al.* 1995; The Bypass Angioplasty Revascularization Investigation (BARI) Investigators 1996). Major ischemic complications, such as death or myocardial infarction, occur with similar frequencies one to five years following either procedure. The main difference being the increased requirement for repeated revascularization in patients who initially underwent PTCA.

1.2 PATHOLOGY OF ATHEROSCLEROSIS.

The normal artery possesses a trilaminar structure comprised of the intima, media and adventitia. The tunica intima consists of a monolayer of endothelial cells lining the luminal aspect. Beneath the endothelium is a condensed layer of extracellular matrix, the basement membrane, composed primarily of type IV collagen with attached proteoglycan molecules specifically of heparin sulphate type. Beyond this in large vessels is a continuous but fenestrated elastin-rich layer, the internal elastic lamina, which provides a boundary to the intima and separates it from the next layer the media. The tunica media is bounded by the internal and external elastic laminae. These laminae are fenestrated sheets of elastic fibres with numerous openings to allow metabolites and cells to pass in either direction. The media consists of spiralling layers of smooth muscle cells attached to one another surrounded by a discontinuous basement membrane and by interspersed collagen fibrils and dermatan sulphate proteoglycans. Outside the media lies the tunica adventitia which is a dense structure containing numerous collagen fibrils, elastic fibres and many fibroblasts, together with some smooth muscle cells. It is a highly vascular tissue and contains many nerve fibres as well.

1.2.1 Lesion Progression In Atherosclerosis.

The intima is the site at which the lesions of atherosclerosis form. It is here within the setting of endothelial dysfunction, a complex interaction of cytokines, growth factors and other biologically active molecules interact with endothelial cells, smooth muscle cells, monocytes, macrophages and platelets to orchestrate the progression of atherosclerosis from fatty streak through to complicated lesion.

1.2.1.1 The fatty streak.

What constitutes the earliest event or events of human atherogenesis is still a matter of debate. A number of animal models suggest that one of the earliest structural changes is the adhesion of monocytes to the endothelial surface followed by entry into the subendothelial tissues (Gerritty 1981; Joris *et al.* 1983; Faggiotto & Ross 1984a). Once within the subendothelial space they can ingest large amounts of lipids and be converted to the characteristic lipid-laden 'foam cells' (Gerritty *et al.* 1979, Faggiotto & Ross 1984a). However, a general consensus exists that the fatty streak is the earliest macroscopically recognisable lesion. These early lesions are slightly raised and start as small round or oval, yellowish dots (1-2 mm in diameter). They tend to occur in rows roughly parallel to the streamlines of the flowing blood and coalesce to form streaks along the axes of the affected artery. Work by Stary (Stary 1989) provided great insight into the progression of these lesions. He undertook detailed studies of the arteries of young subjects, from infancy to 30 years of age, who died suddenly from non-cardiac disease. He found that fatty streaks were present in the coronary arteries of half of the autopsy specimens from children aged 10-14 years. Immunocytochemical studies (Tsudaka *et al.* 1986) have shown that the fatty streak consists mainly of lipid-laden macrophages and T-lymphocytes, together with small and variable numbers of smooth muscle cells.

There is some controversy whether fatty streaks truly represent an initial change in the atherogenic process. Stary himself argues that it may be an adaptive response and not necessarily early atherosclerosis, however, it may act as a sensitised area for later development should the appropriate, or rather inappropriate conditions arise. In support of this not all fatty streaks progress, as can be seen in necroscopy surveys of infants from populations in which advanced atherosclerosis does not develop even though they show large numbers of fatty streaks (Restrepo & Tracy 1975).

1.2.1.2 Fibrolipid plaques.

However, these fatty streaks can evolve into fibro-fatty plaques, in susceptible individuals or those exposed to multiple risk factors (Faggiotto & Ross 1984b). Atherosclerosis progression is associated with changes including the appearance of extracellular lipid, which ultimately develops into the core of the lesion. Smooth muscle cells also migrate and proliferate within the plaque forming a cap over the luminal side of the lipid core. The medial smooth muscle cells elaborate collagen and other extracellular matrix molecules, the plaque size is increased producing what is referred to as a fibrolipid or advanced plaque. The typical fibrolipid plaque or lesion has a core of extracellular lipid together with numerous macrophages and T-lymphocytes which is separated from the media by smooth muscle cells and covered and separated by a thick cap of collagen-rich fibrous tissue containing smooth muscle cells. Some plaques are densely fibrous and contain relatively little lipid, whereas others are rich in lipid deposits and are often associated with different risk factors.

It is the raised fibrolipid or advanced atherosclerotic plaque on which complications develop. They are also associated with the accompanying clinical symptoms. Unlike the fatty streak, the presence of fibrofatty lesions appears to predict the severity and frequency of clinical manifestations of atherosclerosis (Deupree *et al.* 1973). The composition of the fibrolipid plaque dictates its susceptibility to rupture or fissure (Richardson *et al.* 1989); lipid-rich plaques are more likely to rupture with subsequent thrombosis, than the more fibrous plaques, which seem to have some degree of stability conferred upon them as a consequence of their fibrous nature.

1.2.1.3 Complicated lesions.

The plaques may also undergo calcification, which, if it occurs, is deep within the intima close to the base of the plaque and is seen as a thin plate of calcium within the fibrous

tissue. These fibrous plaques which have become involved with thrombosis and/or calcification are often called complicated lesions.

1.2.2 Complications Of Atherosclerosis.

Atherosclerotic plaque fissure or rupture plays a fundamental role in the development of the acute coronary syndromes and clinical symptoms (Falk 1985). It is probable that mild injury to the vessel wall produces transient thrombotic occlusion. While deep vessel injury results in exposure of collagen, lipids and other elements of the vessel media leading to relatively persistent thrombotic occlusion (Willerson *et al.* 1984). Complications of atherosclerosis arise from gradual or sudden occlusion of the affected artery and from haemorrhage (Table 1.2). Gradual occlusion of an artery results in ischaemia or temporary lack of nutrients and oxygen to the cells supplied by that artery. If the affected artery is a coronary artery the outcome would be angina pectoris, the clinical symptoms of which are chest pain or tightness brought about by exertion or emotion, or may occur spontaneously at rest. In other arteries hypertension (renal artery), intermittent claudication (e.g. femoral artery) or dementia (cerebral artery) can result.

Sudden occlusion of an artery may be due to thrombosis and/or embolism. If this occlusion is more than transient or the tissues metabolic requirements cannot be met then the outcome will be cell death or infarction. In the coronary arteries the result could be heart failure, myocardial infarction or sudden death. The pain of myocardial infarction is similar to that of angina pectoris but does not subside with rest or nitrate therapy and may last for several hours. In some arteries the wall becomes weakened by the atherosclerotic plaque and is susceptible to leakage or rupture, with the consequent haemorrhage being life-threatening as in the case of stroke or when an aortic aneurysm bursts.

Table 1.2 Complications of atherosclerosis arising from gradual or sudden occlusion of the affected artery and from haemorrhage.

Artery affected	Symptoms/disease outcome		
	Gradual occlusion	Sudden occlusion (thrombosis, embolism)	Haemorrhage
Coronary	Angina	Myocardial infarction	
Cerebral	Dementia	Stroke	Stroke
Aorta			Aneurysm
Peripheral	Intermittent claudication, gangrene		
Renal	Hypertension		

1.3 CELLULAR PARTICIPANTS OF ATHEROGENESIS.

1.3.1 The Vascular Endothelium.

1.3.1.1 Normal function

The endothelium forms a critical interface between the circulating blood and the artery wall and is the most extensive tissue in the body forms. Endothelial cells are approximately 50 μm in length and 0.5 to 1.2 μm in thickness. As with other eukaryotic cells they contain mitochondria, rough and smooth endoplasmic reticulum, Golgi bodies and lysosomes. Additional structures that seem to be unique to endothelial cells are the tubular bodies referred to as Weibel-Palade bodies (Weibel & Palade 1964). Various functions have been proposed for Weibel-Palade bodies including production of coagulative substances, (Burri & Weibel 1968) containment of factors that can have an effect on blood pressure (Bertini & Santolaya 1970) and as a site of storage of von Willebrand factor (Warhol & Sweet 1984).

The endothelium, once thought to act solely as the internal lining of blood vessels has since been ascribed numerous functions including modulation of inflammation, regulation of vascular tone, both promotion and inhibition of vascular growth and modulation of coagulation.

Table 1.3 Functions of the vascular endothelium

Functions	Mechanisms	References
Regulation of vascular tone	Production of vasodilators including PGI ₂ , EDRF/NO, low levels of oxygen free radicals (OFR). Production of vasoconstrictors including endothelins, angiotensin II, high levels of OFR.	Moncada <i>et al</i> 1976a, Furchgott & Zawadzki 1980, Rubuyani 1988 Yangisawa <i>et al</i> 1988, Masaki <i>et al</i> 1991, Katsue & Vanhoutte 1989
Modulation of coagulation	Procoagulant activity. Regulation of fibrinolysis. Thromboregulation	Bevilacqua <i>et al</i> 1984, Latron <i>et al</i> 1991, Radomski <i>et al</i> 1987, Moncada <i>et al</i> 1977
Immunology and Inflammation	Production and secretion of ILs. Adhesion molecule expression of ICAM-1 and E-selectin	Lukacs <i>et al</i> 1995 Bevilacqua <i>et al</i> 1987
Vascular wall modelling	Promotion of growth by PDGF, bFGF and IGF-1. Inhibition of growth by collagen (type V) and glycosaminoglycans	DiCorleto & Bowen-Pope 1983, Baird & Böhlen 1991, Rechler & Nissley 1991 Cockwell <i>et al</i> 1996

1.3.1.2 The vascular endothelium in atherosclerosis

The normal endothelium has a number of functions including 1) a permeability barrier role; 2) provision of a non-thrombogenic, non-adherent surface; and 3) a source of vasoactive molecules, growth regulatory molecules and connective tissue matrix molecules. Endothelial dysfunction appears to be crucial in the initiation and development of atherosclerosis (Ross & Glomset 1976a; 1976b), a dysfunctional endothelium may result in an alteration in one or more of the functional characteristics. An early sign even before atherosclerosis is evident is the modification of the vasomotor response (Simon *et al* 1993). In atherosclerotic arteries, acetylcholine induces vasoconstriction, compared to the vasodilatory response seen in normal arteries (Ludmer *et al.* 1986). The inability of nitric oxide (NO) to exert its full effect in atherosclerosis (Freiman *et al* 1986), either as a result of degradation and/or reduced synthesis (Verbeuren *et al* 1990). Along with the decreased capacity of the vascular tissue to synthesise prostacyclin (PGI₂), may in part explain the modified vasomotor response. Defective synthesis and/or bioactivity of NO and PGI₂, which normally inhibit platelet adhesion, activation and aggregation (Moncada *et al* 1976a, 1976b; Radomski *et al* 1987), results in the conversion of a non-thrombogenic endothelial surface to one which is prothrombogenic.

The permeability of the endothelium may also be altered in atherosclerotic lesions and lesion prone areas (Bell *et al* 1974a; 1974b; Hoff *et al* 1983). This may lead to leakage of the plasma constituents into the artery wall including low density lipoprotein (LDL). Endothelial cells have the capacity to oxidatively modify plasma-derived LDL within the subendothelial space (Steinbrecher *et al.* 1984; Henriksen *et al* 1982). Oxidised LDL may contribute to the progression of atherosclerosis in numerous ways (refer to section 1.5.2).

Adhesion molecules such as VCAM-1 and ICAM-1 are believed to mediate monocyte adhesion in the developing lesion, and their expression is upregulated in atherosclerosis

(Poston *et al.* 1992; Davies *et al.* 1993). This enhanced expression, which may be a consequence of cytokine release within the atherosclerotic lesion (Bevilacqua *et al.* 1985; Pober *et al.* 1986), could lead to promotion of monocyte adhesion as seen in lesions of atherosclerosis (Gerrity 1981; Faggiotto & Ross 1984a). Cytokines released within the atherosclerotic lesion are also capable of increasing endothelial cell procoagulant activity by promoting production of plasminogen activator inhibitor (Emeis & Kooistra 1986) and platelet-activating factor (Bussolino *et al.* 1986).

Production of inhibitors of migration and proliferation such as NO (Garg & Hassid 1989; Dubey *et al.* 1995), heparin-like substances (Castellot *et al.* 1981) and transforming growth factor β (TGF β) (Hannan *et al.* 1988; Battegay *et al.* 1990) may also be impaired following endothelial injury. Injury could also promote the production of growth factors, including PDGF (DiCorleto & Bowen-Pope 1983; Barrett *et al.* 1984) culminating in the neointimal proliferation observed following balloon injury.

1.3.2 Smooth Muscle Cells.

1.3.2.1 Normal function

Vascular smooth muscle cells are 25-80 μm long. They have a single centrally placed nucleus and within the cytoplasm are mitochondria, rough endoplasmic reticulum, and Golgi complex. Although each smooth muscle cell is anatomically distinct, it is coupled electrically to other cells through gap junctions so that the whole area of smooth muscle behaves as a single unit. At least two different phenotypes are expressed by smooth muscle cells these being termed 'contractile' and 'synthetic' phenotypes respectively (Chamley-Campbell *et al.* 1981; Thyberg *et al.* 1983). The contractile phenotype has extensive myofibrils throughout their cytoplasm and as the name suggests are generally thought to be associated with contractility. In the synthetic phenotype there is a decreased content of myofilaments and an extensive rough endoplasmic reticulum and

Golgi complex has developed. These cells are capable of the formation of various secretory proteins including connective tissue matrix molecules.

1.3.2.2 Smooth muscle cells in atherosclerosis

Smooth muscle cells have been identified by immunocytochemical analysis in both fatty streaks and fibrous plaques (Tsukada *et al.* 1986). Within the locale of the lesion smooth muscle cells may adopt a synthetic phenotype, as phenotypic changes from contractile to synthetic have been demonstrated in various models of vascular injury (Clowes *et al.* 1985, 1986). Smooth muscle cells of a synthetic phenotype can respond to growth factors such as PDGF present within the lesion. Sources of PDGF include endothelial cells (Gajdusek *et al.* 1980; DiCorleto & Bowen-Pope 1983), macrophages (Shimokado *et al.* 1985), and platelets (Ross *et al.* 1974). Smooth muscle cells can also release growth factors such as PDGF and basic fibroblastic growth factor (bFGF), which can act in a autocrine manner to stimulate neighbouring smooth muscle cells or in a paracrine fashion on the adjacent endothelium (Walker *et al.* 1986; Libby *et al.* 1988).

PDGF is both mitogenic and chemoattractant for smooth muscle (Grotendorst *et al.* 1982) which may account for the migration of smooth muscle cells from the media seen in atherogenesis in response to arterial injury. PDGF also induces binding of LDL by increasing the number of LDL receptors (Chait *et al.* 1980), thereby promoting lipid accumulation and foam cell formation. Stimulation of smooth muscle cells by growth factors can also result in the synthesis of collagen (Barnes 1985), dermatan sulphate (Yla-Herttuala *et al.* 1986) and elastin (Ross 1971), altering the extracellular matrix composition of the atherosclerotic plaque. Hence through their proliferative capacity, synthetic ability and the accumulation of lipid, smooth muscle cells can contribute to the size and composition of the atherosclerotic lesion and its progression.

1.3.3 Monocytes And Macrophages.

1.3.3.1 Normal function

Approximately 5% of circulating white blood cells are monocytes. They are large cells of 16-22 μm in diameter with a kidney shaped nucleus and a scattering of delicate azurophilic granules in the cytoplasm. They belong to the mononuclear phagocytic system, a cell lineage which originates in the bone marrow and eventually transforms into tissue macrophages. Monocytes leave the bone marrow within 24 hours of formation, upon which they enter the circulation. They reside here for up to 71 hours by which time they enter the tissues, where they undergo maturation and differentiation to become macrophages.

Monocytes/macrophages participate in the inflammatory process. Monocytes enter the area of inflammation and become transformed into macrophages (literally 'big eaters') whereupon they engulf tissue debris and dead cells as well as micro-organisms. Together with neutrophils, macrophages are the main phagocytes in the body. Both types of phagocytic cells contain specialised organelles that fuse with newly formed phagocytic vesicles (phagosomes), exposing phagocytosed microorganisms to a barrage of enzymatically produced, highly reactive molecules of superoxide (O_2^\bullet) and hypochlorite (HOCl) as well as to a concentrated mixture lysosomal hydrolases. Macrophages are much larger and longer lived than neutrophils.

1.3.3.2 Monocyte adhesion

Monocyte adhesion to the endothelium is a dynamic process involving integral cell membrane proteins on both cells including selectins, integrins and members of the immunoglobulin superfamily (Table 1.4).

1.3.3.2.1 The Adhesion Molecules

Selectins

The selectins are a group of glycoproteins. The selectin family consists of E-selectin, P-selectin and L-selectin. All members of the selectin family contain a NH₂-terminal lectin domain, followed by an epidermal growth factor domain, several complement regulatory repeat sequences, a transmembrane domain and a cytoplasmic domain (McEver 1990; Springer & Lasky 1991; Bevilacqua & Nelson 1993). The selectins mediate the initial attachment of flowing leukocytes to the blood vessel wall, during the capture and rolling step of the adhesion mechanism. It is the lectin domain that is involved in the process of adhesion (Kansas *et al.* 1991; Bevilacqua & Nelson 1993).

Immunoglobulin supergene family.

The Immunoglobulin Supergene Family (IgSF) is the most abundant family of cell surface molecules, accounting for 50% of leukocyte surface glycoproteins. Members include ICAM-1, ICAM-2 and VCAM-1 (Williams 1988). A common trait of all members is that they have repetitive extracellular Ig-like domains, followed by a transmembrane domain and short cytoplasmic sequence. Members of the IgSF are involved in the firm adhesion of leukocytes to and transmigration through the vascular endothelium.

Integrins

Integrins are composed of α and β -subunits which form non-covalent heterodimer complexes. Up to now 14 α and 8 β subunits have been described (Hynes 1987). Members of the β_1 and β_2 subfamilies are expressed on monocytes and mediate leucocyte

binding to endothelial cells and matrix proteins. These members include Leukocyte Function Associated molecule-1 (LFA-1, CD11a/CD18), Mac-1 (CR3, CD11b/CD18) and p150,95 (Leu M5, CD11c/CD18).

1.3.3.2.2 *The Adhesion Process*

Monocyte adhesion can be divided into three phases; tethering, triggering and strong adhesion (Butcher 1991) followed by transendothelial migration (Figure 1.1). Tethering is mediated by the selectin molecules (L-selectin, E-selectin and P-selectin) which by virtue of their long molecular structure extend beyond the surrounding glycocalyx allowing them to capture passing leucocytes in the circulation which express the appropriate receptor (Lasky 1992). Selectins mediate a degree of adhesion sufficient to induce rolling along the vessel wall, but not strong enough to stop leucocytes completely. This transient adhesion allows the leucocytes to sample the local endothelium for the presence of specific factors, which can activate leucocyte integrins, if the required 'triggering' factors are not present the leucocyte will disengage and move on.

Strong adhesion of leucocytes is mediated by integrins, which bind to their counterpart receptor on the endothelium. Integrins do not bind well unless they are activated, referred to as the 'triggering' step. The list of triggers includes tumour necrosis factor (Pober *et al* 1986a, 1986b), intercrines such as interleukin-8 (IL-8) (Rot 1992), macrophage inflammatory protein-1 β (MIP-1 β) (Tanaka *et al.* 1993) and possibly even E-selectin, which appears to be able to both 'trigger' and 'tether' (Lo *et al.* 1991). The pathways used by leucocytes to bind to the activated endothelium depend on the site and nature of the endothelial-activating stimulus. Increased ICAM-1 expression has been observed in atherosclerotic plaques (Poston *et al.* 1992; Davies *et al.* 1993) and also on the endothelium of atherosclerotic lesions (Van der Wal *et al.* 1992). Expression of VCAM-1 has also been demonstrated in human coronary atherosclerosis (Davies *et al.* 1993; O'Brien *et al.* 1993).

Following strong adhesion to the endothelium, monocytes migrate into the tissue (Gerrity 1981; Schwartz *et al.* 1984). The presence of monocyte chemotactic protein-1 (MCP-1) which can be produced by the endothelial cells appears to enhance the process *in vitro* using a co-culture model (Navab *et al.* 1992). Whilst other studies have suggested that transient increases in endothelial permeability accompany leucocyte transmigration (Territo *et al.* 1984; Huber & Weiss 1989).

Studies using monoclonal antibodies and selectin- and ICAM-1- 'knock-out' mice support this proposed sequence of events during monocyte adhesion. Mice lacking P-selectin have leucocytes with reduced ability to roll across the endothelium of mesenteric vessels and a diminished inflammatory response (Bullard *et al.* 1995). A reduction in leucocyte accumulation has also been observed in mice deficient in E-selectin (Labow *et al.* 1994). Single 'knock-out' of either selectin could not prevent leucocyte migration. However, administration of anti-P-selectin antibody to E-selectin 'knock-out' mice completely prevented neutrophil migration (Labow *et al.* 1994), suggesting a complementary relationship between P-selectin and E-selectin in leucocyte rolling and adhesion. ICAM-1 deficient mice also exhibited impaired neutrophil migration in response to bacterial challenge (Sligh *et al.* 1993). A co-operation between selectin and ICAM-1 was supported by the finding that neutrophil migration was totally inhibited in P-selectin/ICAM-1 double-mutants (Bullard *et al.* 1995). These studies indicate the potential importance of cell adhesion molecules in leucocyte adhesion and migration.

1.3.3.3 Monocytes/macrophages in atherosclerosis

The role of monocytes/macrophages in foam cell formation during lesion development in atherosclerosis was suggested many years ago (Poole & Florey 1958). However, in the sixties opinion shifted towards a smooth muscle cell origin, mainly because electron microscopy had shown that smooth muscle cells were recognisable within the intima of

atherosclerotic lesions (Geer 1965). More recent evidence, particularly from the use of monoclonal antibodies, which recognise monocyte-derived macrophages, confirmed that foam cells of human atherosclerotic lesions are derived from blood monocytes (Mitchinson & Ball 1982; Aqel *et al.* 1985; Gown *et al.* 1986).

Although movement of leucocytes from the mainstream of blood to the affected tissue is a key and essential event in mediating the inflammatory event, any abnormal or uncontrolled leucocyte infiltration can contribute to the pathology of atherosclerosis. Monocyte adhesion is one of the earliest changes in the generation of the atherosclerotic plaque and can be seen experimentally within two weeks after pigs (Geritty 1981), primates (Faggiotto & Ross 1984a) or rats (Joris *et al.* 1983) are fed an atherogenic diet.

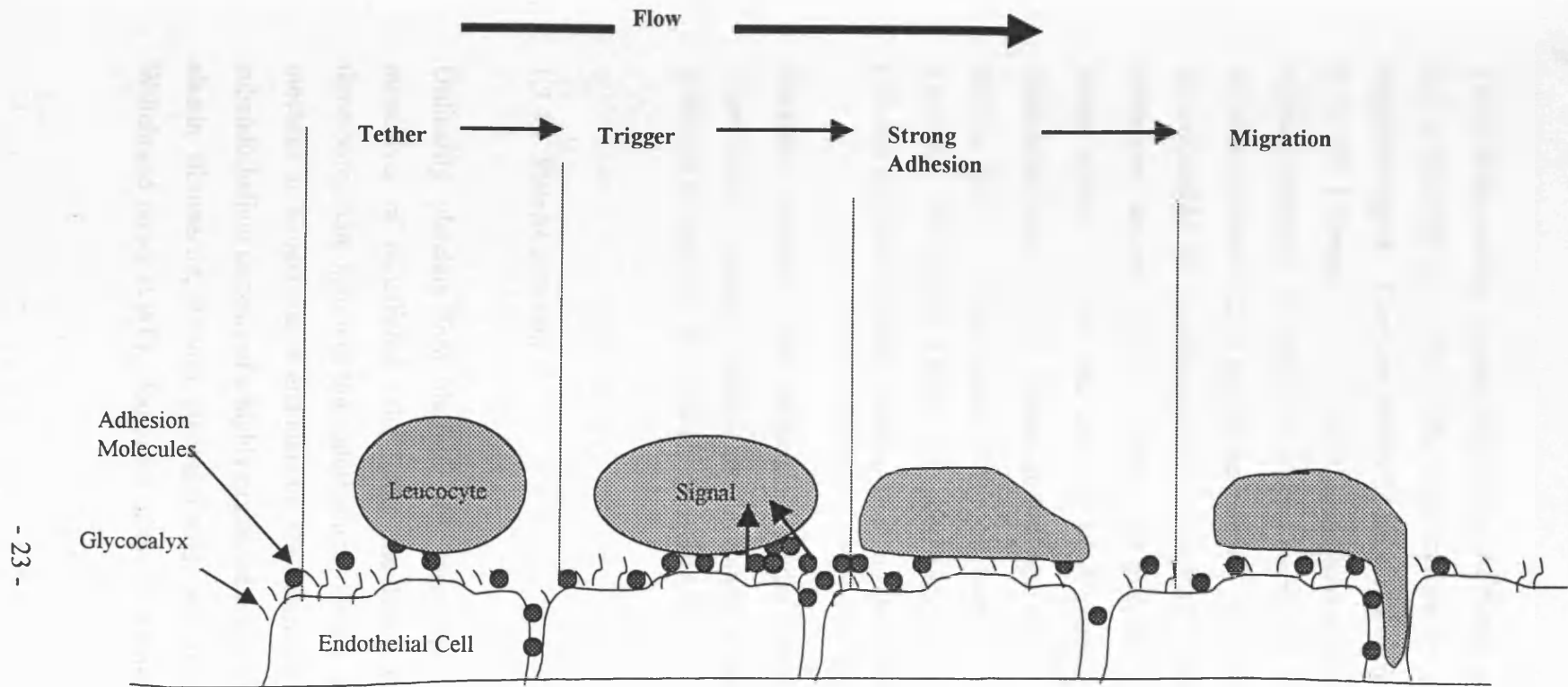
Increased monocyte adhesion is observed over lesion prone areas and injured endothelium (Hansson *et al.* 1980,1981). Adherent monocytes can undergo transendothelial migration into the subintima. Within the subintima, they may differentiate to become activated macrophages and ingest modified LDL. The resulting cells are called foam cells and are the major constituent of the fatty streak. Hence monocyte-derived macrophages can contribute to the bulk of the lesion by the accumulation of lipid (Brown & Goldstein 1983). Once activated, macrophages are capable of releasing a vast range of substances, including tumour necrosis factor (TNF), interleukin-1 (IL-1), enzymes, arachidonic acid metabolites, reactive oxygen radicals and growth factors including PDGF (Johnston & Kitagawa 1985; Halme 1989; Ross *et al.* 1990; Ziegler-Heitrock *et al.* 1992).

Growth factors including PDGF, which is both chemoattractant and mitogenic for smooth muscle cells (Grotendorst *et al.* 1982; Ross *et al.* 1974), can be produced by macrophages within the lesion (Ross *et al.* 1990). IL-1 and TNF are important cytokines produced by activated macrophages that can induce the expression of adhesion molecules promoting leucocyte-endothelial cell adhesion (Bevilacqua *et al.* 1984, 1985; Pober 1988). IL-1 is also capable of increasing endothelial cell production of procoagulant activity

(Bevilacqua *et al.* 1984, 1985), plasminogen activator inhibitor (Emmeis & Kooistra 1986) and platelet-activating factor (Bussolino *et al.* 1986) resulting in increased thrombogenicity of the endothelium contributing to the thrombotic complications of atherosclerosis. Macrophages also release many catabolic enzymes (Nathan 1987) including collagenase and elastase which may play a part in the connective tissue necrosis believed to occur at the base of the plaque. Oxygen radicals produced and released by macrophages can further oxidatively modify LDL (Cathcart *et al.* 1985, 1989; Leake & Rankin 1990) and can contribute to plaque progression through its various effects including foam cell formation and cytotoxicity (Shatos *et al.* 1991).

Adhesion Molecule	Cells on which expressed	Role	Ligand	Cells on which ligand is expressed	References
SELECTIN FAMILY					
E-Selectin	Endothelial cells	Mediates neutrophil adhesion to endothelium during capture and rolling	Sialyl Lewis carbohydrates	Monocytes	Bevilacqua <i>et al</i> 1987, 1989, DiCorleto & delaMotte 1989, Phillips <i>et al</i> 1990
L-Selectin	Neutrophils, Monocytes	Mediates neutrophil and monocyte binding to the endothelium during capture and rolling	Sialyl Lewis carbohydrates	Endothelial cells	Kansas 1992, Bery <i>et al</i> 1992, Lawrence & Springer 1991,
P-Selectin	Platelets, Endothelial cells, Megakaryocytes	Mediates adhesion of neutrophils and monocytes to activated platelets and endothelial cells	LNF III	Neutrophils, Monocytes, Platelets	Hattori <i>et al</i> 1989, McEver 1990, Larsen <i>et al</i> 1990
IMMUNOGLOBULIN FAMILY					
ICAM-1	Endothelial cells, Monocytes	Important in the adhesion of monocytes and neutrophils to activated endothelium during activation, flattening and extravasation	LFA-1 (CD11a/CD18) Mac-1 (CD11b/CD18)	Monocytes, Neutrophils	Rothlein <i>et al</i> 1986, Staunton <i>et al</i> 1988, Diamond <i>et al</i> 1990
VCAM-1	Endothelial cells	Mediates adhesion of monocytes to endothelium during activation, flattening and extravasation	VLA-4 (CD49d/CD29)	Lymphocytes, Monocytes	Osborn <i>et al</i> 1989, Elices <i>et al</i> 1990, Jonjic <i>et al</i> 1992

Table 1.4 Adhesion molecules involved in leucocyte adhesion to the vascular endothelium



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Figure 1.1 Sequential steps in adhesion of monocytes to the endothelium.

Firstly the flowing leucocytes is tethered and brought into contact with the endothelial wall by selectin mediated interaction. Tethering allows cytokine triggering of strong adhesion to the vessel wall. Subsequent migration is directed by chemokines, and possibly other cytokines (adapted from Adams & Shaw 1994)

1.3.4 Platelets.

Platelets the smallest formed elements of the blood are ovoid discs approximately 2 to 4 μm in diameter produced in the bone marrow by fragmentation of the cytoplasm of megakaryocytes. They are present in the circulation at concentration of approximately $2.5 \times 10^{11} \text{ l}^{-1}$ (range $1.5 - 4 \times 10^{11} \text{ l}^{-1}$), and have a lifespan of 7-10 days. Surrounding the platelet cytoplasm is a trilaminar plasma membrane with numerous invaginations, which increase the total surface area of the platelet. A circumferential skeleton of microtubules is responsible for maintenance of the normal circulating discoid shape. Within the cytoplasm, several types of granules are present: most numerous are α -granules and dense bodies. Among the contents of α -granules are platelet factor 4 (PF4), β -thromboglobulin (β -TG), platelet-derived growth factor (PDGF), fibrinogen and other clotting factors. Dense bodies contain calcium, serotonin and two adenine nucleotides: adenosine diphosphate (ADP) and adenosine triphosphate (ATP). The platelet also contains glycogen granules, lysosomes, RNA and mitochondria.

Platelets contribute to the maintenance of the normal circulation of blood through the preservation of vascular integrity and the control of haemorrhage after injury, through the processes of adhesion, activation and aggregation.

1.3.4.1 Platelet adhesion.

Ordinarily platelets flow through the vasculature without adhering to the intact monolayer of endothelial cells lining the blood vessels, as a consequence of the thromboresistant nature of the endothelium. Following vessel injury, whether chemical, mechanical, exogenous or endogenous, the subendothelial matrix may be exposed. The subendothelium consists of a highly organised matrix of molecules that includes collagen, elastin, fibronectin, laminin, glycosaminoglycans, thrombospondin, vitronectin and von Willebrand factor (vWF). Exposure of the components of the subendothelium triggers

platelet adhesion, the first step in the process of haemostasis (Harker & Ross 1979). A hypothetical model of platelet adhesion has been described (Sixma *et al.* 1995) which comprises an initial contact stage, a stabilisation phase, a platelet activation phase, platelet spreading and finally platelet aggregation. Coverage of the exposed site is mediated by a number of platelet surface receptors, most commonly those from the integrin family. These have high affinity for the adhesive glycoproteins found in the subendothelium and pathologic lesions. Platelets contain at least five different integrins, each with its own specificity for one or more extracellular matrix proteins. Platelets also carry other receptors of such as glycoprotein (Gp) Ib/IX a receptor for vWF and Gp IV a receptor for thrombospondin (Kieffer & Phillips 1990). Under conditions of high shear, discoid platelets utilise a mobile receptor complex, GpIb-IX, to adhere to subendothelial collagen fibres saturated with the plasma protein, von Willebrand factor (vWF) and provide the initial contact between platelets and the vessel wall (Kroll *et al.* 1991). The Gp IIb/IIIa integrin, in addition to its function in platelet aggregation, has a secondary role in platelet adhesion (Hantgan *et al.* 1990) under conditions of low shear, or under high shear when a monolayer of platelets has already formed on the denuded surface. Other glycoproteins that may contribute to platelet adhesion, include the integrin Gp Ia/IIa, which appears to be a principal platelet receptor for collagen (Saelman *et al.* 1994) and Gp IV, which may play a part in platelet-collagen interactions (Tandon *et al.* 1989).

A stabilisation phase follows this initial contact and the cells extend long pseudopodial extensions (White 1987). Other ligand receptor interactions help stabilise the platelet against the shear stress of the flowing blood including fibronectin and its receptor, collagen and Gp Ia/IIa and perhaps laminin and GpIc/IIa and thrombospondin and its receptor.

1.3.4.2 Platelet activation.

Platelet activation following adhesion is associated with the stimulation of several metabolic pathways, changes in the shape of the platelets, activation of Gp IIb/IIIa receptor and induction of procoagulant activity. The signal initiated by receptor occupancy by vWF and other platelet agonists such as thrombin, ADP, thromboxane A₂ (TXA₂) or collagen, which can be classified as weak or strong agonists, is then transmitted by the cytoplasmic domain of the receptor, frequently through guanosine triphosphate-binding regulatory proteins (G proteins). Two major pathways have been defined (Figure 1.2). The first of these is activation of membrane-bound phospholipase C (PLC) which stimulates the phosphoinositide pathway via hydrolysis of the membrane phospholipid, phosphatidyl inositol 4,5-bisphosphate (PIP₂). Cleavage of PIP₂ produces two second messengers inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DG) which result in the release of calcium from the platelet dense tubular system and activation of protein kinase C (PKC) respectively. Together they promote protein phosphorylation, platelet granule secretion and expression of the fibrinogen receptor, Gp IIb/IIIa, on the platelet surface. The other pathway involved is activation of phospholipase A₂ (PLA₂) which cleaves membrane phospholipids to release arachidonate. which is enzymatically oxygenated by cyclooxygenase and 12-lipoxygenase to produce TXA₂ and 12-HETE respectively. ADP and TXA₂, released as a result of the platelet release reaction, bind to their respective platelet receptors to further amplify the platelet activation process.

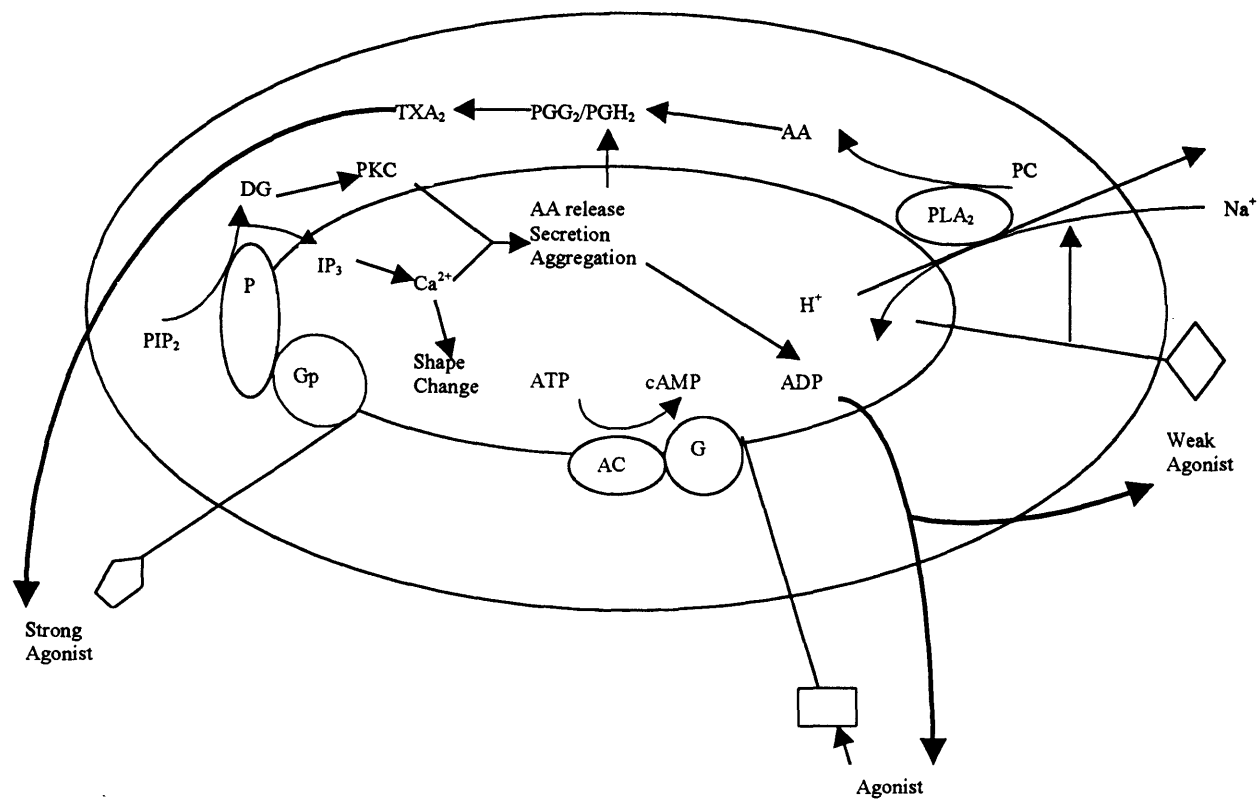


Figure 1.2 Platelet activation by physiologic agonists results in amplification events. Amplification occurs either (a) by release of preformed platelet constituents (e.g. stored in granules) or (b) by *de novo* production of active compounds (adapted from Kroll & Schafer 1989).
Bold lines represent amplification of initial stimulus

1.3.4.3 Platelet aggregation

Irrespective of the agonist, the final common pathway leading to the formation of the platelet plug is aggregation. Platelet membrane changes occur as a result of platelet activation and degranulation. Most importantly, the separate membrane glycoproteins IIb and IIIa are complexed to form a heterodimer (GpIIb/IIIa), this becomes available for interaction with its ligands i.e. fibrinogen, vWF, fibronectin and vitronectin. The interaction of fibrinogen with active GpIIb/IIIa orchestrates the binding of one activated platelet to another in the process of aggregation. The binding of fibrinogen occurs via specific RGD (arginine-glycine-aspartic acid) sequences. Fibrinogen contains 2 RGD sequences per half molecule; they are located on the A α chain of this dimeric protein. When two activated platelets with functional GpIIb/IIIa receptors each bind the same fibrinogen molecule, a fibrinogen bridge is created between the two platelets (Figure 1.3). The surface of each platelet has about 50,000 widely distributed GpIIb/IIIa fibrinogen binding sites. Therefore, numerous activated platelets are recruited to the aggregate via a dense network on intercellular fibrinogen bridges. This final event in the process of haemostasis produces an occlusive platelet thrombus at the site of vascular injury.

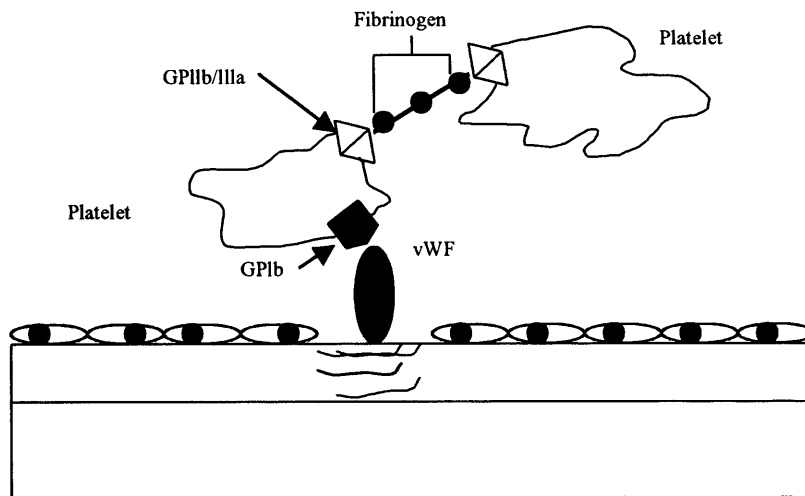


Figure 1.3 Platelet aggregation mediated by the linkage of activated platelets by fibrinogen, which binds to its receptors in the platelet GpIIa/IIIa complex via tripeptide RGD (arginine-glycine-aspartic acid) sequences located on the A α chains of dimeric fibrinogen.

1.3.4.4 Platelets in atherosclerosis

The role of platelet adhesion, activation and aggregation as well as being essential in haemostasis also play important roles in both the progression and associated thrombotic complications of atherosclerosis.

1.3.4.4.1 Platelets in atherosclerotic lesion development

As early as 1852 Carl von Rokintansky proposed the encrustation hypothesis of atherosclerosis which was later modified by Duguid in 1946. This hypothesis suggests that prothrombotic determinants play a role in atherosclerosis, and was further modified into the response to injury hypothesis (Ross & Glomset 1976a; 1976b). Ross and Glomset proposed that platelets contribute to early atherosclerotic lesion development, by adhering to the endothelium overlying fatty streaks (Ross & Glomset 1976a; 1976b; Hoak 1988). This endothelium may appear healthy, but was proposed to be dysfunctional with increased permeability and other alterations, which may lead to spontaneous accumulation of platelets and development of mural thrombi (Nachman 1992). Endothelial injury may range from alterations in cell-surface constituents through to loss of endothelial cover. Platelet adherence to a dysfunctional endothelium may not be a common event (Ross 1986) and may only occur if the endothelium is denuded. Ross (1993) suggests that platelets do not play a pivotal role in early atherosclerotic lesion development, more likely they are involved in the later stages of lesion development and the associated thrombotic complications.

Adhesion to damaged or disrupted endothelium results in platelet activation and subsequent release of granule contents including PDGF, TXA₂ and ADP. Release of TXA₂ and ADP can act to amplify platelet activation and aggregation at the site of injury, while PDGF can promote both smooth muscle migration (Grotendorst 1982) and proliferation (Ross *et al.* 1974) contributing to the development of the lesion. The

importance of platelet adhesion and the release reaction has been demonstrated in pigs lacking vWF, which is important in platelet adhesion. These pigs resist aortic atherosclerosis even in the presence of hypercholesterolaemia (Fuster *et al.* 1978), however, coronary atherosclerosis may occur (Griggs *et al.* 1981).

Platelets may also act as a source of cholesterol for macrophages, due to their high levels of free cholesterol. This ability of platelets to serve as cholesterol donors for macrophages increases in proportion to plasma cholesterol levels, however, platelets may affect foam cell formation in the absence of severe hypercholesterolaemia (Mendelsohn & Loscalzo 1988). The release of reactive oxygen species by platelets may also contribute to atherosclerotic lesion development (Finazzi-Agrò *et al.* 1982; Salvenimi & Botting 1990).

1.3.4.4.2 Thrombotic complications of atherosclerosis

A possible further role of platelets in atherosclerosis may follow plaque rupture or fissure. This results in thrombus formation, and subsequently the thrombus may become incorporated into the plaque, resulting in an increase in the local intimal thickening either by the bulk of the thrombus itself or because of the platelet-driven proliferation of smooth muscle cells. Carstairs (Carstairs 1965) was the first to identify platelet masses within atherosclerotic lesions using immunofluorescence. Other work has shown that platelet antigens are found only in raised lesions and not in fatty streaks (Woolf & Carstairs 1969). It does appear that incorporation of mural thrombi frequently occurs in relation to established atherosclerotic plaque. Further evidence for thrombus formation and incorporation as part of plaque progression is provided by the use of platelet-specific monoclonal antibodies. They have been used to demonstrate that, in advanced plaques, fibrin and fibrin related products were found in the intima, neointima and even in the deeper medial layer. Fibrin and fibrin related products were also found in small quantities in early lesions and in normal arteries (Bini *et al.* 1989).

Platelet-fibrin thrombi also play an important role in the complications of atherosclerosis. Individuals whose death was the result of a myocardial infarction are usually found to have occlusive thrombi in the coronary artery that supplied the infarcted region (Chandler *et al.* 1974; Davies *et al.* 1976). It is likely that when injury to the vessel wall is mild, thrombotic occlusion is transient, as occurs in unstable angina. Whilst, deep vessel injury secondary to rupture and ulceration results in exposure of collagen, lipids and other elements of the vessel media, leading to relatively persistent thrombotic occlusion and myocardial infarction (Fuster *et al.* 1990; Willerson *et al.* 1984; Fuster *et al.* 1988).

1.4 THEORIES OF ATHEROGENESIS.

A number of theories have been postulated to explain the origin of atherosclerotic disease. Present theories of the pathogenesis of atherosclerosis are related to earlier proposals made by Virchow (1856), von Rokitansky (1852), Duguid (1946) and Anitschkow (1913). Virchow suggested that low-grade injury to artery wall resulted in an inflammatory response which in turn caused increased passage and accumulation of plasma constituents within the intima of the artery. Rokitansky's idea, modified later by Duguid was that mural thrombi occurring at sites of arterial injury could be incorporated into the lesions and promote lesion progression. Anitschow and colleagues observed that the lesion development of rabbits fed a diet of egg yolk was identical to that in man. With further work he became convinced that there would be 'no atheroma without cholesterol'.

1.4.1 Response-To-Injury Hypothesis.

In 1976 Ross & Glomset combined the ideas of Virchow, Rokitansky and Duguid with improved knowledge of cellular and molecular biology of the artery wall into a theory termed the 'response to injury hypothesis'. According to the 'response to injury hypothesis', endothelial injury or dysfunction can be considered to be an initiating event in atherogenesis (Ross & Glomset 1976a; 1976b). Possible mediators of injury include hypercholesterolaemia (Simon *et al.* 1993), hypertension (Lockette *et al.* 1986), free radicals (Shatos *et al.* 1991), cigarette smoking (Ball & Turner 1974), viruses, such as herpes viruses (Hajjar *et al.* 1986) or other organisms such as chlamydia (Kuo *et al.* 1993). Injury to the endothelium can be manifested in a number of ways including: interference with its permeability barrier role (Bell *et al.* 1974a; 1974b; Hoff *et al.* 1983); alteration in the nonthrombogenic properties of the endothelial surface (Stehbens 1992); promotion of its procoagulant properties (Emmeis & Kooistra 1986; Bussolino *et al.*

1986); enhanced leucocyte adhesion (Hansson *et al* 1980, 1981) and modification of the vasomotor response and release of vasoactive substances (Ludmer *et al* 1986; Verbeuren *et al* 1990).

A pathophysiological classification of three types of vascular injury has been proposed (Ip *et al.* 1990). Type I injury is characterised by alteration of endothelial cells without significant morphological changes. Such changes may result in increased permeability (Bell *et al.* 1974a), increased mitotic activity (Wright 1972) and endothelial turnover (Caplan & Schwartz 1973) at areas where atherosclerotic plaques usually develop. Type II injury on the other hand is characterised by endothelial denudation with superficial intimal damage, in which platelet deposition with or without thrombosis can be seen (Davies *et al.* 1989). Type III damage is characteristic of more advanced atherosclerosis and relates to deep intimal and medial damage. Vascular damage of this magnitude can be seen after disruption of lipid-rich plaques. This disruption, resulting in obstructive thrombosis, appears to be the principal mechanism leading to unstable angina and acute myocardial infarction.

1.4.2 Oxidative Modification Hypothesis.

Hypercholesterolaemia is a known risk factor for atherosclerosis and coronary heart disease and clinical intervention studies have demonstrated the beneficial effects of lowering cholesterol levels (Shepherd *et al.* 1995). As has already been mentioned the fatty streak is one of the earliest lesions of atherosclerosis at the centre of which is the accumulation of cholesterol within macrophages to form 'foam cells'. With the discovery by Brown and Goldstein of the LDL receptor (Brown & Goldstein 1976) it seemed possible that this was the mechanism by which LDL was taken up by macrophages (Brown *et al.* 1981). However, macrophages in culture incubated with LDL failed to take up adequate LDL to cause cholesteryl ester accumulation, also individuals completely lacking LDL receptors show the most advanced atherosclerosis so an alternative mechanism was obviously in use. Goldstein and Brown suggested that

modification of LDL (in this case acetylation) was a prerequisite for macrophage uptake and cholesterol accumulation (Goldstein *et al.* 1979) by an alternative receptor, the scavenger receptor.

Acetylation of LDL does not occur *in vivo*, however, LDL may be modified by other means. One such modification is the oxidation of LDL. Oxidised LDL (Ox LDL) possesses pro-atherogenic properties (see section 1.5.2) which prompted Steinberg and colleagues (1989) to propose the 'oxidative modification hypothesis'. This basically states that in the presence of elevated plasma LDL, the concentration of LDL within the intima will be increased, some of which may become oxidised. Ox LDL can act as a chemoattractant of monocytes and also possesses the ability to inhibit the egress of resident macrophages and thereby act to promote recruitment of monocytes into the lesion. Within the arterial wall monocytes are modified to form macrophages which rapidly take up the oxidised LDL to produce the characteristic foam cell. This hypothesis provided a mechanism to link elevated plasma LDL with accelerated development of atherosclerosis.

The cytotoxicity of Ox LDL may provide a means by which the 'response to injury' hypothesis and the 'oxidative modification' hypothesis can be linked. An increase in plasma lipoproteins and principally Ox LDL may result in toxic injury to the endothelium. This injury may result in the increased adhesion of monocytes observed at sites throughout the arterial tree in hypercholesterolaemia (Navab *et al.* 1994)

1.5 OXIDISED LDL IN ATHEROSCLEROSIS.

Reactive oxygen species (ROS) involved in atherosclerosis include free radicals (an atom or molecule that contains one or more unpaired electrons), hydrogen peroxide and singlet oxygen. Five normal cellular processes result in the formation of ROS; the mitochondrial electron transport system, purine metabolism, neutrophil activation, phagocytosis and the synthesis of prostaglandins through the arachidonic acid cascade. A series of defence mechanisms exist to protect tissues against ROS attack, the major ones being superoxide dismutase (SOD), catalase, peroxidase enzymes, glutathione, transition binding proteins and antioxidant vitamins C and E. However, when these fail, either as a consequence of excessive free radical production or decreased scavenging, damage to tissues or molecules occurs. ROS can damage biomolecules including proteins (Greenwald & Moy 1980), polysaccharides (Kohen *et al* 1993), deoxyribonucleic acid (DNA) (Schuessler & Jung 1989) and polyunsaturated fatty acids (PUFAs) of both cell membranes and lipoproteins.

1.5.1 Oxidative Modification Of LDL.

LDL is a complex spherical particle consisting of a central core composed of cholesteryl ester and triglycerides, surrounded by an outer shell of phospholipid molecules and free cholesterol, embedded in which is apolipoprotein B, the protein recognised by the LDL receptor. One of the initial events in the oxidation of LDL is the lipid peroxidation chain reaction driven by free radicals (Figure 1.4). It begins when an initiating radical abstracts a hydrogen atom from one of the polyunsaturated fatty acids (PUFAs) contained in the LDL lipids. The PUFA radical reacts with molecular oxygen producing a lipid peroxy radical which in turn abstracts a hydrogen atom from an adjacent PUFA, yielding a lipid hydroperoxide and a new PUFA radical. Ultimately a radical initiated lipid peroxidation chain reaction has occurred. The propagation phase follows in which the PUFAs are rapidly oxidised to lipid hydroperoxides, the rate of this step depends upon the number of

antioxidants within the LDL particle which act to scavenge the lipid peroxy radicals and compete with the chain propagation (Esterbauer *et al.* 1991b). A decomposition phase follows, during which the lipid hydroperoxides break down to yield a range of products including aldehydes, hydrocarbon gases, epoxides and alcohols. The aldehydes formed including malondialdehyde and 4-hydroxynonenal (Esterbauer *et al.* 1987) react with the ϵ -amino acid of lysine residues on apo B, resulting in Schiff base formation and subsequent increase of negative charge on LDL (Steinbrecher 1987), which permits recognition by the scavenger receptor.

What constitutes the initiating radical remains unclear, although candidates include superoxide anions from endothelial or smooth muscle cells and monocytes (Heinecke *et al.* 1986; Hirarmatsu *et al.* 1987), autooxidised thiols produced by smooth muscle cells (Heinecke *et al.* 1993) and cellular enzymes such as 15-lipoxygenase (Parthasarathy *et al.* 1989). LDL can be oxidatively modified by a variety of mechanisms and at present, no consensus opinion is held on the predominant mechanism of LDL oxidation in vivo.

LDL from patients with proven coronary artery disease has been shown to have elevated oxidised cholesterol content and increased susceptibility to oxidation (Liu *et al.* 1992; Miwa *et al.* 1995). This susceptibility of LDL to oxidation has been demonstrated to be inversely related to coronary atherosclerosis (Regnström *et al.* 1992) and can be reduced by antioxidant supplementation (Mosca *et al.* 1997).

1.5.2 Role Of Oxidised LDL In Atherosclerosis.

Upon entering the arterial wall from the luminal surface, LDL may be subject to oxidative modification by endothelial cells (Henriksen *et al.* 1982; Steinbrecher *et al.* 1984), smooth muscle cells (Heinecke *et al.* 1986) and resident monocytes and macrophages (Parthasarathy *et al.* 1986; Hirarmatsu *et al.* 1987). The minimally modified LDL formed (Liao *et al.* 1991) can exert a variety of effects on cells. These include induction of adhesion molecule expression on endothelial cells (Berliner *et al.* 1990), secretion of monocyte chemotactic protein (MCP-1) (Cushing *et al.* 1990) and macrophage colony stimulating factor (M-CSF) by endothelial cells (Rajavashisth *et al.* 1990). Together these events result in increased monocyte binding to endothelium and subsequent migration into the subendothelial space, where monocytes are promoted to differentiate into macrophages. The mature macrophage is capable of further modification of minimally modified LDL to a more oxidised form, which can ultimately be taken up by the scavenger receptor, resulting in foam cell formation.

Ox LDL is also chemotactic for monocytes, attributable to its lysolecithin content (Quinn *et al.* 1987, 1988). Also it inhibits migration of macrophages and thereby prevents them re-entering the circulation (Quinn *et al.* 1985). Ox LDL and its products are also cytotoxic to a variety of cells, including endothelial cells, smooth muscle cells and macrophages (Hessler *et al.* 1979; Morel *et al.* 1984; Hughes *et al.* 1994; Reid & Mitchinson 1993). The observed cytotoxicity of Ox LDL could promote endothelial dysfunction and the evolution of the fatty streak into a more advanced lesion as suggested in the 'response to injury' hypothesis. One indication of an early response to injury may be the stimulation of PGI₂ production by human vascular endothelial cells, which, occurs following incubation with Ox LDL (Triauf *et al.* 1988). This effect of Ox LDL on prostacyclin production appears to depend upon the extent of oxidation of LDL, with LDL containing low levels of lipid peroxides stimulating PGI₂ synthesis, while more extensively Ox LDL inhibited PGI₂ synthesis (Zhang *et al.* 1990).

Other effects of Ox LDL include modulation of the production of growth factors such as PDGF (Fox *et al* 1987) and TNF- α (Hamilton *et al* 1990) and inflammatory mediators, such as IL-1 (Frostegård *et al* 1992). Ox LDL may also affect the coagulation pathway by inducing tissue factor (Drake *et al.* 1991) and plasminogen activator inhibitor-1 (Latron *et al.* 1991), inhibiting nitric oxide production (Tanner *et al.* 1991) and promoting platelet aggregation (Ardlie *et al.* 1989).

1.5.3 Evidence For Oxidised LDL In Atherosclerosis.

Several lines of evidence support the existence of Ox LDL in vivo. Ceroid pigment, a complex of oxidised lipids and proteins, which has been demonstrated in atherosclerotic lesions and also within macrophages (Mitchinson *et al* 1990). LDL derived from atherosclerotic lesions has been isolated, characterised and shown to include Ox LDL (Palinski *et al.* 1989; Yla-Herttuala *et al.* 1989). Immunocytochemical evidence using antibodies to various epitopes on Ox LDL have shown staining of atherosclerotic lesions in both animals and humans (Haberland *et al* 1988; Palinski *et al.* 1989; Rosenfeld *et al* 1990). Autoantibodies against Ox LDL have also been detected in plasma of patients and of Watanabe and New Zealand rabbits (Palinski *et al.* 1989). The presence of autoantibodies against Ox LDL has been positively correlated with the progression of atherosclerosis, as manifested by carotid artery stenosis (Salonen *et al.* 1992).

1.6 ANTIOXIDANTS AND ATHEROSCLEROSIS.

Compelling evidence to support the 'Oxidative Modification of LDL' hypothesis of atherosclerosis is the demonstration of the presence of Ox LDL in atherosclerotic lesions and that certain antioxidants can inhibit the progression of atherosclerosis. Although some mechanisms by which this may occur have been demonstrated, there is still work to be done in this area.

1.6.1 Antioxidants.

Molecules with the ability to 'scavenge' free radicals can be referred to as antioxidants, including probucol, butylated hydroxytoluene (BHT), glutathione, superoxide dismutase (SOD), α -tocopherol, ascorbic acid and β -carotene. Discussed in greater detail here are α -tocopherol and ascorbic acid.

1.6.1.1 α -Tocopherol

Vitamin E (from the Greek *tokos* – childbirth, the verb *pherein* – to bring forth and *ol* was added to indicate the alcohol nature of the compound), is an essential fat soluble vitamin, the importance of which was first recognised by Evans & Bishop (1922). The richest dietary sources of vitamin E are vegetable oils, in descending order wheat germ, sunflower seed, palm, rapeseed and other oils. The absorption of vitamin E is relatively poor, only 20 - 40% of a test dose is normally absorbed from the small intestine, in mixed lipid micelles along with other dietary lipids. Vitamin E is then incorporated into chylomicrons; the liver takes up the chylomicron remnants, which is the main organ for storage and excretion. Since vitamin E is transported in lipoproteins secreted by the liver, the plasma concentration depends to a great extent on total plasma lipids. The recommended dietary intake of vitamin E is 10 International Units (IU) per day (Wretling 1982).

The most widely accepted biological function of vitamin E is its antioxidant property, though it may also have a role in the prevention of degenerative disorders. Vitamin E is the generic term for the tocopherols and the tocotrienols, within each class there are the α , β , δ and γ - tocopherols or tocotrienols. α -tocopherol, the biologically and chemically most active form of vitamin E, is the principal lipid soluble antioxidant in tissues and plasma (Figure 1.5). It is also the predominant antioxidant in the LDL particle.

1.6.1.2 Ascorbic Acid

Vitamin C is a simple sugar of molecular weight 176 kDa that exists in only one form in nature, which is termed L-ascorbic acid. However, it can exist in two oxidation states: 1) the reduced form of L-ascorbic acid itself accounting for the bulk of the vitamin and 2) the reversibly oxidised form, dehydroascorbic acid, only small amounts of which can be found (Figure 1.5). Fresh fruit, especially blackcurrant and citrus fruits, and leafy green vegetables are rich dietary sources of vitamin C. Absorption of vitamin C occurs in the intestine, followed by distribution into the blood where it is taken up unevenly in the tissues. The recommended dietary intake for vitamin C is 60 mg per day for adults, though factors including the contraceptive pill and smoking may increase these requirements.

Vitamin C is a water soluble antioxidant, which acts as a first line of defence in the plasma (Frei *et al* 1989). It has a number of biological functions including roles in collagen synthesis (Peterkofsky 1991), the biosynthesis of carnitine and neuroendocrine peptides (Diliberto *et al.* 1991) and the conversion of bile acids to cholesterol (Ginter 1973). Vitamin C has also been demonstrated to regenerate α -tocopherol from its chromanoxyl radical form (Packer *et al.* 1979) to yield tocopherol and the dehydroascorbic acid, the result of which is to restore the radical-scavenging activity of tocopherol (Figure 1.5).

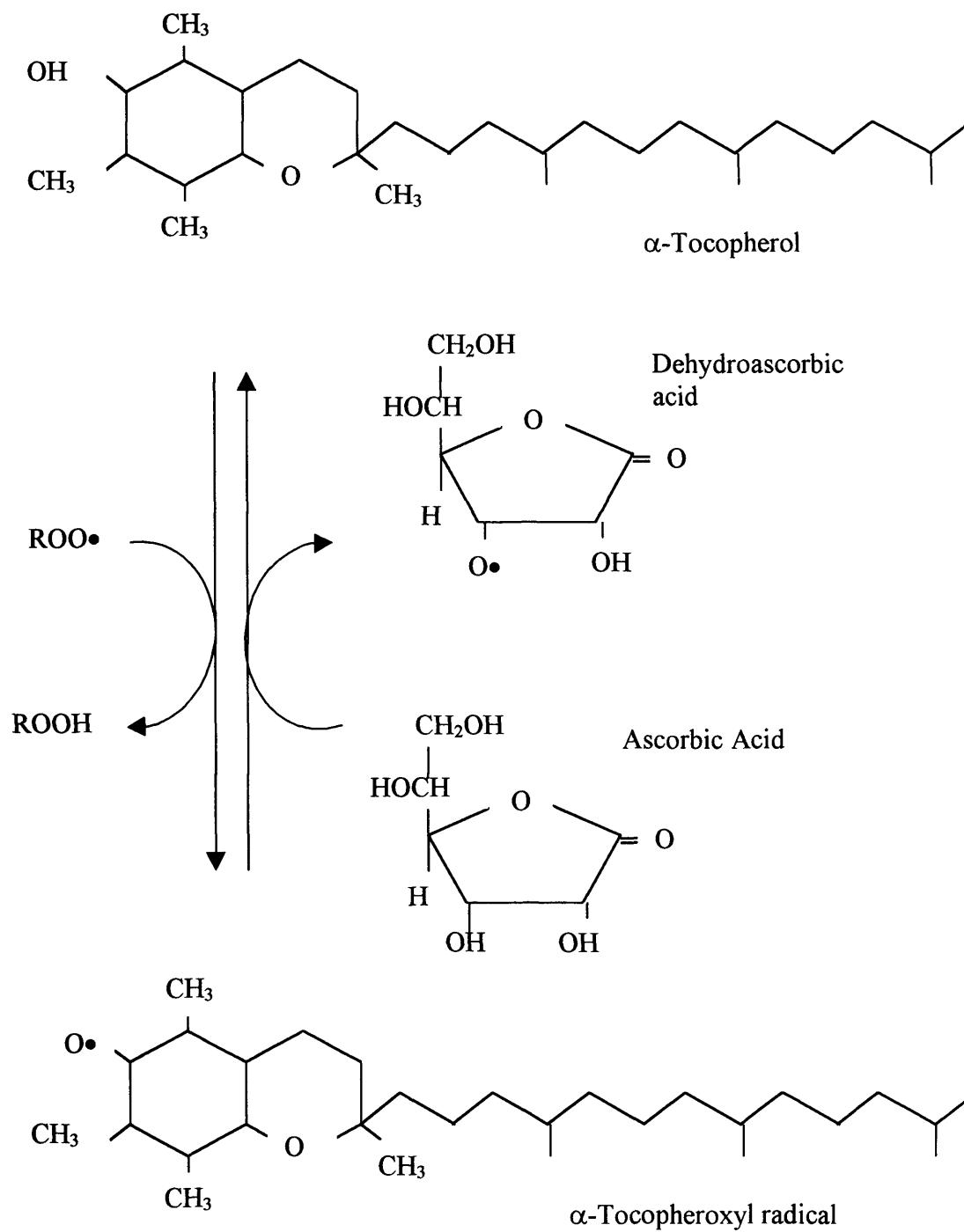


Figure 1.5 The role of α -tocopherol as a chain-breaking antioxidant, and the reduction of the tocopheryl radical by vitamin C.

1.6.2 Effects Of Antioxidants In Atherosclerosis.

1.6.2.1 Animal studies

Atherosclerotic lesions appear to occur with increased frequency in rodents, piglets and non human primates that have chronic marginal deficiencies of vitamin E (Janero 1991). A number of studies in animals have demonstrated that antioxidants inhibit the development of atherosclerosis. Probucol, a lipid-soluble cholesterol lowering drug with potent antioxidant properties, fed to rabbits resulted in less extensive lesions compared to a cholesterol-matched control group (Carew *et al.* 1987). Probucol also has the ability to reduce the early fatty streak lesions in the aorta of atherosclerotic rabbits (Kita *et al.* 1987; Daugherty *et al.* 1989), cholesterol-fed monkeys (Sasahara *et al.* 1994) and rats (Shankar *et al.* 1989). Antioxidants, including probucol and vitamin E, can inhibit neo-intimal thickening in the cholesterol-fed rabbit and pig (Williams *et al.* 1992; Ferns *et al.* 1993; Freyschuss *et al.* 1993; Schneider *et al.* 1993).

Vitamin E supplementation has beneficial effects on atherosclerosis in animal models. These effects include decreased progression and even regression of atherosclerosis in male monkeys (Verlangieri *et al.* 1992), reduced plasma lipid peroxides and aortic intimal thickening in restricted ovulatory hens (Smith and Kummerow 1989) and protection against restenosis in the cholesterol-fed rat (Konneh *et al.* 1995) and rabbit (Lafont *et al.* 1995). However, other studies failed to reveal a beneficial effect of vitamin E (Dam 1944; Moses *et al.* 1952; Godfried *et al.* 1989).

Vitamin C deficiency has been shown to cause atherosclerotic lesions in guinea pigs (Willis 1953; Ginter *et al.* 1969). Subsequent studies have shown that vitamin C supplementation can significantly reduce atherosclerotic plaque formation in guinea pigs (Willis 1957; Ginter *et al.* 1969; Sharma *et al.* 1988) and rabbits (Verlangieri *et al.* 1977)

and has even resulted in regression of atherosclerotic lesions among hypercholesterolaemic rats (Altman *et al.* 1980).

1.6.2.2 Human studies

1.6.2.2.1 Epidemiological evidence

Several epidemiological trials suggest that populations with a high intake of vitamin E have a lower risk of cardiovascular heart disease. Gey *et al.* have shown an inverse correlation between plasma vitamin E levels and mortality from ischaemic heart disease in middle aged men (aged 40-59 years) (Gey *et al.* 1991). A case-controlled Scottish study of a heavy smoking male population (aged 35-54 years) showed that vitamin E levels are inversely related to risk of angina after adjustment for a variety of risk factors including age, smoking, blood pressure, lipids and relative weight (Riemersma *et al.* 1991).

In the largest study to date, Stampfer *et al.* (1993) reported that in nurses (aged 34-59 years) who were free from diagnosed cardiovascular disease and cancer at baseline, women in the highest quintile of the cohort for vitamin E consumption had a relative risk factor of 0.66 (95% CI, 0.50, 0.87). Similar findings were also seen in men (aged 40-75 years) free of prevalent cardiovascular disease, diabetes or high cholesterol, men in the highest quintile of vitamin E intake had an age-adjusted relative risk of coronary disease of 0.59 (95% CI, 0.47, 0.75) (Rimm *et al.* 1993). Others including Salonen *et al.* have found no association between plasma vitamin C or E and prevalent ischaemic heart disease (Salonen *et al.* 1988).

Low plasma and tissue concentrations of ascorbate have been identified as a risk factor for atherosclerosis. A Scottish study reported that the risk of developing angina is increased with low plasma levels of vitamin C (Riemersma *et al.* 1991). Two large

epidemiological studies have shown a significant inverse correlation exists between plasma ascorbate and cardiovascular mortality (Gey *et al.* 1993; Engstrom *et al.* 1992). Furthermore a recent Finnish study demonstrated an increased risk of myocardial infarction (MI) with vitamin C deficiency (Nyyssönen *et al.* 1997). Concentrations of ascorbate in atheromatous aortas are lower than in control vessels (Dubick *et al.* 1987), whilst smokers, diabetics and patients with coronary disease all have lower concentrations of plasma ascorbate (Ramirez & Flowers 1980; Stankova *et al.* 1984; Chow *et al.* 1986). The data on vitamin C are far from conclusive. In both the Nurses health study and the Health Professionals study, the use of vitamin C supplements was not significantly associated with the risk of coronary events (Stampfer *et al.* 1993; Rimm *et al.* 1993).

1.6.2.2.2 Supplementation studies

The most direct way to ascertain whether antioxidants are beneficial in the prevention and/or treatment of cardiovascular disease is to conduct randomised placebo-controlled trials. Probucol supplementation (500 mg per day) has proved effective in reducing the rate of restenosis after coronary balloon angioplasty, as assessed by angiography (Watanabe *et al.* 1996; Tardif *et al.* 1997). The anti-atherogenic effects of probucol have proved much less convincing in the Probucol Quantitative Regression Swedish Trial (PQRST), where probucol supplementation along with a low cholesterol diet and cholestyramine therapy had no more regression than patients receiving a low-cholesterol-diet and cholestyramine alone (Walldius *et al.* 1994).

Several trials examining the effects of vitamin E in angina failed to demonstrate any benefit (Rinzler *et al.* 1950; Anderson & Reid 1974; Gillilan *et al.* 1977). Vitamin E supplementation has produced an improvement in patients suffering from intermittent claudication as a result of peripheral atherosclerosis (Williams *et al.* 1971) and a reduction in the restenosis rate in patients following percutaneous transluminal coronary angioplasty (PTCA), however, this reduction just failed to reach significance ($p = 0.06$)

(DeMaio *et al.* 1992). A recent study on patients with coronary atherosclerosis has shown that supplementation with α -tocopherol resulted in a significant reduction in the risk of non-fatal MI, however, there was a non-significant increase in cardiovascular deaths in the α -tocopherol groups (Stephens *et al.* 1996). Hence the effects of α -tocopherol are far from proven with regard to cardiovascular disease and are still worthy of investigation.

1.6.3 Potential Anti-Atherogenic Effects Of Antioxidants.

1.6.3.1 Oxidation of LDL

Oxidative modification is believed to play an important role in the pathogenesis of atherosclerosis (Steinberg *et al.* 1989) and as such the propensity of LDL to oxidation is of particular relevance. Various antioxidants including probucol (Parthasarathy *et al.* 1986b) and vitamin E (Esterbauer *et al.* 1991a) can increase the resistance of LDL to oxidation *in vitro*. Supplementation studies have confirmed the ability of probucol (Masana *et al.* 1991; Reaven *et al.* 1992) and vitamin E to increase the resistance of LDL to oxidation, while β -carotene has not proved so effective (Princen *et al.* 1992; Reaven *et al.* 1993). A combination antioxidant supplement has also produced a reduction in lipid peroxidation in healthy volunteers (Calzada *et al.* 1995), men with low antioxidant status (Salonen *et al.* 1991) and patients with established cardiovascular disease (Mosca *et al.* 1997).

1.6.3.2 Platelets.

Vitamin E was first demonstrated to inhibit platelet aggregation in 1974 (Higashi *et al.* 1974), since then other investigators have reported similar results *in vitro* on collagen, ADP, epinephrine, arachidonic acid (AA) and thrombin induced platelet aggregation (Fong 1976; Agradi *et al.* 1981; Srivastava 1986; Violi *et al.* 1990; Dierichs & Maschke

1993) and spontaneous platelet aggregation (Kakishita *et al.* 1990). Supplementation with vitamin E has provided conflicting results. In healthy volunteers a small but significant inhibitory effect on collagen induced aggregation was observed in women, in men, however, this failed to reach significance (Steiner 1983). While others have failed to see an inhibitory effect (Stampfer *et al.* 1988). Studies in men with low antioxidant status (Salonen *et al.* 1991), heart transplant recipients (DeLorgeril *et al.* 1994) and hyperlipoproteinaemics (Szczeklik *et al.* 1985) have all shown a significant inhibition of platelet aggregation. Vitamin E supplementation has also been shown to alter platelet adhesion (Steiner 1983, Jandak *et al.* 1988), the platelet release reaction (Steiner & Anastasi 1976), membrane fluidity (Steiner 1981), calcium release (Butler *et al.* 1979), PKC activity (Freedman 1996) and AA metabolism (Chan *et al.* 1986; Pritchard *et al.* 1986a).

Ascorbic acid has been shown to inhibit platelet aggregation and metabolism (Cordova *et al.* 1982). Inhibition of platelet aggregation and adhesion followed oral administration of vitamin C in coronary artery disease patients (Bordia & Verma 1985). Therefore vitamin C may also be of importance in the prevention of chronic thromboatherosclerotic disease of the arteries.

1.6.3.3 Monocytes.

Monocyte and macrophage recruitment is vital to atherosclerotic lesion progression. α -tocopherol has been shown *in vitro* to inhibit monocyte adhesion to human umbilical vein endothelial cells (HUVEC), as a result of decreased surface expression of E-Selectin (Faruqi *et al.* 1994). Similarly monocytes taken from subjects supplemented with α -tocopherol exhibited decreased adhesion to resting and activated endothelial cells (Devaraj *et al.* 1996). These monocytes exhibited decreased release of reactive oxygen species, lipid oxidation and IL-1 β secretion. Probucol has also been shown to inhibit monocyte adhesion to the vascular endothelium of the cholesterol-fed rabbit (Ferns *et al.*

1993), while vitamin C has been shown to reduce the increased monocyte adhesiveness seen in smokers (Weber *et al.* 1996).

1.6.3.4 Vascular Endothelium

Endothelial injury or dysfunction may be the initiating event in atherogenesis as described in the 'response to injury' hypothesis. Endothelial cells and macrophages loaded with α -tocopherol or probucol are protected from the cytotoxic effects of Ox LDL (Evensen *et al.* 1983; Kuzuya *et al.* 1991) a potential mediator of injury. One form of endothelial dysfunction the reduced endothelium-derived nitric oxide mediated vascular relaxation seen in cholesterol-fed rabbits, can be prevented by the administration of α -tocopherol (Keaney *et al.* 1993; Andersson *et al.* 1994; Stewart-Lee *et al.* 1994; Anderson *et al.* 1995), β -carotene (Keaney *et al.* 1993) or probucol (Simon *et al.* 1993; Keaney *et al.* 1995). Vitamin C has also been shown to improve the endothelium-dependent vasomotor capacity of coronary artery disease patients (Levine *et al.* 1996). Antioxidants including α -tocopherol (Faruqi *et al.* 1994) and probucol (Kaneko *et al.* 1996) have the capacity to inhibit cell adhesion molecule expression on stimulated endothelial cells, which may prevent or reduce monocyte or macrophage accumulation at lesion-prone areas.

1.6.3.5 Smooth muscle cells

Smooth muscle cell proliferation in the arterial intima is important in the formation of intermediate and advanced lesions of atherosclerosis and during restenosis following percutaneous transluminal coronary angioplasty. Vitamin E and more specifically RRR- α -tocopherol (Boscoboinik *et al.* 1991a; Azzi *et al.* 1995) can inhibit smooth muscle cell proliferation in a dose-dependent manner. It is suggested that this anti-proliferative effect is independent of its antioxidant properties and rather is an effect on signal transduction, namely an inhibition of protein kinase C activity (Boscoboinik *et al.* 1991a; Azzi *et al.* 1995).

1.7 OBJECTIVES

The aims of this thesis were to examine the effects of supplementation of the antioxidant vitamins C and E, with particular regard to platelet and monocyte function, in subjects at coronary risk. Preliminary studies were undertaken to investigate the effects of α -tocopherol and ascorbic acid on platelets and monocytes *in vitro*, the findings of which are recounted in Chapter 3. Hypercholesterolaemia, a risk factor for atherosclerosis, is a condition in which both platelet and monocyte function may be altered (Carvalho *et al* 1974; Bath *et al* 1991a). Chapter 4 describes the effect on monocyte adhesion and platelet aggregation following vitamin E supplementation at 400 IU per day in newly diagnosed hypercholesterolaemics. Elevated blood pressure is associated with increased risk of coronary artery disease (MRFIT Research Group 1982). Chapter 5 reports the effects of vitamin C supplementation on platelet and monocyte adhesion and soluble adhesion molecule levels, in elderly hypertensive and normotensive subjects. Finally, vitamin E supplementation of patients with angiographically defined coronary artery disease who underwent routine percutaneous transluminal coronary angioplasty was examined and the results of this study are detailed in Chapter 6.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

Materials and their suppliers are listed in appendix I

2.2 METHODS

2.2.1 Measurement Of Plasma Lipid Levels.

Blood was obtained by venepuncture from subjects following an overnight fast. Plasma was obtained from the blood samples that were collected into lithium-heparin Monovette® tubes (1.5 U heparin ml⁻¹) (Sarstedt, Leicester, UK) by centrifuging at 1500 x g for 10 minutes at 4°C. Plasma was stored at 4°C in the dark, prior to measurement of plasma total- and HDL cholesterol, and triglycerides, which was normally performed the same day. Additional plasma was stored in the dark at -70°C.

Measurements of plasma total- and HDL-cholesterol, and triglycerides were made using a Kodak Ektachem 700XR Analyser C series (Eastman Kodak Company, Rochester, USA). If a subject's plasma triglyceride levels were ≤ 4.0 mmol l⁻¹, plasma LDL cholesterol concentrations were estimated using the Friedewald formula (Friedewald *et al* 1972) (see appendix III for formula).

Measurements taken for the study of elderly hypertensive and normotensive subjects were made using the Hitachi 717EB analyser by the colorimetric method by Simbec Enhanced pathology, Merthyr Tydfil, UK. Total cholesterol and triglycerides were determined using enzymatic CHOD-PAP and GPO-PAP respectively. HDL cholesterol was measured in the supernatant following phosphotungsten-magnesium precipitation. Plasma LDL-cholesterol was estimated using the Friedewald formula (Friedewald *et al*. 1972) (see appendix III for formula).

2.2.2 Measurement Of Plasma Antioxidant Vitamin Levels By HPLC.

2.2.2.1 α -tocopherol.

Plasma was obtained from a fasting blood sample collected into a 5.5ml lithium heparin Monovette® (1.5 Units Heparin ml⁻¹) and centrifuged at 1500 x g for 10 minutes at 4°C (Megafuge 1.0R, Heraeus Sepatech, Kalkberg, Austria). The plasma collected was stored in the dark at -70°C until analysis.

Two hundred microlitres of internal standard (α -tocopherol acetate 20 mg l⁻¹) was added to 200 μ l of plasma or appropriate working standard (α -tocopherol 20 mg l⁻¹) in a glass test tube. To each tube 400 μ l of hexane was then added and the tubes were vortex mixed for 1 minute. The tubes were then centrifuged for 5 minutes at 13,000 rpm (MSE, Micro-Centaur, Loughbrough, UK). The organic upper phase was transferred to a solvent resistant plastic screw top tube and the solvent dried down at 40°C with nitrogen. The residue was reconstituted in 100 μ l of absolute ethanol and transferred to autosampler vials.

Plasma α -tocopherol was determined by the method of Bieri *et al* (1979). The HPLC system comprised an Isocratic LC pump 250 (Perkin Elmer, Beaconsfield, Bucks, UK), an ISS-100 autosampler set at 30 μ l (Perkin Elmer, Beaconsfield, Bucks, UK) equipped with a 100 μ l sampling loop (Rheodyne) and a Spectra system UV2000 detector (Thermo-separation products, UK). The detection wavelength employed was 285 nm. The separation was carried out on a Spherisorb column (250 x 4.6 mm, particle size 5 μ) (Phase Sep, Clywd, UK) using methanol (HPLC grade). A flow rate of 1.3 ml min⁻¹ was used. Under these conditions the chromatogram was completed in about 20 minutes, with an α -tocopherol peak retention time of 12 minutes and α -tocopherol acetate peak retention time of 18 minutes. The peaks obtained were integrated using PC 1000 software (Thermo-separation products, UK) and a value for peak area calculated. From the peak

area values a ratio of α -tocopherol peak area to α -tocopherol acetate was calculated. A standard curve was plotted of peak area ratio value against α -tocopherol concentration. The α -tocopherol concentration of the plasma samples were determined from this standard curve if the r^2 (coefficient of determination) value was >0.95 .

2.2.2.2 Ascorbic acid.

Plasma was obtained from a fasting blood sample collected into a 4ml potassium EDTA Monovette® (1.6 mg ml⁻¹) and centrifuged at 1500 x g for 10 minutes at 4°C (Megafuge 1.0R, Heraeus Sepatech, Kalkberg, Austria). Immediately after harvesting the plasma, ascorbic acid was stabilised by the addition of an equal volume of 10% (w/v) metaphosphoric acid, this sample was vortex mixed and centrifuged at 1500 x g for 10 min at 4°C (Megafuge 1.0R, Heraeus Sepatech, Kalkberg, Austria). The clear supernatant was removed and stored in the dark at -70°C for up to 1 month prior to analysis.

Plasma ascorbic acid was measured by HPLC using a modification of the method of Lunec & Blake (1985). The HPLC system comprised a Constametric® 3200 solvent delivery system (Thermo-separation products, UK), a 100 μ l sampling loop (Rheodyne) and a Spectromonitor® 3100 variable wavelength detector (Milton Roy). The detection wavelength employed was 254 nm. The separation was carried out on a LiChrospher® 100 NH₂ column (244 x 4mm, particle size 5 μ m) (Merck, Darmstadt, Germany) using an eluent of acetonitrile: 25 mmol l⁻¹ citric acid/ sodium citrate buffer glacial acetic acid at ratio of 85:15:0.1 (v/v). The flow rate used was 1.0 ml min⁻¹. Under these conditions the chromatogram was completed in about 15 minutes, with an ascorbic acid peak retention time of 6-7 minutes. Peaks were recorded using an R50 chart recorder (Perkin Elmer, Beaconsfield, Bucks, UK) with a chart speed of 30 cm h⁻¹. The peak heights were then

determined manually and the ascorbic acid concentration determined from an ascorbic acid standard curve of peak height vs ascorbic acid concentration ($\mu\text{mol l}^{-1}$).

2.2.2.3 Retinol.

Plasma was obtained from a fasting blood sample collected into a 5.5ml lithium heparin Monovette® (1.5 Units Heparin ml^{-1}) and centrifuged at $1500 \times g$ for 10 minutes at 4°C (Megafuge 1.0R, Heraeus Sepatech, Kalkberg, Austria). The plasma collected was stored in the dark at -70°C until analysis.

Two hundred microlitres of internal standard (retinyl acetate 2.0 mg l^{-1}) was added to 200 μl of plasma or appropriate working standard (retinol 1.75 mg l^{-1}) in a glass test tube. To each tube 400 μl of hexane was then added and the tubes were vortex mixed for 1 minute. The tubes were then centrifuged for 5 minutes at 13,000 rpm (MSE, Micro-Centaur, Loughbrough, UK). The organic upper phase was transferred to a solvent resistant plastic screw top tube and the solvent dried down at 40°C with nitrogen. The residue was reconstituted in 100 μl of absolute ethanol and transferred to autosampler vials.

Plasma retinol was determined by the method of Bieri *et al* (1979). The HPLC system comprised of an Isocratic LC pump 250 (Perkin Elmer, Beaconsfield, Bucks, UK), an ISS-100 autosampler set at 30 μl (Perkin Elmer, Beaconsfield, Bucks, UK) equipped with a 100 μl sampling loop (Rheodyne) and a Spectra system UV2000 detector (Thermo-separation products, UK), the detection wavelength employed was 325 nm. The separation was carried out on a Spherisorb column (250 x 4.6 mm, particle size 5 μ) (Phase Sep, Clywd, UK) using methanol (HPLC grade). A flow rate of 1.3 ml min^{-1} was used. Under these conditions the chromatogram was completed in less than 10 minutes. The retinol and retinyl acetate peak retention times were 3 minutes and 6 minutes respectively. The peaks obtained were integrated using PC 1000 software (Thermo-separation products, UK) and a value for peak area calculated. From the peak area values a ratio of retinol peak area to retinyl acetate was calculated. A standard curve was plotted

of peak area ratio value against retinol concentration. The retinol concentration of the plasma samples were determined from this standard curve if the r^2 (coefficient of determination) value was >0.95 .

2.2.2.4 Evaluation of measurement of antioxidant vitamins by HPLC.

Plasma levels of α -tocopherol, retinol and ascorbic acid were measured by HPLC methods that had been previously validated within our laboratory.

2.2.2.4.1 *α -tocopherol and retinol.*

An internal standard was added to both samples and standards in order to eliminate any errors which may arise from pipetting or the evaporation of solvents. With every batch of samples processed a quality control (QC) was measured in duplicate. The assay was rejected if the value obtained for the QC was outside the range for mean ± 2 standard deviations of the expected value. All standards and samples were processed in duplicate and values accepted if the difference between these duplicates was less than 10%. Any samples or standards that failed this criterion were remeasured. Typical HPLC chromatograms for α -tocopherol and retinol analysis are shown in Figure 2.1 and 2.2 respectively.

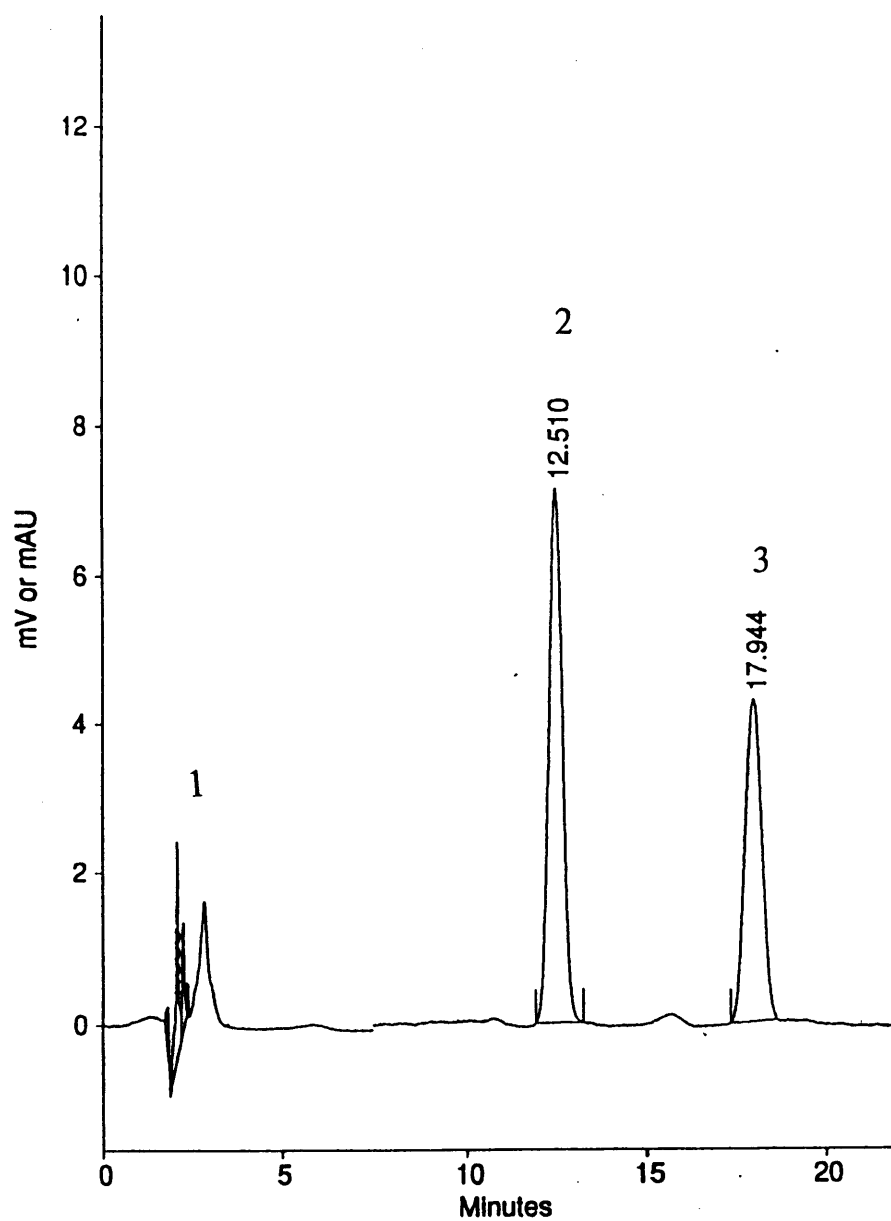


Figure 2.1 Analysis of plasma α -tocopherol. Column packing, Spherisorb (5 μ m); column dimensions, 250 x 4.6 mm internal diameter; eluent, methanol (hplc grade); flow rate, 1.3 ml min⁻¹; column temperature, ambient; detection, UV at 285 nm. Solutes 1, solvent; 2, α -tocopherol; 3, α -tocopheryl acetate.

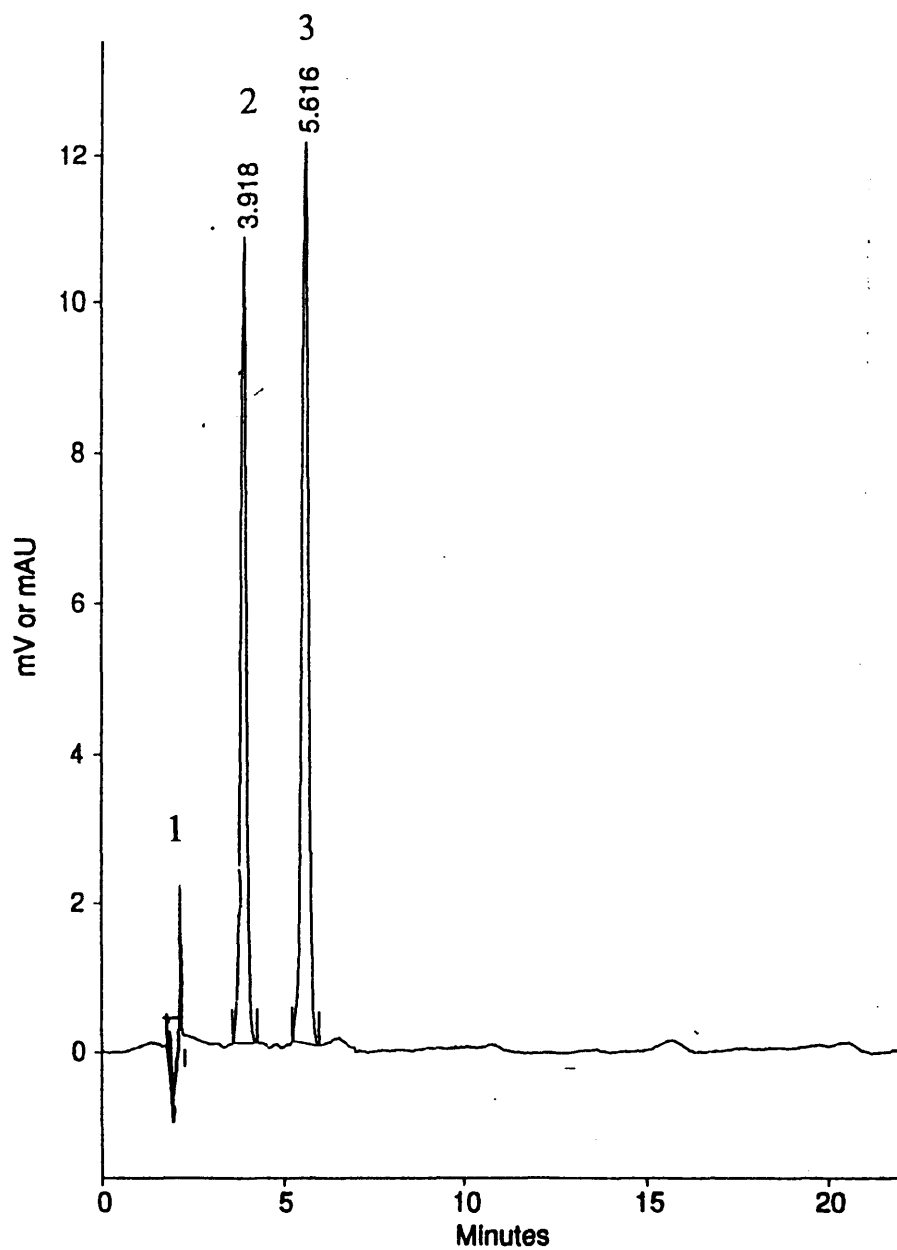


Figure 2.2 Analysis of plasma retinol. Column packing, Spherisorb (5 μm); column dimensions, 250 x 4.6 mm internal diameter; eluent, methanol (hplc grade); flow rate, 1.3 ml min^{-1} ; column temperature, ambient; detection, UV at 325 nm. Solutes 1, solvent; 2, retinol; 3, retinyl acetate.

The concentrations of α -tocopherol and retinol within a sample were determined using a standard curve, which was produced by plotting concentration of α -tocopherol or retinol (mg l^{-1}) against α -tocopherol/ α -tocopheryl acetate peak area ratio or retinol/retinyl acetate peak area ratio respectively, using Graphpad Prism software (Graphpad Inc, USA).

2.2.2.4.2 Ascorbic acid.

For the measurement of ascorbic acid all standards and samples were processed in duplicate and values accepted if the difference between these duplicates was less than 10%. A typical chromatogram obtained for ascorbic acid measurement is shown in Figure 2.3. To determine the concentration of ascorbic acid within a plasma sample a standard curve was plotted of peak height (mm) against ascorbic acid concentration ($\mu\text{mol l}^{-1}$), using Graphpad Prism software (Graphpad Inc, USA).

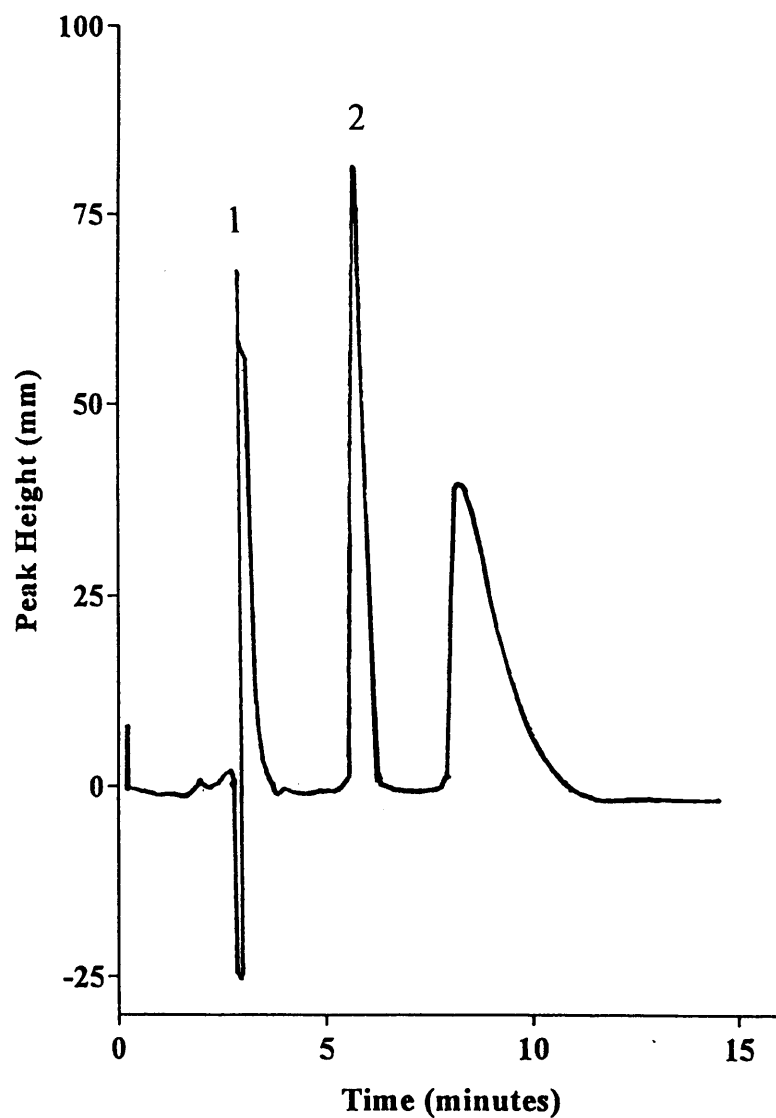


Figure 2.3 Analysis of plasma ascorbic acid. Column packing, LiChrospher® (5 μm); column dimensions, 244 x 4 mm internal diameter; eluent, acetonitrile/ 25 m mol l⁻¹ citric acid/ sodium citrate buffer/ glacial acetic acid (85: 15:0.1); flow rate, 1.0 ml min⁻¹; column temperature, ambient; detection, UV at 285 nm. Solutes 1, solvent; 2, ascorbic acid.

2.2.3 Platelet Function Studies.

2.2.3.1 Platelet rich plasma (PRP) preparation.

Whole blood anticoagulated with 1/10th volume of 3.8 % (w/v) trisodium citrate was centrifuged at 200 x g for 20 minutes using a Megafuge 1.0R centrifuge (Heraeus Sepatech, Austria) and the upper platelet-rich layer was collected.

2.2.3.2 Washed platelet preparation.

Whole blood anticoagulated with 1/10th volume of 3.8 % (w/v) trisodium citrate was centrifuged at 200 x g for 20 minutes using a Megafuge 1.0R centrifuge (Heraeus Sepatech, Austria) and the upper platelet-rich layer was collected. Apyrase (final concentration 10 $\mu\text{g ml}^{-1}$) and prostacyclin (final concentration 0.33 $\mu\text{g ml}^{-1}$) were added, to prevent premature platelet activation, followed by centrifugation at 800 x g for 15 minutes. The platelet pellet which resulted was resuspended in Ca^{2+} -free Tyrodes buffer (see appendix II). For measurement of platelet membrane fluidity at this stage the platelets underwent another two washing steps that involved centrifugation at 800 x g for 15 minutes and were finally resuspended in Ca^{2+} free tyrodes buffer. The platelet count was determined using a haemocytometer, and was subsequently adjusted to 3×10^{11} platelets l^{-1} . There was negligible leucocyte contamination of the platelet suspension upon examination by microscopy. This suspension was allowed to equilibrate at ambient temperature for 1 hour prior to use.

2.2.3.3 Platelet aggregation monitored by conventional aggregometry.

In vitro assessment of platelet aggregation was based upon the method of Born (1962). The prepared platelet-rich plasma (PRP), was transferred into a labelled plastic tube using a plastic transfer pipette, capped and retained at room temperature for the duration of the test. Platelet-poor plasma (PPP) was prepared by centrifugation of a portion of the blood at $1500 \times g$ for 10 minutes at 20°C , again the PPP was transferred to a labelled tube using a plastic pipette and kept at room temperature. The assay was performed between 30 minutes and 3 hours after the test blood sample was drawn. The platelet count of the PRP was determined using a haemocytometer and adjusted to $3 \times 10^{11} \text{ l}^{-1}$ with autologous PPP. The Payton Scientific aggregation module, model 300-5B (Ion trace Inc, Ontario, Canada) was calibrated so that the PRP gave 10% and the PPP gave 90% light transmission, measured using a SE120 BBC Goerz Metrawatt Chart recorder (Fisons instruments, Loughbrough, UK). A $500 \mu\text{l}$ aliquot of PRP was added to a glass aggregation cuvette containing a stir bar rotating at 900 rpm in the aggregation module maintained at a temperature of 37°C . As a test for spontaneous aggregation the platelet response was monitored for 10 minutes with the chart recorder at a speed setting of 10 mm min^{-1} . Subsequent $500 \mu\text{l}$ aliquots were placed in aggregation cuvettes with stir bars and run for 3 minutes at 37°C with stirring at 900 rpm, during which time the baseline was monitored, before the addition of $5 \mu\text{l}$ of the appropriate concentration of agonist. The platelet aggregatory response was monitored for a minimum of 5 minutes.

2.2.3.4 Evaluation of conventional platelet aggregometry.

In order to standardise the aggregation procedure the following conditions were maintained throughout:

- 1) Platelet concentration of $3 \times 10^{11} \text{ l}^{-1}$
- 2) PRP stored at room temperature, warmed to 37°C for 3 minutes prior to addition of agonist

- 3) Calibration of aggregometer prior to use
- 4) Constant stirring speed of 900 rpm
- 5) All tests completed within 3 h of sample having been taken.

When all these conditions were applied, based upon five preliminary samples the intra-assay coefficient of variation was calculated to be 10.3 %.

A typical aggregometry tracing records the light transmission prior to, and following addition of an agonist for platelet aggregation such as collagen (Figure 2.4). From this tracing a variety of parameters can be measured including the maximum change in light transmission (Tmax), the maximum rate of aggregation (i.e. the tangent to the curve measured in mm per unit time, also termed the 'slope value') and the length of the lag phase (Figure 2.4).

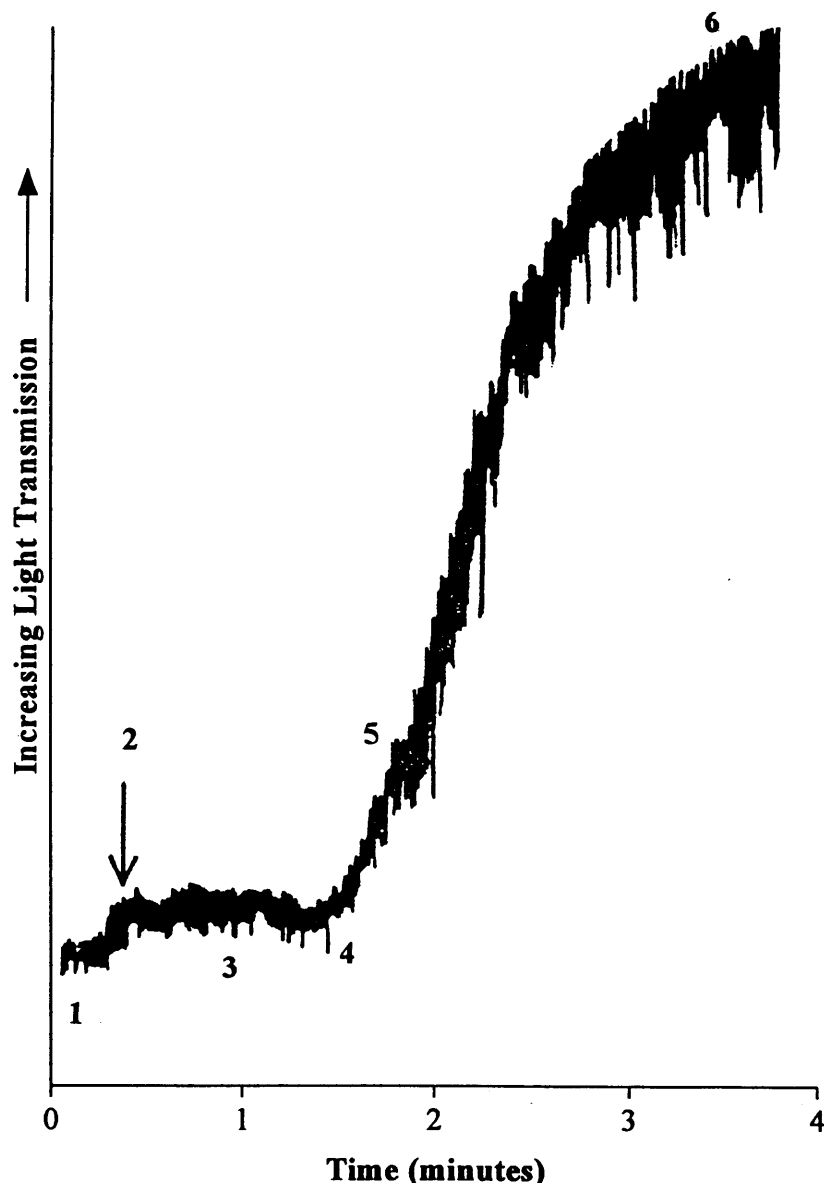


Figure 2.4 A typical collagen induced aggregation tracing.

Several components of which can be described:

- 1) Baseline, the oscillating trace on maximum absorption which represents discoid platelets polarising the light through the unaggregated suspension.
- 2) Addition of the agonist marked by a deflection in the tracing as the light beam is interrupted.
- 3) The lag phase before the commencement of aggregation, a characteristic feature of collagen induced aggregation, the length of which at a given agonist concentration depends on the responsiveness of the platelets.
- 4) Platelet shape change from discoid to spherical form.
- 5) Initiation/progression of aggregation with corresponding increase in light transmission.
- 6) Maximum aggregatory response at which the light transmission reaches a maximum and levels off.

2.2.3.5 Platelet aggregation using a microtitre plate technique.

Platelet aggregation was measured in a washed platelet suspension (prepared as section 2.2.3.1) using the method of Fratantoni & Poindexter (1990). The time course of platelet aggregation was monitored in flat bottom 96 well microtitre plates by following the change in optical density using an automated Anthos HTIII plate reader (Labtech International, East Sussex, UK). Increasing concentrations of thrombin were added to quadruplicate wells at a final concentration ranging from 0-2000 Units l⁻¹. Calcium chloride was added to each well at a final concentration of 0.033 μ mol l⁻¹. Platelet suspension (135 μ l) at a concentration of 3×10^{11} platelets l⁻¹ in Ca²⁺ free tyrodes buffer was then added to each well giving a final total volume per well of 150 μ l. The initial absorbance at 405 nm of each well was measured, and subsequent readings taken over the following 20 minutes at one minute intervals. The microtitre plate was maintained at 37°C throughout the experiment, and between readings shaken at the maximum speed setting. The time taken for the optical density (OD) of each well to reach 0.200 was calculated using the Biolise software package (Labtech International). Using this information a dose response curve was plotted, and the EC₅₀ Units l⁻¹ (UI⁻¹) determined.

2.2.3.6 Evaluation of microtitre plate technique.

As in conventional platelet aggregometry in an attempt to standardise the aggregation procedure certain conditions were consistently applied.

- 1) Washed platelets prepared at a concentration of 3×10^{11} l⁻¹
- 2) Platelets were stored at room temperature and warmed to 37°C prior to use
- 3) Platelet shaking was always performed at maximum setting of plate reader
- 4) Change in optical density of each well was determined at 405 nm
- 5) All tests were completed within 2 h of the blood sample being taken

The Anthos HTIII automatic plate reader contains three agitation programs, the details of these agitation programs (amplitude, direction, period) are proprietary and could not be obtained from the manufacturer. The maximum agitation setting was employed; this resulted in the microtitre plate being shaken for 50 seconds before each reading, which was sufficient to support platelet aggregation. The change in optical density at 405 nm over time within a single well of the microtitre plate following the addition of thrombin at a final concentration of 500 U l⁻¹ is shown in Figure 2.5.

From such curves obtained using thrombin concentrations ranging between 0 – 2000 U l⁻¹ the maximum slope could be determined using the Biolise software which would have been analogous to the slope value of aggregation as measured in an aggregometer. However, the maximum slopes calculated by the computer package were sometimes anomalous. Therefore another parameter was used as a measure of the rate of platelet aggregation, this being the time in seconds (s) taken for the optical density (OD) within each well to reach a value of 0.200. The optical density value 0.200 was selected as preliminary studies had shown that in >95% of the samples this OD was reached within 20 minutes at all thrombin concentrations. Using this parameter and employing the conditions detailed above the intra-assay and inter-assay variabilities were 6.9% and 15.8% respectively. The values calculated for time at which OD=0.200 were then used to plot a dose-response curve of log thrombin concentration against time taken for OD to reach 0.200 (s), which was typically sigmoidal (Figure 2.6) and from which the thrombin EC₅₀ could be determined.

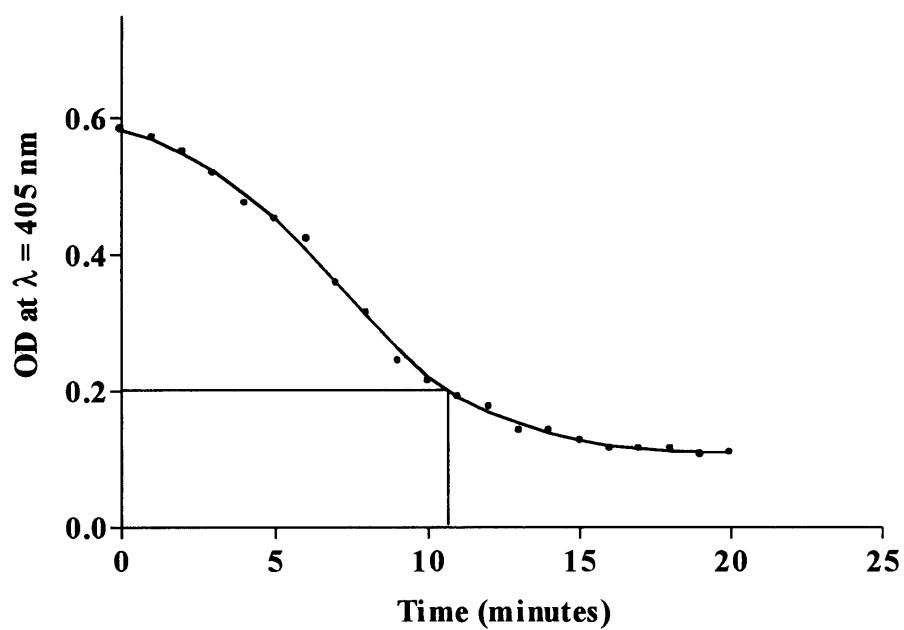


Figure 2.5 Optical density (OD) at $\lambda = 405$ nm over time (min) following addition of thrombin at a final concentration of 500 U l^{-1} . Values are means \pm SEM of quadruplicates from a typical experiment.

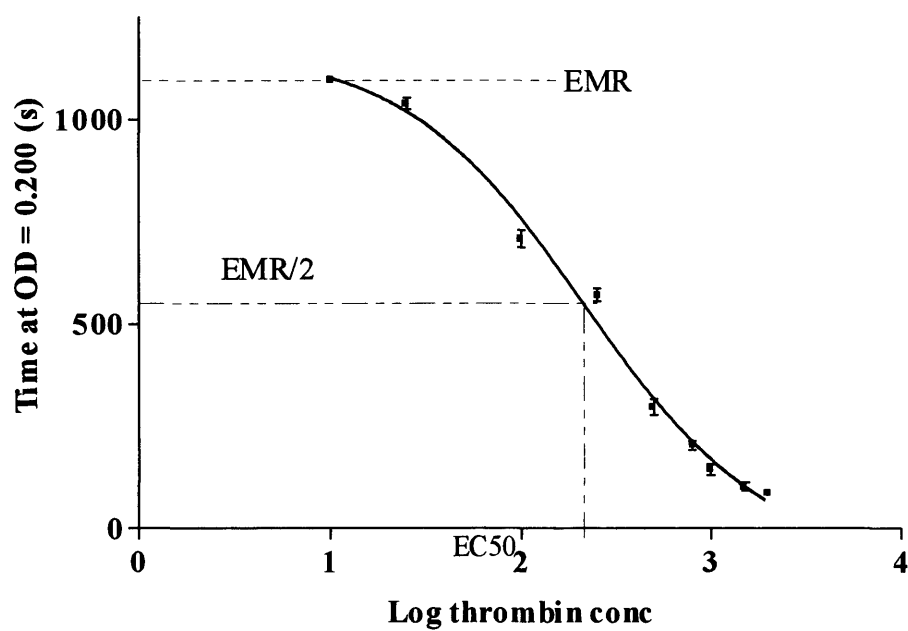


Figure 2.6 Dose-response curve of thrombin-induced aggregation. Values are expressed as mean \pm SEM. EMR = estimated maximum response.

2.2.3.7 Measurement of platelet adhesion

Washed platelets were prepared as previously described and the concentration adjusted to 1×10^{11} platelets l^{-1} in Ca^{2+} free tyrodes buffer. Platelet adhesion was measured according to the method of Bellavite *et al* (1994). This method is based on the determination of the acid phosphatase activity of platelets by using the substrate *p*-nitrophenylphosphate. One hundred microlitres of the platelet suspension was added to plastic and collagen coated ($20 \mu\text{g ml}^{-1}$, overnight at 4°C) wells of a 96 well microtitre plate and incubated at 37°C for 1 hour. Nonadherent platelets were removed by manually washing the plates twice with phosphate buffered saline (PBS) using a multichannel pipette. One hundred and fifty microlitres of 0.1 mol l^{-1} citrate buffer, pH 5.4, containing 0.1% (v/v) Triton-X-100 and 5 mmol l^{-1} *p*-nitrophenol phosphate was rapidly added to each well, and the plate incubated for 1 hour at room temperature. The reaction was then terminated by the addition of $50 \mu\text{l}$ of 4 mol l^{-1} NaOH. The absorbance of each well was determined using an Anthos HTIII automatic plate reader (Labtech International, East Sussex, UK) set at a wavelength of 450 nm. The percentage adhesion was calculated using Biolise software (Labtech International, East Sussex, UK) with reference to a standard curve containing a known number of platelets, prepared for each batch.

2.2.3.8 Evaluation of platelet adhesion method.

Non specific adhesion to tissue culture plastic microwells and specific adhesion to collagen coated microwells was measured according to the method of Bellavite *et al* (1994). Eight preliminary samples were used for the determination of intra-assay and inter-assay coefficients of variation. These were found to be 5.4 % and 9.4 % respectively.

Increasing volumes of a washed platelet suspension to a concentration of $1 \times 10^{11} \text{ l}^{-1}$ was found to produce a linear standard curve (Figure 2.7) from which percentage adhesion was calculated. The activity of acid phosphatase, an enzyme located in the lysosomes of

platelets, was unaffected by thrombin stimulation. This is demonstrated in Figure 2.8 by the comparison of standard curves produced from platelets stimulated with thrombin at concentration of 250 U l^{-1} and from unstimulated platelets, there was no significant difference between these curves. Centrifugation of the stimulated platelet suspension did not affect the activity of acid phosphatase as shown in Figure 2.9, here a standard curve of platelets which had been centrifuged prior to lysis is superimposed on those which were not centrifuged prior to lysis. No significant difference was found between the two curves, suggesting that the enzyme remains cell bound and is not released following activation. Together these show that measurement of acid phosphatase whose activity is stable independently of stimulation and is not released during aggregation, is a reliable and reproducible marker of platelet number.

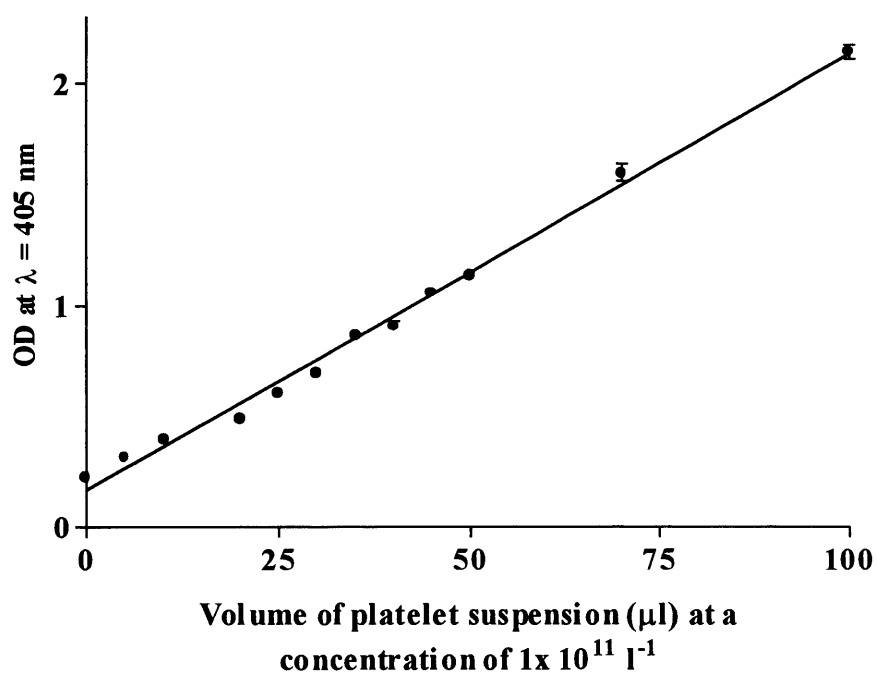


Figure 2.7 Acid phosphatase activity was assessed by optical density (OD) at 450 nm with increasing volumes (μl) of washed platelets at a count of $1 \times 10^{11} \text{ l}^{-1}$. Values are expressed as mean \pm SEM of triplicates from a typical experiment.

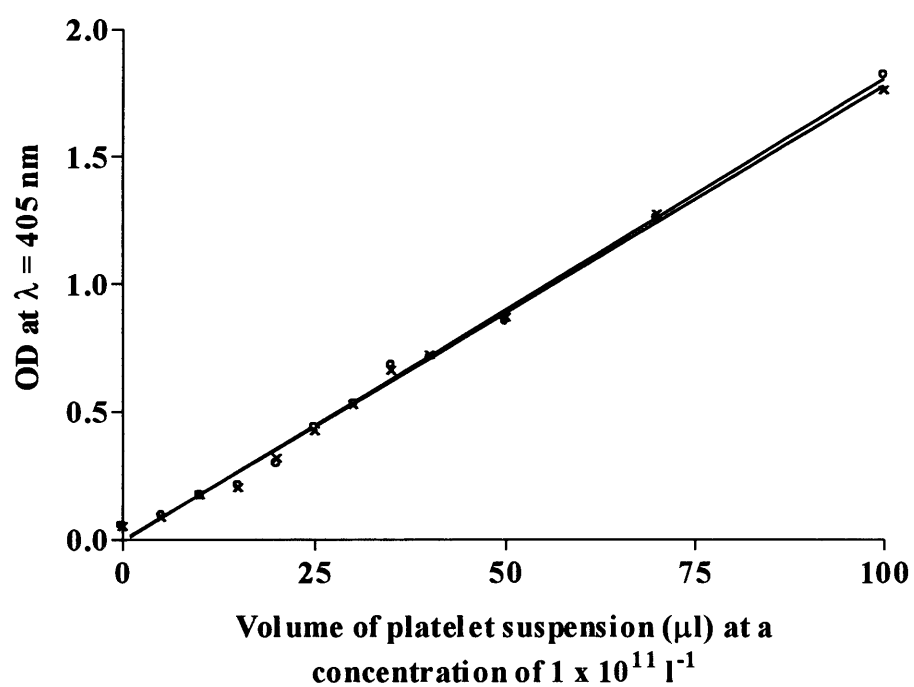


Figure 2.8 Acid phosphatase activity of increasing volumes (μl) of thrombin stimulated (250 U l^{-1}) (x) and unstimulated (o) washed platelets at a count of $1 \times 10^{11} \text{ l}^{-1}$. Values are expressed as mean \pm SEM.

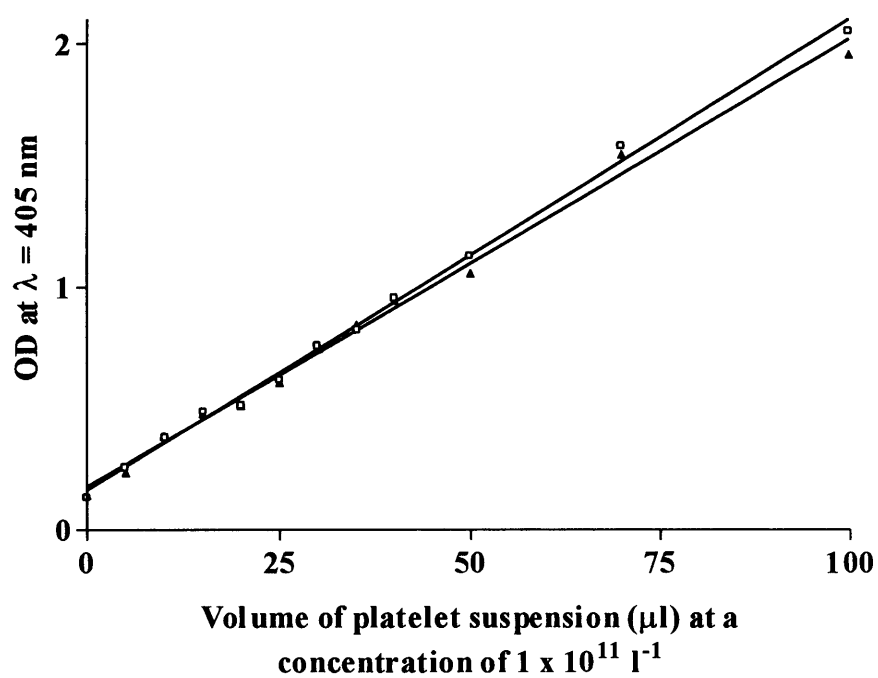


Figure 2.9 Acid phosphatase activity of increasing volumes (μl) of thrombin stimulated (250 U l^{-1}) washed platelets at a count of $1 \times 10^{11} \text{ l}^{-1}$ prior to (\blacktriangle) or following centrifugation (\square) at $1500 \times g$ for 10 minutes. Values are expressed as mean \pm SEM.

2.2.3.9 Measurement of platelet membrane microviscosity (η).

Thrice washed platelets prepared as previously described (section 2.2.3.1) were made up to a concentration of 1×10^{11} platelets l^{-1} in Ca^{2+} free tyrodes buffer. An equal volume of the platelet suspension was added to $1 \mu\text{mol } l^{-1}$ 1,6-diphenyl-1,3,5-hexatriene (DPH) solution ($2 \text{ mmol } l^{-1}$ in tetrahydrofuran stock solution diluted 1 in 2000 immediately prior to use in Ca^{2+} free tyrodes) and incubated at 37°C for 35 minutes according to the method of Shattil & Cooper (1976). The fluorescence intensities with vertically polarised excitation light were measured at the following combinations of polarising light i.e. $0^{\circ}0^{\circ}$, $0^{\circ}90^{\circ}$, $90^{\circ}90^{\circ}$ and $90^{\circ}0^{\circ}$ using a Perkin-Elmer LS-3 fluorescence spectrophotometer fitted with a Heidolph unit and a temperature controlled cell holder set at 37°C . From the average fluorescence polarisation P , corrected for the grating factor G , measured for each sample, fluorescence anisotropy, r , was calculated according to the standard formula (see appendix III) from which the apparent microviscosity (η) was calculated.

2.2.3.10 Evaluation of measurement of membrane microviscosity.

The microviscosity of platelet membranes was assessed by measuring the fluorescence anisotropy (r) of the membrane incorporated fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH) incubated at 37°C for 30 minutes according to the method of Shattil & Cooper (1976). Under these conditions incorporation of this probe has been previously demonstrated to reach stable equilibrium (Shattil & Cooper 1976). All measurements were made in quadruplicate. In each experiment control samples of DPH suspensions and unlabeled platelets were examined, the light intensities of which were less than 3% of total fluorescence and hence were neglected. An intra-assay variability of 8.7% was calculated based upon samples measured in quadruplicate from six healthy volunteers, the average microviscosity of which was determined as $2.1 \pm 0.08 P$.

2.2.4 Measurement Of Monocyte Adhesion.

2.2.4.1 Mononuclear cell isolation.

Whole blood anticoagulated with 1/10th volume of 3.8% (w/v) trisodium citrate was centrifuged at 200 x g for 20 minutes and the upper platelet-rich layer was removed without disturbing the 'buffy-coat'. The blood was restored to its original volume with PBS, and 5 ml aliquots overlaid onto Histopaque®-1077. Blood mononuclear cells were prepared as previously described (Bøyum 1968). In brief the tubes were centrifuged at 400 x g for 30 minutes, the mononuclear cell layer was recovered and washed twice in 5 volumes of PBS. Immediately prior to use, the cells were resuspended in serum-free RPMI 1640 medium at a concentration of $6 \times 10^9 \text{ l}^{-1}$. Prior to use, cell viability was assessed by trypan blue exclusion, and on all occasions viability exceeded 95%. The content of contaminating platelets was low, with a ratio of platelets: mononuclear cells being <1.

2.2.4.2 EA-hy-926 cell culture.

EA-hy-926 is hybrid cell line produced as a result of the fusion of Human Umbilical Vein Endothelial Cells (HUVEC) with the human epithelial line, A549, originally derived from lung carcinoma (Edgell *et al* 1983). The EA-hy-926 cell line has been shown to retain a number of properties of endothelial cells including expression of von Willebrand Factor, the capacity to produce prostacyclin (Suggs *et al* 1986) and fibrinolytic properties (Emeis & Edgell 1988). The adhesive properties of this cell line have also been investigated and found to be similar to those of HUVEC (Thornhill *et al* 1993).

Cultured EA-hy-926 cells were serially passaged (1:3 split ratio) using trypsin (0.05% w/v) / (0.02% w/v) EDTA solution and grown to confluence in T75 tissue culture flasks

with Dulbecco's modified Eagle's medium (containing 4.5 g l⁻¹ glucose, 100 µmol l⁻¹ hypoxanthine, 0.4 µmol l⁻¹ aminopterin and 16 µmol l⁻¹ thymidine) with 10% heat-inactivated foetal calf serum, at 37°C in a humidified atmosphere of 5% CO₂/air. For the adhesion assay the EA-hy-926 cells were used between passages 30-40 and grown to confluence (3-4 days) on 96-well flat bottom microtitre plates. Prior to use in the adhesion assay cells were washed twice with RPMI-1640 medium.

2.2.4.3 Measurement of monocyte adhesion.

Mononuclear cell (MNC) suspensions in serum-free RPMI were added to wells containing confluent endothelial monolayers (6×10^5 MNC per well), or cell-free wells (6×10^5 MNC per well) in 96 well microtitre plates. The plates were incubated at 37°C for 30 minutes, and non-adherent cells removed by washing twice with PBS. Monocyte specific adherence was determined by a modification of the method described by Bath et al (1989). This method relies on conversion of a colourless substrate tetramethyl benzidine (TMB) to a blue product by the action of monocyte-specific myeloperoxidase activity. The cells contained in each well were lysed in 100 µL hexadecyltrimethylammonium bromide (0.5% in PBS; pH 5.0) at 37°C for 60 minutes. A fresh solution of TMB (0.1 mg ml⁻¹ in 0.05M phosphate citrate buffer (pH 6.0) containing 0.03% sodium perborate) was added to each well and the plate incubated for 10 minutes at room temperature. The reaction was stopped by the addition of 50 µl of 2.5 mol l⁻¹ sulphuric acid and the absorbance was measured at 450 nm using an Anthos HTIII microplate reader. A standard curve of cell number versus absorbance was constructed for each batch of MNCs and absolute adhesion calculated by reference to this curve.

2.2.4.4 Evaluation of monocyte adhesion method.

Monocyte adhesiveness was measured by the method of Bath *et al* (1989) which had previously been validated for use within our laboratory. Eight preliminary samples were used for the determination of intra-assay and inter-assay coefficients of variation. These were found to be 6.67 % and 10.4 % respectively. Increasing volumes of a mononuclear cell suspension at a count of $6 \times 10^9 \text{ l}^{-1}$ was found to produce a consistently linear relationship (Figure 2.10). Despite the addition of a mononuclear cell suspension which contained monocytes, lymphocytes and neutrophils, only the adhesion of the monocytes was measured by the substrate TMB, which determined the activity of the monocyte-specific enzyme, myeloperoxidase. The degree of monocyte adhesion was expressed as a percentage, which was calculated using a standard curve produced for each batch of monocytes.

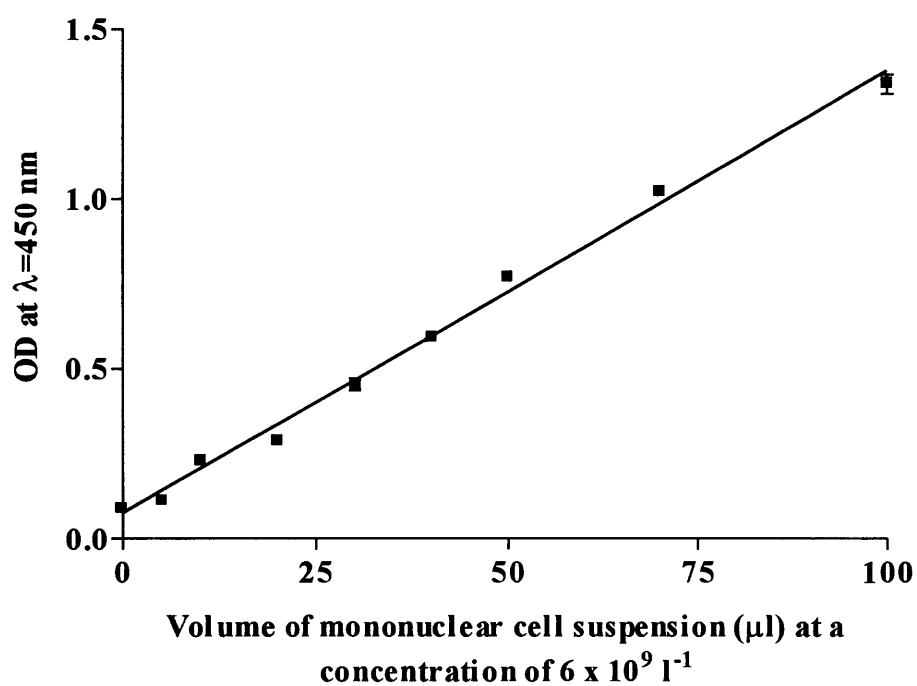


Figure 2.7 Myeloperoxidase activity as assessed by optical density (OD) at 450 nm with increasing volumes (μl) of mononuclear cell suspension at a count of $6 \times 10^9 \text{ l}^{-1}$. Values are expressed as mean \pm SEM of triplicates from a typical experiment.

2.2.5 Measurement Of Plasma Levels Of Soluble Adhesion Molecules.

2.2.5.1 Soluble ICAM-1.

Plasma levels of soluble ICAM-1 (sICAM-1) were determined using a Parameter human sICAM-1 ELISA kit (R&D Systems, Abingdon, Oxon, UK). One hundred microlitres of Anti-ICAM-1-HRP-conjugate was added to each well of the 96 well microtitre plate provided, that had previously been coated with murine monoclonal antibody to human sICAM-1. Following this 100 µl of either the standards provided, diluted sample (20 fold in sample diluent provided) or diluted Parameter serum control (20 fold in sample diluent) was added to the appropriate wells in duplicate. The plate was then covered with a plate sealer and incubated at room temperature for 1.5 hours. The plate was washed 6 times using an Anthos AW1, automatic plate washer (Anthos Labtech instruments, Salzburg, Austria), any residual liquid was removed by tapping the inverted plate firmly on clean paper towels. 100 µl of the substrate provided was added to each well in and the plate covered with a plate sealer and incubated at room temperature for 30 minutes. At the end of the incubation period 100 µl of the stop solution provided was added to each well in the same order as the substrate. The absorbance of each well was determined within 30 minutes using an Anthos HTIII plate reader (Anthos Labtech instruments, Salzburg, Austria) set at 450 nm with a correction wavelength of 620 nm. The correction wavelength was employed to ensure the absorbance due to the plastic microtitreplate does not interfere with the optical density reading. The concentration of sICAM-1 was determined from the standard curve using BIOLISE software (Labtech, Uckfield, UK).

2.2.5.2 Soluble E-selectin.

Plasma levels of soluble E-selectin (sE-selectin) were determined using a Parameter human sE-selectin ELISA kit (R&D Systems, Abingdon, Oxon, UK). One hundred

microlitres of Anti-E-selectin-HRP-conjugate was added to each well of the 96 well microtitre plate provided, that had previously been coated with murine monoclonal antibody to human sE-selectin. Following this 100 μ l of either the standards provided, diluted sample (20 fold in sample diluent provided) or diluted Parameter serum control (20 fold in sample diluent) was added to the appropriate wells in duplicate. The plate was then covered with a plate sealer and incubated at room temperature for 1.5 hours. The plate was washed 6 times using an Anthos AW1, automatic plate washer (Anthos Labtech instruments, Salzburg, Austria), any residual liquid was removed by tapping the inverted plate firmly on clean paper towels. One hundred microlitres of the substrate provided was added to each well in and the plate covered with a plate sealer and incubated at room temperature for 30 minutes. At the end of the incubation period 100 μ l of the stop solution provided was added to each well in the same order as the substrate. The absorbance of each well was determined within 30 minutes using an Anthos HTIII plate reader (Anthos Labtech instruments, Salzburg, Austria) set at 450 nm with a correction wavelength of 620 nm. The concentration of sE-selectin was determined from the standard curve using BIOLISE software (Labtech, Uckfield, UK).

2.2.5.3 Soluble L-selectin.

Plasma levels of soluble L-selectin (sL-selectin) were determined using a Parameter® human sL-selectin ELISA kit (R&D Systems, Abingdon, Oxon, UK). One hundred microlitres of either the standards provided, diluted sample (100 fold dilution in sample diluent provided) or diluted Parameter® control serum (100 fold dilution in sample diluent) were added in duplicate to the appropriate wells of the 96 well microtitre plate provided, that had previously been coated with murine monoclonal antibody to human sL-selectin. The plate was then covered with a plate sealer and incubated at room temperature for 1 hour. Following this incubation period 100 μ l of Anti-L-selectin HRP-conjugate was added to each well of the microtitre plate, which was then incubated at room temperature for 30 minutes. The plate was then washed 6 times using an Anthos

AW1 automatic plate washer (Anthos Labtech Instruments, Salzburg, Austria), any residual liquid was removed by tapping the inverted plate firmly on clean paper towels. To each well 100 μ l of the TMB substrate provided was then added and the plate covered with a plate sealer and incubated at room temperature for 30 minutes. One hundred microlitres of stop solution (acid solution) was then added to each well. The absorbance of each well was determined within 30 minutes using an Anthos HTIII plate reader (Anthos Labtech Instruments, Salzburg, Austria) set at 450 nm with a correction wavelength of 620 nm. The concentration of sL-selectin in the samples and control serum was calculated from the standard curve using BIOLISE software (Labtech, Uckfield, UK).

2.2.5.4 Soluble P-selectin.

Quantitative determination of human soluble P-selectin (sP-selectin) was performed using an immunoassay kit (R&D Systems, Abingdon, Oxon, UK) according to the following provided protocol. All samples and the parameter control serum were diluted 1 in 20 using sample diluent provided prior to assay. 100 μ l of either standard, sample or parameter control serum was added in duplicate to the appropriate wells of a 96 well microtitre plate that had been previously coated with a murine monoclonal antibody to human sP-selectin. To each well 100 μ l of diluted anti sP-selectin HRP-conjugate was added. The microtitre plate was then covered with a plate sealer and incubated for 1 hour at room temperature. Following this incubation period the microtitre plate was washed three times with the wash buffer provided, using an automated Anthos AW1 automatic plate washer (Anthos Labtech Instruments, Salzburg, Austria). Following the washing process any excess liquid was removed from the plate by tapping the inverted microtitre plate firmly on a clean paper towel. Then 100 μ l of TMB solution was added to each well and the plate left to incubate for 15 minutes at room temperature. The reaction was terminated by the addition of 100 μ l of sulphuric acid solution. The optical density of each well was determined within 30 minutes using an automated Anthos HTIII plate

reader (Anthos Labtech instruments, Salzburg, Austria) set to 450nm. The concentration of sP-selectin in the samples and control serum was calculated from the standard curve using BIOLISE software (Labtech, Uckfield, UK).

2.2.5.5 von Willebrand factor (vWF).

Plasma levels of von Willebrand Factor (vWF) were determined using a Shield von Willebrand Factor Activity Test (Shield Diagnostics Ltd., Dundee, UK), a quantitative direct enzyme immunoassay in which a purified monoclonal antibody which recognises a functional epitope on the vWF antigen is used. The characteristics of this antibody have been documented (Goodall *et al* 1985). The samples for measurement were diluted (1 in 20) with the vWF diluent provided prior to assay. The calibrator and controls were reconstituted in 0.5 ml dH₂O and allowed to stand for 30 minutes with intermittent swirling. The controls were then diluted 1 in 20 with the vWF diluent. The calibrator was then diluted 1 in 10, from this 1 in 10 stock a series of doubling dilutions were prepared to give a decreasing range of calibrator values, all dilutions were prepared using the vWF diluent. To the appropriate wells of a microtitre plate, previously coated with a preparation of purified murine anti-vWF IgG monoclonal antibody, 100 µl of either calibrator, control or sample were added in duplicate, the plate was then incubated at room temperature for 1 hour. Following this incubation period the microtitre plate was washed five times using an Anthos AW1 automatic plate washer (Anthos Labtech Instruments, Salzburg, Austria), any residual liquid was removed by tapping the inverted plate firmly on clean paper towels. To each well of the microtitre plate 100 µl of the working strength vWF conjugate was added (conjugate concentrate was diluted 1 in 500, 5-10 minutes before use), the plate was then incubated at room temperature for 1 hour. Next the microtitre plate was washed five times using an Anthos AW1 automatic plate washer (Anthos Labtech Instruments, Salzburg, Austria), any residual liquid was removed by tapping the inverted plate firmly on clean paper towels. To all wells of the microtitre plate 100 µl of TMB substrate (diluted 1 in 5 with substrate buffer, 5-10 minutes before use) was added, the plate was then incubated for 5 minutes at room

temperature. Following which, 100 μl of stop solution (2 mol l^{-1} H_2SO_4) was added to each well. The absorbance of each well was determined within 30 minutes using an Anthos HT III plate reader (Anthos Labtech Instruments, Salzburg, Austria) set at 450 nm.

2.2.5.6 Evaluation of measurement of soluble adhesion molecules.

Measurement of soluble adhesion molecules in plasma were performed using commercially available ELISA kits, the inter-assay and intra-assay variation of which were $<10\%$. A vial of control serum was provided with each kit, the data obtained was only used if the value obtained for the QC fell within the expected range (assigned value ± 2 SD), indicating the validity of the assay performed. All samples and standards were performed in duplicate and the data only utilised if the difference between the two values was less than 10%.

2.2.6 Plasma Protein Determination By Lowry Assay.

The amount of protein within the plasma was determined according to the method of Lowry *et al* (1951). Briefly plasma was diluted 50 fold in dH_2O and 5, 10 and 20 μl were added to plastic tubes (Sarstedt, Leicester, UK), in duplicate. To another set of tubes 5, 10 and 20 μl of 1 mg ml^{-1} Bovine Serum Albumin (BSA) was added in duplicate to act as standards. Solutions of 2% Na_2CO_3 in 0.4% NaOH (w/v)/ 1% CuSO_4 (w/v)/ 2% potassium sodium tartrate (w/v) were mixed in the following ratio 10 ml/ 0.1 ml/ 0.1 ml respectively. To all tubes 1 ml of the above solution was added, vortex mixed and incubated at room temperature for 10 minutes. Next 100 μl of 50% (v/v) Folin-Ciocalteu phenol reagent in dH_2O was added to each tube, vortex mixed and incubated at room temperature for 30 minutes. The absorbance was read at 650 nm on a UV-160A UV-

Visible spectrophotometer (Shimadzu Corporation, Japan). The protein concentration of the plasma was determined from the BSA standard curve.

CHAPTER 3

**EFFECTS OF α -TOCOPHEROL AND ASCORBIC ACID
ON PLATELET AND MONOCYTE FUNCTION *IN VITRO*.**

3.1 INTRODUCTION

Platelets participate in the development of atherosclerotic lesions and are intimately involved in its thrombotic complications. Alteration to the endothelium or a break in its continuity can promote platelet adhesion, which generally precedes aggregation, the adherence of platelets to each other. The antioxidant vitamins C and E inhibit both platelet adhesion and aggregation (Agradi et al 1981; Cordova et al 1982; Bordia & Verma 1985; Jandak et al 1988). The mechanism by which this occurs remains to be conclusively determined.

The present study was designed to investigate the effect of α -tocopherol and ascorbic acid on platelet adhesion and the effect of α -tocopherol on platelet aggregation and platelet membrane fluidity. Platelet adhesion was measured by the method of Bellavite *et al* (1994) which quantitates the number of platelets adhering to collagen coated or tissue culture plastic wells by the activity of the enzyme acid phosphatase. Platelet aggregation was monitored using two methods. The first was the conventional method described by Born (1962) which involves following the changes in light transmission with the use of a temperature controlled aggregation module linked to a chart recorder. The alternative method is based upon that of Fratantoni & Poindexter (1990) in which the time course of platelet aggregation is monitored in flat bottom 96 well microtitre plates. An automatic plate reader is utilised with the temperature maintained at 37°C throughout the experiment, between readings the plate is shaken. This method has a number of advantages over conventional

aggregometry. Firstly, as many as 96 samples can be analysed, allowing a more complex experimental design. Secondly, all samples are studied within the same time period allowing meaningful comparisons between the conditions. Finally, the output is suitable for immediate computer analysis and not subject to interpretation error as in conventional aggregometry.

Like all mammalian cells, the surface membrane of platelets is composed of lipid and protein to which the fluid mosaic model proposed by Singer & Nicolson (1972) applies. Individual lipid molecules are able to diffuse freely within lipid bilayers and the composition of this bilayer affects its fluidity (Shattil & Cooper 1976). The easiest method to measure fluidity is by steady state fluorescence depolarisation of the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH) which serves as a convenient probe of the fluidity (or microviscosity) of the lipid environment in which it resides (Shinitzky & Barenholz 1974). The measured data reflects the structural order of the membrane lipids, from which apparent microviscosity (η) can be calculated.

Monocytes also play an important role in the pathogenesis of atherosclerosis. Adhesion of monocytes to the endothelium of lesion prone regions of the vasculature is one of the earliest visible events in animal models of atherosclerosis (Gerrity 1981; Faggitto *et al* 1984a; Joris *et al* 1983). Monocytes bind to the endothelium through plasma membrane associated cellular adhesion molecules (Butcher 1991). A dysfunctional endothelium may exhibit enhanced expression of these adhesion molecules along with increased secretion of cytokines and growth factor (Poston *et al* 1992; Davies *et al* 1993). Together they may act to attract, activate and cause transendothelial migration of monocytes into the intima of the vessel wall. Once within the intima they can take up oxidised LDL and form the characteristic lipid laden foam cell of atherosclerotic lesions. Monocyte adhesion to endothelial cells *in vitro* therefore serves as a useful model that simulates the early cellular events in the

pathophysiology of atherosclerosis. Antioxidants both *in vitro* and following supplementation in animals and man have been demonstrated to decrease monocyte-endothelial cell adhesion (Ferns *et al* 1993; Faruqi *et al* 1994; Devaraj *et al* 1996). This may be one of the mechanisms by which antioxidants and more specifically vitamin E exerts an anti-atherogenic effect. In the present study we have examined the effect of pre-incubation of monocytes and endothelial cells with α -tocopherol on monocyte adhesiveness to EA-hy-926 endothelial cells and tissue culture plastic microwells using the method of Bath *et al* (1989).

3.2 MATERIALS AND METHODS

Materials and their suppliers are listed in appendix I

Details of methods employed during this study are described in section 2.2

3.2.1 Examination Of The Effect Of α -Tocopherol On Platelet Aggregation *In Vitro*.

3.2.1.1 Conventional Aggregometry Method.

The effect of α -tocopherol on platelet aggregation was examined as follows. Aliquots of 500 μ l of platelet-rich plasma (PRP), prepared as described in section 2.2.3.1, were pre-incubated at 37°C in a Payton aggregometer (Ion Trace Inc, Canada) for 5 minutes with α -tocopherol at 100 or 200 μ mol l⁻¹ (dissolved in absolute ethanol) or 1% ethanol. Platelet aggregation was then monitored according to a standard aggregometry technique as detailed in section 2.2.3.3, the agonists employed were collagen 10, 5 and 2.5 μ g ml⁻¹ and adenosine diphosphate (ADP) at concentrations of 10, 5 and 2.5 μ mol l⁻¹.

3.2.1.2 Microtitre Plate Method.

Washed platelet suspensions at a concentration of 3×10^{11} l⁻¹ (refer to section 2.2.3.2 for preparation details) were incubated at ambient temperature in sealed plastic containers for 1 hour with either α -tocopherol at 100 or 200 μ mol l⁻¹ (dissolved in absolute ethanol) or 1% ethanol. Aggregation was then monitored according to the method of Fratantoni & Poindexter (1990) (refer to section 2.2.3.4 for details).

3.2.2 Examination Of The Effect Of α -Tocopherol On Platelet Adhesion *In Vitro*.

Washed platelets at a concentration of $1 \times 10^{11} \text{ l}^{-1}$ (refer to section 2.2.3.2. for preparation details) were incubated at ambient temperature for 1 hour with either α -tocopherol at a final concentration of 100 or 200 $\mu\text{mol l}^{-1}$ (dissolved in absolute ethanol) or 1% ethanol. Adhesion was then measured according to the method of Bellavite *et al* (1994) described in section 2.2.3.7. During the adhesion procedure thrombin at a final concentration of 250 U l^{-1} was added to the appropriate wells, the adhesion values obtained from these wells were categorised as 'stimulated' adhesion. At this concentration of thrombin, <20% of the platelets adhered as small aggregates, but a consistently elevated adhesion value was obtained. To all other wells, an equivalent volume of Ca^{2+} free Tyrodes buffer (refer to appendix II for composition) was added and the adhesion values obtained from these wells categorised as 'resting' adhesion. The percentage platelet adhesion was calculated using Biolise software (Labtech International, East Sussex, UK) with reference to a standard curve prepared for each condition as previously described in section 2.2.3.7.

3.2.3 Examination Of The Effect Of α -Tocopherol On Platelet Membrane Microviscosity (η) *In Vitro*.

α -tocopherol at a final concentration of 100 or 200 $\mu\text{mol l}^{-1}$ (dissolved in ethanol) or 1% ethanol was added to platelet-rich plasma (prepared as described in section 2.2.3.1) and incubated at ambient temperature for 1 hour. Following this incubation period washed platelets at a count of $1 \times 10^{11} \text{ l}^{-1}$ were prepared (refer to section 2.2.3.2 for preparation details) and the membrane microviscosity (η) of these platelets determined as in described in section 2.2.3.9.

3.2.4 Examination Of The Effect Of α -Tocopherol On Monocyte Adhesion *In Vitro*.

A mononuclear cell suspension at a concentration of $6 \times 10^9 \text{ l}^{-1}$ was prepared as previously described in section 2.2.4.1. EA-hy-926 cells grown to confluence in 96 well microtitre plates (refer to section 2.2.4.2 for cell culture conditions) were incubated with RPMI containing $100 \mu\text{mol l}^{-1}$ α -tocopherol (dissolved in ethanol) or an RPMI/ethanol control for 1 hour at 37°C . Mononuclear cells were then added to washed EA-hy-926 cells and monocyte adhesion measured according to the method of Bath *et al* (1989) (refer to section 2.2.4.3 for details). Monocytes at a concentration of $6 \times 10^9 \text{ l}^{-1}$ were also incubated at 37°C for 1 hour with $100 \mu\text{mol l}^{-1}$ or a 1% ethanol control. The adhesion of these monocyte to endothelial cells and tissue culture plastic was then measured as above.

3.2.5 Examination Of The Effect Ascorbic Acid On Platelet Adhesion *In Vitro*.

Washed platelets at a concentration of $1 \times 10^{11} \text{ l}^{-1}$ (refer to section 2.2.3.2 for preparation details) were incubated at room temperature for 1 hour with either 1 or 2 mg l^{-1} ascorbic acid (dissolved in dH_2O). A significant fall in pH resulted from the addition of ascorbic acid at both 1 and 2 mg ml^{-1} . To compensate for this alteration in pH a control was employed, the pH of which was adjusted to the pH of the platelet suspension to which ascorbic acid had been added, by the addition of 5 mol l^{-1} citric acid. Platelet adhesion was then measured according to the method of Bellavite *et al* (1994) described in section 2.2.3.7. During the adhesion procedure thrombin at a final concentration of 250 U l^{-1} was added to the appropriate wells, the adhesion values obtained from these wells were categorised as 'stimulated' adhesion. To all other wells, an equivalent volume of Ca^{2+} free Tyrodes buffer (refer to appendix II for composition) was added and the adhesion values obtained from these wells categorised as 'resting' adhesion.

The percentage platelet adhesion was calculated using Biolise software (Labtech International, East Sussex, UK) with reference to a standard curve prepared for each condition.

3.2.6 Statistical Analysis.

Statistical analysis was performed using Instat software (Graphpad Inc., USA) on a PC. Significance was assessed using ANOVA and paired t-tests. $p < 0.05$ was considered statistically significant. Results are expressed as mean \pm SEM.

3.3 RESULTS

3.3.1 Effects Of Vitamin E On Platelet Aggregation *In Vitro*.

3.3.1.1 Conventional aggregometry method.

α -tocopherol resulted in a significant decrease in both the slope value ($p < 0.0001$, ANOVA, $n=5$) and the T_{max} ($p=0.0069$, ANOVA, $n=5$) on collagen induced aggregation (Table 3.1, Figures 3.1 and 3.2). Inhibition of the slope value ($LTU\ min^{-1}$) was significant at 100 and 200 $\mu mol\ l^{-1}$ ($p < 0.001$ vs 1% ethanol), as was the inhibitory effect on the maximum light transmission (T_{max}) ($p < 0.05$ vs 1% ethanol and $p < 0.01$ vs 1% ethanol respectively). Whilst incubation with α -tocopherol significantly inhibited only the slope value of ADP induced aggregation ($p=0.04$, ANOVA, $n=4$), which was only significant at 200 $\mu mol\ l^{-1}$ ($p < 0.05$ vs 1% ethanol) (Table 3.2, Figures 3.3 and 3.4). A reduction was also seen in the value for T_{max} but this failed to reach significance ($p=0.0706$, ANOVA, $n=4$).

Table 3.1 Slope (LTU min⁻¹) and Tmax (%) of platelet aggregation in response to 2.5, 5.0 and 10 µg ml⁻¹ collagen, following pre-incubation with 1% ethanol, 100 µmol l⁻¹ or 200 µmol l⁻¹ α-tocopherol for 5min at 37°C. Values are expressed as mean ± SEM.

	Ethanol control	α-tocopherol 100 µmol l ⁻¹	α-tocopherol 200 µmol l ⁻¹
Slope (LTU min ⁻¹)			
2.5 µg ml ⁻¹	25.3 ± 3.13	16.4 ± 2.05	12.0 ± 0.50
5.0 µg ml ⁻¹	37.9 ± 4.38	29.4 ± 3.83	25.0 ± 4.32
10.0 µg ml ⁻¹	49.3 ± 4.49	40.6 ± 5.31	34.0 ± 3.18

Tmax (%)			
2.5 µg ml ⁻¹	40.6 ± 1.61	20.7 ± 1.60	18.3 ± 2.5
5.0 µg ml ⁻¹	56.5 ± 3.76	45.6 ± 4.62	37.0 ± 3.49
10.0 µg ml ⁻¹	60.4 ± 4.13	54.2 ± 3.77	38.9 ± 3.37

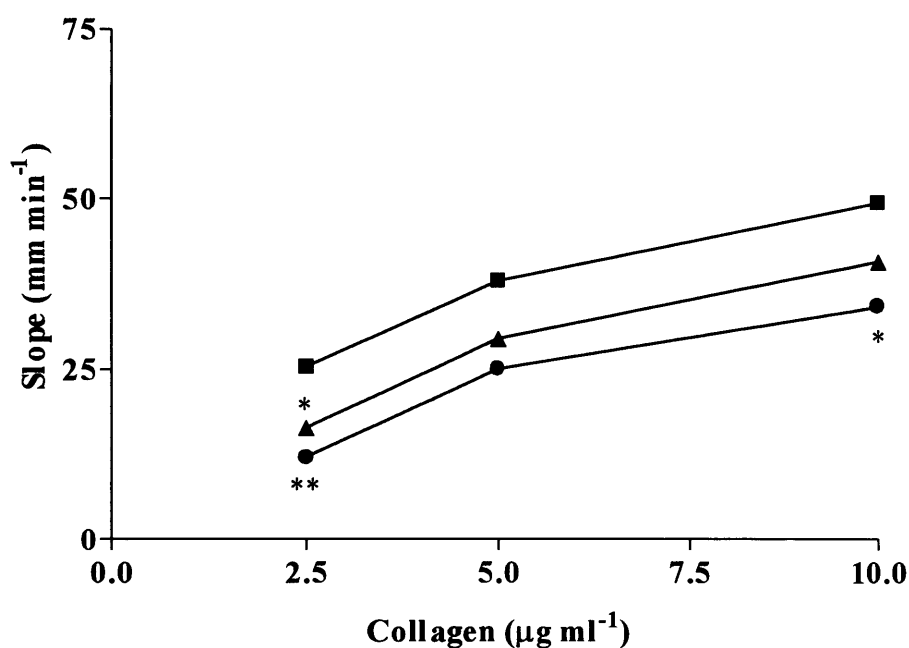


Figure 3.1 Rate of platelet aggregation (LTU min^{-1}) in response to different concentrations of collagen. Platelet rich plasma at a count of $3 \times 10^{11} \text{ l}^{-1}$ pre-incubated with either 1% ethanol (■), $100 \mu\text{mol l}^{-1}$ α -tocopherol (▲) or $200 \mu\text{mol l}^{-1}$ α -tocopherol (●) for 5 min at 37°C . The final collagen concentrations were 2.5, 5.0 and $10 \mu\text{g ml}^{-1}$. Each point and vertical bar represent the mean \pm SEM of 5 experiments. * $p < 0.05$, ** $p < 0.01$.

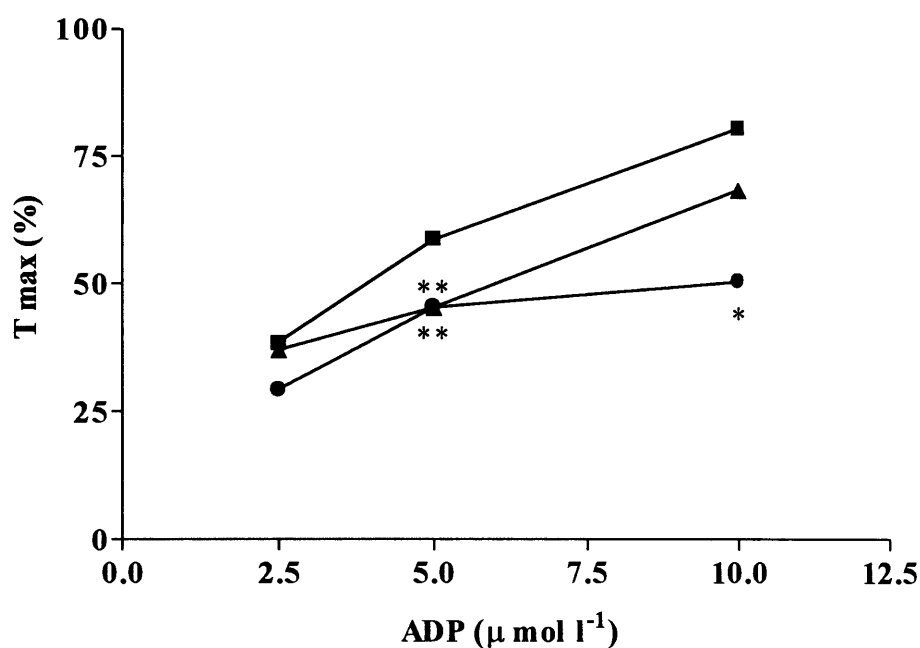


Figure 3.2 Extent of platelet aggregation (%) in response to different concentrations of collagen. Platelet rich plasma at a count of $3 \times 10^{11} \text{ l}^{-1}$ pre-incubated with either 1% ethanol (■), $100 \mu \text{mol l}^{-1}$ α -tocopherol (▲) or $200 \mu \text{mol l}^{-1}$ α -tocopherol (●) for 5 min at 37°C . The final collagen concentrations were 2.5, 5.0 and $10 \mu \text{g ml}^{-1}$. Each point and vertical bar represents the mean \pm SEM of 5 experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Table 3.2 Slope (LTU min⁻¹) and Tmax (%) of platelet aggregation in response to 2.5, 5.0 and 10 µmol l⁻¹ ADP, following pre-incubation with 1% ethanol, 100 µmol l⁻¹ or 200 µmol l⁻¹ α-tocopherol for 5min at 37°C.

	Ethanol control	α-tocopherol 100 µmol l ⁻¹	α-tocopherol 200 µmol l ⁻¹
Slope (LTU min ⁻¹)			
2.5 µmol l ⁻¹	51.0 ± 5.06	47.0 ± 5.92	38.3 ± 5.81
5.0 µmol l ⁻¹	71.2 ± 2.02	53.8 ± 2.06	52.8 ± 1.96
10.0 µmol l ⁻¹	85.5 ± 5.58	65.3 ± 2.91	48.7 ± 4.91

Tmax (%)			
2.5 µmol l ⁻¹	38.5 ± 5.96	37.0 ± 6.35	29.3 ± 4.75
5.0 µmol l ⁻¹	58.2 ± 2.35	45.3 ± 3.0	45.3 ± 2.13
10.0 µmol l ⁻¹	80.5 ± 4.09	68.3 ± 5.44	50.3 ± 4.01

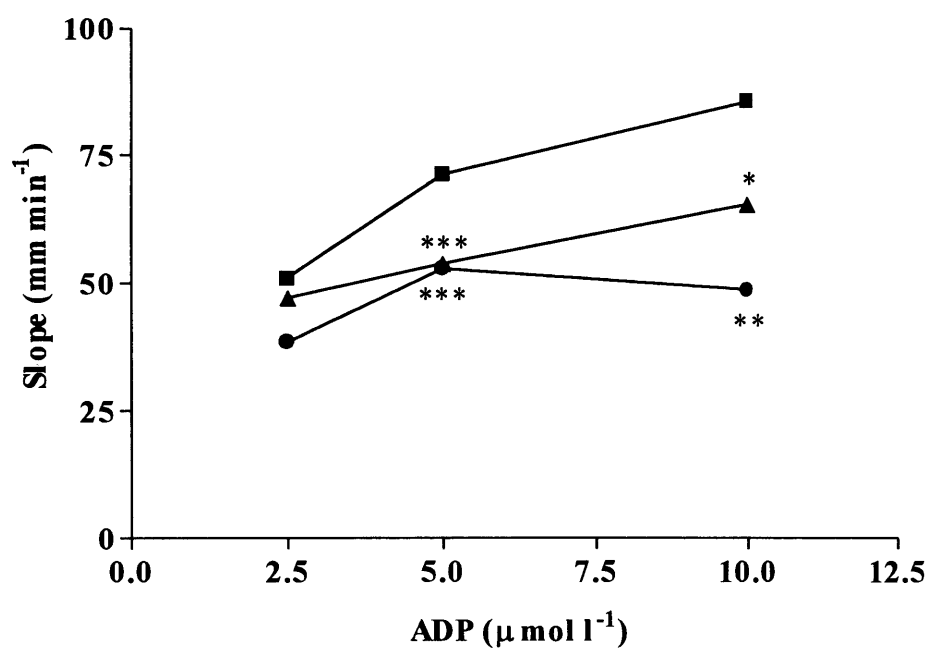


Figure 3.3 Rate of platelet aggregation (LTU min^{-1}) in response to different concentrations of ADP. Platelet rich plasma at a count of $3 \times 10^{11} \text{ l}^{-1}$ pre-incubated with either 1% ethanol (\blacksquare), $100 \mu\text{mol l}^{-1}$ α -tocopherol (\blacktriangle) or $200 \mu\text{mol l}^{-1}$ α -tocopherol (\bullet) for 5 min at 37°C . The final ADP concentrations were 2.5, 5.0 and $10 \mu\text{mol l}^{-1}$. Each point and vertical bar represent the mean \pm SEM of 4 experiments. ** $p < 0.01$.

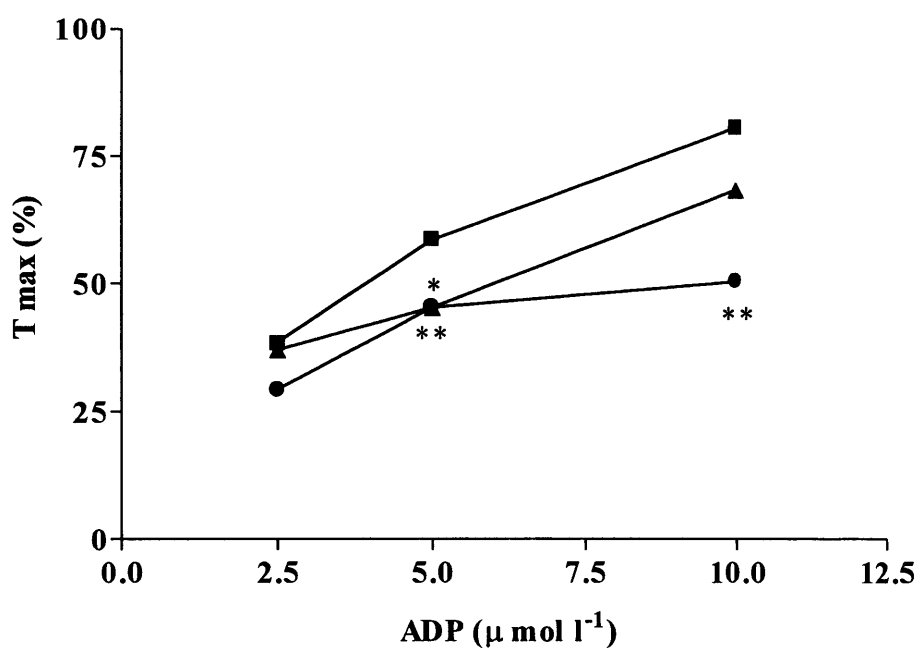


Figure 3.4 Extent of platelet aggregation (%) in response to different concentrations of ADP. Platelet rich plasma at a count of $3 \times 10^{11} \text{ l}^{-1}$ pre-incubated with either 1% ethanol (\blacksquare), $100 \mu \text{mol l}^{-1}$ α -tocopherol (\blacktriangle) or $200 \mu \text{mol l}^{-1}$ α -tocopherol (\bullet) for 5 min at 37°C . The final ADP concentrations were 2.5, 5.0 and $10 \mu \text{mol l}^{-1}$. Each point and vertical bar represent the mean \pm SEM of 4 experiments. * $p < 0.05$, ** $p < 0.01$.

3.3.1.2 Microtitre plate method.

Pre-incubation of washed platelets with $100 \mu\text{mol l}^{-1}$ α -tocopherol produced a 12% increase in the mean EC_{50} (U l^{-1}), however this increase failed to reach statistical significance (257 ± 40 Vs 289 ± 40 , $n=5$, $p>0.05$). A significant 48% increase in mean EC_{50} (U l^{-1}) was observed following incubation with $200 \mu\text{mol l}^{-1}$ α -tocopherol (257 ± 40 Vs 380 ± 62 , $n=5$, $p=0.035$) (Figure 3.5). This increase in EC_{50} represents a significant inhibition of platelet aggregation *in vitro* by α -tocopherol at a concentration of $200 \mu\text{mol l}^{-1}$.

3.3.2 Effect Of α -Tocopherol On Platelet Adhesion *In Vitro*.

The adhesion of resting (89.7 ± 2.9 vs 90.5 ± 2.9 , $n=8$, $p>0.05$) and stimulated (93.4 ± 2.1 vs 97.1 ± 2.6 , $n=8$, $p>0.05$) platelets pre-incubated with α -tocopherol at a final concentration of $100 \mu\text{mol l}^{-1}$ were not significantly different to that of the 1% ethanol control (Figure 3.6). Incubation with a higher concentration of $200 \mu\text{mol l}^{-1}$ had no significant effect on resting platelet adhesion (86.8 ± 2.5 vs 84.0 ± 2.5 , $n=11$, $p>0.05$) but produced a significant inhibition of thrombin (250 U l^{-1}) stimulated adhesion (79.5 ± 4.7 vs 88.7 ± 3.6 , $n=11$, $p=0.02$) (Figure 3.7).

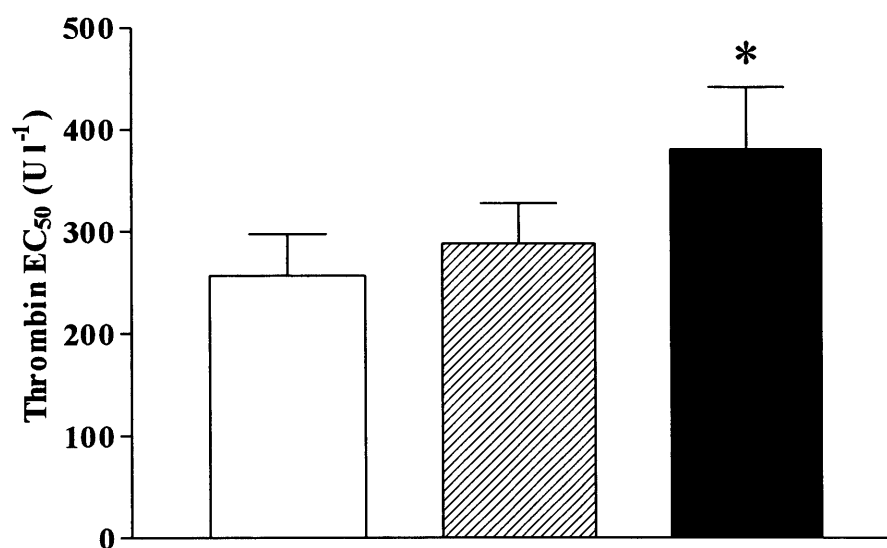


Figure 3.5 Effect of pre-incubation with 1% ethanol (□), α -tocopherol 100 $\mu\text{mol l}^{-1}$ (▨) and 200 $\mu\text{mol l}^{-1}$ (■) on the EC_{50} (U l^{-1}) of thrombin induced aggregation ($n=5$). Data are expressed as means, with SEM shown by vertical error bars. * $p<0.05$ vs 1% ethanol

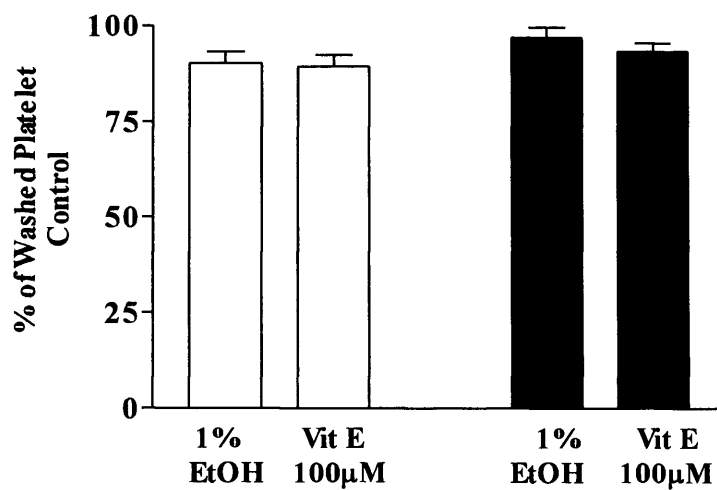


Figure 3.6 Effect of pre-incubation of platelets with α -tocopherol at 100 $\mu\text{mol l}^{-1}$ or 1% ethanol on resting (\square) and stimulated (\blacksquare) platelet adhesion. Data are expressed as means, with SEM shown by vertical error bars.

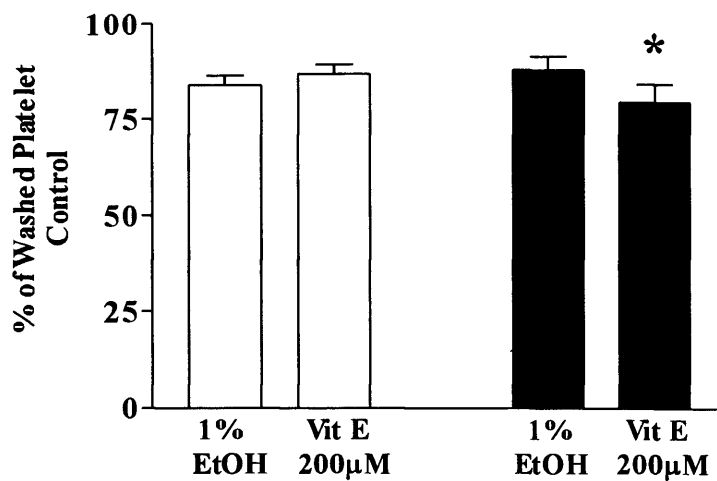


Figure 3.7 Effect of pre-incubation of platelets with α -tocopherol at 200 $\mu\text{mol l}^{-1}$ or 1% ethanol on resting (\square) and stimulated (\blacksquare) platelet adhesion. Data are expressed as means, with SEM shown by vertical error bars. * $p < 0.05$ vs 1% ethanol.

3.3.3 Effect Of α -Tocopherol On Platelet Membrane Microviscosity *In Vitro*.

Washed platelets prepared from platelet-rich plasma which had been pre-incubated with α -tocopherol at $200 \mu\text{mol l}^{-1}$ for 1 hour at 37°C displayed a significant decrease ($p=0.012$, $n=5$) in membrane microviscosity as measured at 37°C . α -tocopherol loaded platelets had a membrane microviscosity of 2.26 ± 0.07 P compared to 2.66 ± 0.12 P to platelet incubated with 1% ethanol as a control (Figure 3.8).

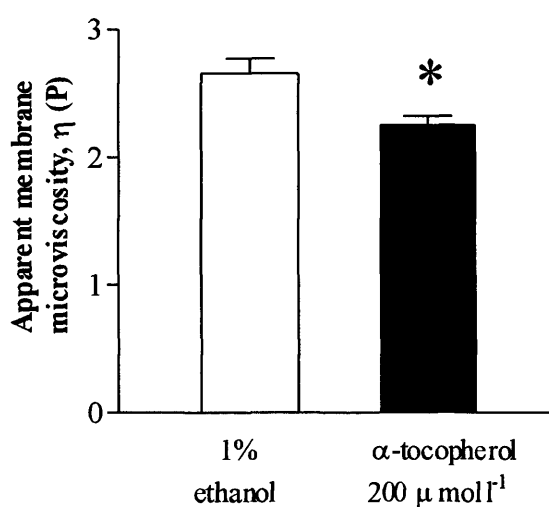


Figure 3.8 Effect of pre-incubation of platelets with α -tocopherol at $200 \mu\text{mol l}^{-1}$ (■) or 1% ethanol (□) on platelet membrane microviscosity ($n=5$). Data are expressed as means, with SEM shown by vertical error bars. * $p<0.05$ vs 1% ethanol.

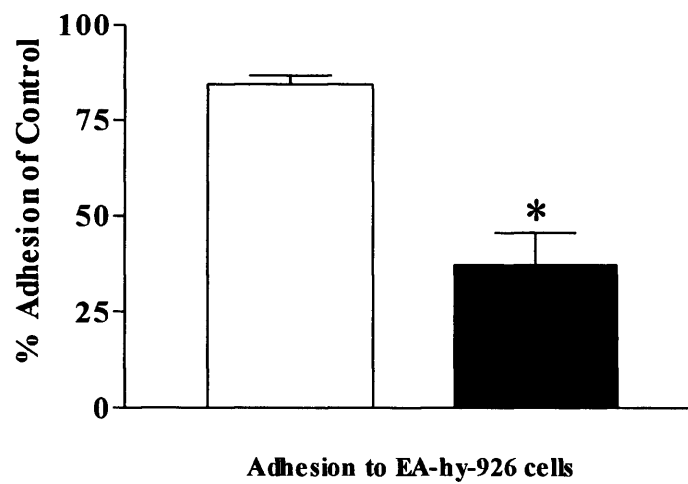
3.3.4 Effect Of α -Tocopherol On Monocyte Adhesion *In Vitro*.

Pre-incubation of EA-hy-926 endothelial cells for 1 hour in RPMI containing $100 \mu\text{mol l}^{-1}$ α -tocopherol significantly inhibited subsequent monocyte adhesion (Mean inhibition = 56.2%, $n=3$, $p<0.05$) (Figure 3.9a). In contrast, incubation of monocytes with $100 \mu\text{mol l}^{-1}$ α -tocopherol during the adhesion procedure did not significantly inhibit MNC adhesion to EA-hy-926 cells (Mean inhibition = 18%, $n=8$, $p>0.05$), but significantly reduced monocyte adhesion to tissue culture plastic (Mean inhibition = 27%, $n=9$, $p<0.01$) (Figure 3.9b).

3.3.5 Effect Of Ascorbic Acid On Platelet Adhesion *In Vitro*.

Pre-incubation of platelets with ascorbic acid at a final concentration of 1 mg ml^{-1} or those platelets where the pH of the suspension was adjusted to an equivalent pH did not produce any significant effects on adhesion to collagen coated wells, expressed as a percentage of a washed platelet control (Figure 3.10). Pre-incubation of platelets with ascorbic acid at 2 mg ml^{-1} resulted in decreased adhesion compared to the washed platelet control, however, this reduction was not significantly different to that produced by altering the pH (Figure 3.11).

a)



b)

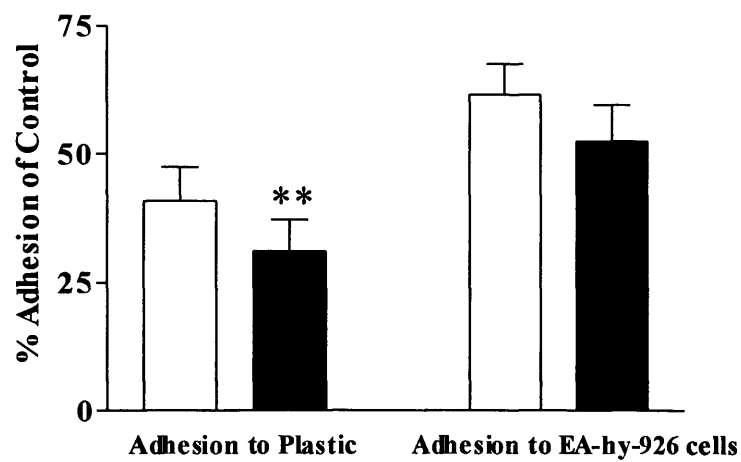


Figure 3.9 Effect on monocyte adhesion of a) pre-incubation of EA-hy-926 cells with 0.05% ethanol (□) or 100 $\mu\text{mol l}^{-1}$ vitamin E (■) and b) co-incubation of monocyte suspension with 0.05% ethanol (□) or 100 $\mu\text{mol l}^{-1}$ vitamin E (■). Data are expressed as means, with SEM shown by vertical error bars. * $p < 0.05$ vs 1% ethanol, ** $p < 0.01$ vs ethanol..

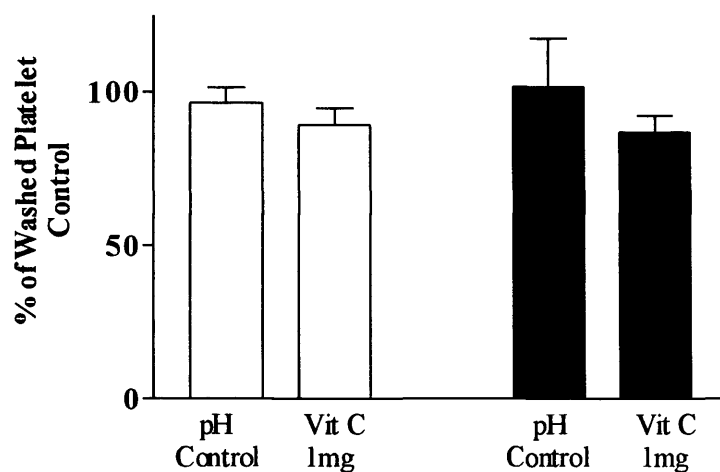


Figure 3.10 Effect of pre-incubation of platelets with ascorbic acid at 1 mg ml^{-1} or incubation at an equivalent pH on resting (\square) and stimulated (\blacksquare) platelet adhesion. Data are expressed as means, with SEM shown by vertical error bars.

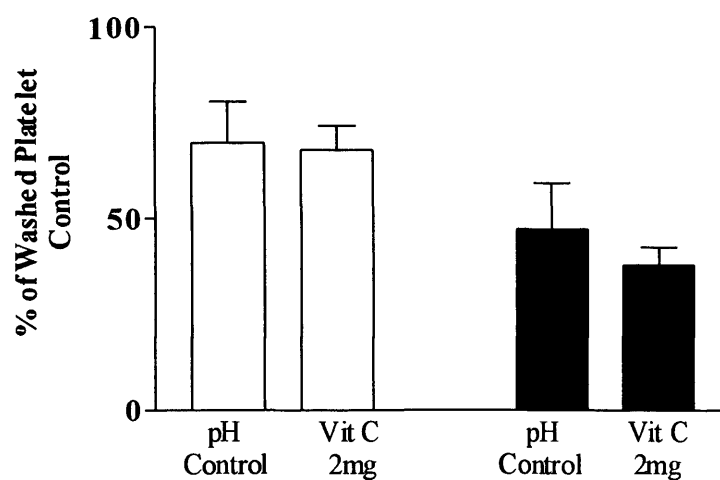


Figure 3.11 Effect of pre-incubation of platelets with ascorbic acid at 2 mg ml^{-1} or incubation at an equivalent pH on resting (\square) and stimulated (\blacksquare) platelet adhesion. Data are expressed as means, with SEM shown by vertical error bars.

3.4 DISCUSSION

3.4.1 α -Tocopherol Inhibits Platelet Aggregation *In Vitro*.

In agreement with other studies α -tocopherol had an inhibitory effect on platelet aggregation *in vitro* (Fong 1976; Srivastava 1986; Violi *et al* 1990). This inhibitory effect was observed on collagen-, ADP- and thrombin-induced platelet aggregation, suggesting the mechanism by which α -tocopherol exerts its inhibitory effect is late in the aggregation pathway. Two different techniques were employed in these studies both of which produced similar results. At a concentration of 200 $\mu\text{mol l}^{-1}$ α -tocopherol can inhibit platelet aggregation. From the results obtained by standard aggregometry we can see that α -tocopherol significantly reduces both the slope value and the maximum light transmission, which indicates that α -tocopherol has the ability to inhibit both the rate and the extent of aggregation. With the microtitre plate technique pre-incubation with α -tocopherol produced a significant increase in the EC₅₀ of thrombin induced aggregation, which indicates a decreased susceptibility of platelets to aggregate. The measurement of aggregation by dose-response assessment is considered a more powerful method than that of single or a few concentrations of an agonist due to the considerable inter donor variability of response (Calzada *et al* 1997). Overall the results obtained through both these methods confirm the anti-aggregatory effect of α -tocopherol *in vitro*. However, these *in vitro* results do not always translate into an equally potent inhibition of platelet aggregation when measured *ex vivo* following supplementation studies.

3.4.2 α -Tocopherol Inhibits Stimulated Platelet Adhesion *In Vitro*.

Platelets pre-incubated with α -tocopherol at a concentration of $200 \mu\text{mol l}^{-1}$ displayed significantly reduced adhesive properties upon stimulation with thrombin at a concentration 250 U l^{-1} , however, resting adhesion was unaffected at this concentration. Pre-incubation with α -tocopherol at $100 \mu\text{mol l}^{-1}$ did not affect either resting or stimulated platelet adhesion. There have been reports that vitamin E supplementation at doses ranging from 400 IU to 1600 IU per day can inhibit platelet adhesion *ex vivo* (Steiner 1983; Jandak *et al* 1988), however, the effects of α -tocopherol on platelet adhesion *in vitro* have not been investigated. The reduced adhesiveness observed following vitamin E supplementation has been attributed to a reduction in the size and number of pseudopodia formed following activation (Steiner 1991). Pseudopodia formation is very rapid and is the first recognisable event following platelet activation. Therefore if α -tocopherol the predominant and most biologically active isomer of vitamin E has the same affect, the pre-incubation of platelets with α -tocopherol may reduce the size and number of the pseudopodia produced by thrombin stimulation resulting in the reduced adhesiveness of the platelets as seen in this study. However, as some of the platelets did form small aggregates following stimulation with 250 U l^{-1} , one cannot ignore the possibility that this presumed inhibition of adhesion could actually be an inhibition of aggregation by α -tocopherol.

3.4.3 α -Tocopherol Increases Membrane Fluidity *In Vitro*.

Both membrane anisotropy and membrane microviscosity are inversely related to membrane fluidity (Shinitzky & Barenholz 1978). Therefore the significant decrease in membrane microviscosity observed following incubation with α -tocopherol is representative of an increase in platelet membrane fluidity. This is in agreement with the work of Steiner (1981) in which platelets incubated with

α -tocopherol at 1 mmol^{-1} at 37°C had reduced apparent microviscosity compared to a control platelet suspension. Cholesterol incorporation into the platelet membrane produces increased sensitivity to platelet agonists and is accompanied by a reduction in membrane fluidity (Shattil & Cooper 1976). Thus an increase in membrane fluidity may contribute to the antiaggregatory actions of vitamin E. Incorporation of vitamin E into the platelet membrane may affect its physical state and hence influence the lipid protein interactions of the membrane which occur during platelet aggregation (Diplock & Lucy 1973).

3.4.4 α -Tocopherol Inhibits Monocyte Adhesion *In Vitro*.

Monocyte adhesion to the arterial wall prior to transendothelial migration is an important early event in the development of atherosclerotic lesions (Gerrity 1981). We have seen that monocyte adhesion *in vitro* was significantly inhibited following pre-incubation of endothelial cells with α -tocopherol. This inhibition may be a result of decreased endothelial cell adhesion molecule expression, especially E-selectin (Faruqi *et al* 1994; Martin *et al* 1997). This decreased expression of adhesion molecules may be as a result of the effect of α -tocopherol on the activity of NF κ B. Antioxidants have been demonstrated to inhibit activation of NF κ B, though Faruqi *et al* (1994) failed to observe such an effect. Pre-incubation of monocytes with α -tocopherol prior to measurement of adhesion also resulted in a decreased adhesion to tissue culture plastic, whilst adhesion to endothelial cells was unaffected. This decreased adhesion to tissue culture plastic may be a result of reduced monocyte adhesion molecule expression, though this was not examined here. Supplementation of healthy volunteers has been demonstrated to inhibit monocyte adhesion to endothelial cells (Devaraj *et al* 1996), though little work has been performed on the effect on α -tocopherol on monocytes *in vitro*. These results suggest that α -tocopherol is capable of modulating monocyte adhesion both by effects on the endothelium

and through effects on the monocyte itself and it may be through these mechanisms that α -tocopherol can reduce atherosclerotic lesion development.

3.4.5 Ascorbic Acid Does Not Affect Platelet Adhesion.

Despite previous reports of the ability of ascorbic acid to inhibit platelet adhesion and aggregation (Cordova *et al* 1982; Bordia & Verma 1985), in this study no significant effect of ascorbic acid on platelet adhesion *in vitro* was observed. However a major problem encountered was the change in pH which occurred following the addition of ascorbic acid at 1 or 2 mg l⁻¹. In an attempt to control for this an equivalent batch of washed platelets, the pH of which was adjusted to the value obtained following addition of ascorbic acid was prepared. The addition of 2 mg ml⁻¹ ascorbic acid and alteration of washed platelets to the equivalent pH both resulted in decreased adhesion compared to the washed platelet control. The decrease in adhesion produced by ascorbic acid was not significantly different to that produced by the alteration in pH. Previously the effect of vitamin C on platelet adhesion was examined *ex vivo* following oral administration of 1g of ascorbic acid three times daily which resulted in a significant reduction in platelet adhesiveness (Bordia & Verma 1985). Only the effect of ascorbic acid addition to PRP on platelet aggregation has been examined *in vitro* (Cordova *et al* 1982), in this study the change in pH never exceeded 0.06 even at 10 mg ml⁻¹. This is in contrast to the effect of ascorbic acid addition on pH in our study, the addition of 1 mg ml⁻¹ to a washed platelet suspension produced a change of 0.84, indicating an overload of the buffering capacity. Therefore a solution with a greater buffering capacity or even PRP may have enabled the effect of ascorbic acid to be observed more effectively.

CHAPTER 4**VITAMIN E SUPPLEMENTATION OF
HYPERCHOLESTEROLAEMIC SUBJECTS.****4.1 INTRODUCTION**

Hypercholesterolaemia is now a well-established risk factor for atherosclerosis. Evidence for the role of plasma cholesterol as a determinant of coronary heart disease (CHD) risk is derived from a variety of sources. In the 'Seven Countries Study', Keys (1980) found that the incidence of CHD was high in countries where median cholesterol levels were high and correspondingly low in countries where median cholesterol levels were low. Even within populations higher levels of blood cholesterol were associated with higher rates of CHD mortality (Rose & Shipley 1986). Using this and other evidence the so-called cholesterol hypothesis was formulated, stating that elevated plasma levels of cholesterol were causally related to the development of CHD and lowering the plasma cholesterol would reduce the CHD risk.

Support for this view has come from major intervention trials, two of the most recent and largest are the Scandinavian Simvastatin Survival Study (4S) (1994) and the West of Scotland Coronary Prevention Study (Shepherd *et al.* 1995). They showed that lowering plasma cholesterol and more specifically LDL-cholesterol reduces the risk of subsequent coronary events in those men and women who have a preexisting CHD and in high-risk men who have no overt evidence of disease. Intervention guidelines by the British Hyperlipidaemia Association (Shepherd *et al.* 1987), classify the levels of cholesterol in the range less than 5.2 mmol l⁻¹ as associated with the lowest level of risk, 5.2 - 6.5 mmol l⁻¹ as low to moderate risk, 6.5 - 7.8 mmol l⁻¹ as moderate to high risk and levels greater than 7.8 mmol l⁻¹ as very high risk with treatment depending on the presence of other risk factors for CHD.

Hypercholesterolaemia may contribute to the development of atherosclerosis in a number of ways including effects on the endothelium, platelets and monocytes. Platelets play an essential role in the progression of atherosclerosis and later in the thromboembolic complications (Packham & Mustard 1986). Hypercholesterolaemia is often accompanied by enhanced platelet responses to aggregatory factors (Carvalho *et al* 1974; Latta *et al* 1994; Opper *et al* 1995). Enhanced platelet activity may be a result of cholesterol incorporation into the platelet membrane (Chetty & Naran 1992), or a direct effect of LDL on platelets (Aviram & Brook 1983; Hassal *et al* 1983), due to the high affinity LDL receptors which platelets possess (Koller *et al* 1982; Hassal *et al* 1990).

Accumulation of lipid-laden macrophages is an important event in the development of atherosclerosis and involves the adhesion of circulating monocytes to the vascular endothelium and subsequent recruitment into the subendothelial space. It has been demonstrated that cholesterol-rich lipoproteins enhance the adhesion of monocytes to cultured endothelial cells *ex vivo* (Foxall *et al* 1990) and *in vitro* (Alderson *et al* 1986). Monocytes isolated from hypercholesterolaemic patients also exhibit altered functional behaviour including increased adhesiveness (Bath *et al* 1991a; Lösche *et al* 1992; Stragliotto *et al* 1993), phagocytic activity (Lösche *et al* 1992) and prostaglandin production (Stragliotto *et al* 1993).

Supplements of vitamin E, a potent lipid soluble antioxidant, have been shown to be associated with reduced risk of coronary heart disease in men and women (Rimm *et al* 1993; Stampfer *et al* 1993). Vitamin E has also been shown to reduce mortality of patients with established coronary heart disease (Stephens *et al* 1996). The mechanism(s) by which vitamin E exerts its effect(s) is unclear, but inhibition of platelet aggregation (Steiner 1983; Kakishita *et al* 1990; Violi *et al* 1990; Salonen *et al* 1991), platelet adhesion (Steiner 1983; Jandak *et al* 1988) and monocyte adhesion (Devaraj *et al* 1996) have been demonstrated.

In the following study the effect of vitamin E supplementation on platelet aggregation and monocyte adhesion *ex vivo*, in patients with primary hypercholesterolaemia was investigated. The patients studied were newly diagnosed as hypercholesterolaemic, they did not have a current smoking habit or suffer from hypothyroidism or diabetes mellitus. These exclusion criteria were employed due to their effects on platelet and monocyte function (Lederman *et al* 1982; Ford & Carter 1990; Beswick *et al* 1991; Winocour 1994; Costa Rosa *et al* 1995). Monocyte adhesion *ex vivo* to EA-hy-926 cells was assessed by the method of Bath *et al* (1989). Platelet aggregation was measured using the microtitre plate technique of Fratantoni & Poindexter (1990). A placebo-controlled trial design was employed, with the supplementation with placebo (soybean oil) preceding that of vitamin E, due to the retention of vitamin E within tissues following supplementation (Ingold *et al* 1987).

4.2 STUDY DESIGN

4.2.1 Materials And Methods.

Materials and their suppliers are listed in appendix I

Details of methods employed during this study are described in section 2.2

4.2.2 Subjects.

Patients were recruited from those newly referred to the Lipid Clinic at Glenfield General Hospital, Leicester, with a diagnosis of primary hypercholesterolaemia (serum cholesterol $> 5.7 \text{ mmol l}^{-1}$, but $< 10 \text{ mmol l}^{-1}$ and serum triglycerides $< 3.3 \text{ mmol l}^{-1}$). Exclusion criteria included: a positive recent smoking habit, diabetes mellitus, or hypothyroidism. Patients taking antihypertensive or diuretic therapy, steroids or vitamin supplements were also excluded. Ethical Committee approval was sought from the Local Ethics Committee, and approved prior to the start of the trial. Patients were asked to read an information sheet, briefly explaining the experimental protocol, at the same time they were told they would be taking either the active vitamin E capsules, or 'dummy' capsules for six weeks, at which point the capsules would be switched. Written informed consent was obtained from all patients.

4.2.3 Vitamin Supplementation.

Patients were then given a supply of placebo capsules (containing soybean oil) for six weeks. This was followed by a period of six weeks during which vitamin E was taken at a dose of 400 IU per day

4.2.4 Blood Sampling.

Blood samples for baseline lipid levels (total cholesterol, triglycerides and HDL cholesterol) and antioxidant vitamin (α -tocopherol, retinol and ascorbate) concentrations were taken following a 12 h, overnight fast. LDL cholesterol was calculated according to the Friedewald formula (Friedewald *et al* 1972). Baseline assessment of monocyte adhesion and platelet aggregation was performed according to the methods of Bath *et al* (1989) and Fratantoni & Poindexter (1990) respectively. Further blood samples were taken every three weeks for the duration of the study, for the assessment of the parameters listed above.

4.2.5 Statistical Analysis.

Statistical significance was analysed by unpaired Student's t-test and analysis of variance with Tukey's post hoc test for normally distributed data and by Mann-Whitney U test and the Kruskal-Wallis test for data which is not normally distributed. Data is presented as mean \pm SEM, median and range or actual numbers. $p < 0.05$ was considered statistically significant. Analyses were performed using InStat software (Graphpad Inc, USA) on a PC.

4.3 RESULTS

4.3.1 Patients Characteristics.

A total of 28 subjects (male:female ratio 15:13); mean age 55.2 ± 2.14 years (range 33-73 years) were recruited into the study. Their clinical characteristics are summarized in Table 4.1. Of the patients recruited 15 had pure hypercholesterolaemia, 13 had mixed hyperlipidaemia.

Table 4.1 Basal characteristics of hypercholesterolaemic subjects. Values expressed as mean \pm SEM or mean (range).

n	28
mean age	55.2 ± 2.14
M : F ratio	15 : 13
Pure hypercholesterolaemia	15
Mixed hyperlipidaemia	13
Lipoprotein Profile	
mean total serum cholesterol (mmol l ⁻¹)	7.1 ± 0.15
mean HDL cholesterol (mmol l ⁻¹)	1.3 ± 0.05
mean LDL cholesterol (mmol l ⁻¹)	4.9 ± 0.15
mean total triglycerides (mmol l ⁻¹)	1.7 (0.8,4.1)
Plasma antioxidant concentrations	
Vitamin A (mg l ⁻¹)	0.71 ± 0.02
Vitamin E (mg l ⁻¹)	19.39 ± 0.95
Vitamin C (μ mol l ⁻¹)	60.81 ± 4.82
Cell function	
Monocyte adhesion to ECs (%)	12.15 ± 1.36
Monocyte adhesion to plastic (%)	71.0 ± 3.16
Platelet aggregability to Thrombin (EC ₅₀) (UI ⁻¹)	286 ± 33

4.3.2 Effect Of Vitamin E On Plasma Lipid And Antioxidant Vitamin Levels.

There were no significant changes in mean plasma total-, calculated LDL-, or HDL-cholesterol, triglycerides, or ascorbic acid and retinol during treatment with placebo or vitamin E supplementation (Table 4.2 and Figures 4.1 and 4.2). Although plasma α -tocopherol levels were not affected by treatment with placebo, they increased significantly following 3 weeks treatment with vitamin E ($38.4 \pm 1.68 \text{ mg l}^{-1}$ vs $20.4 \pm 0.99 \text{ mg l}^{-1}$, $p < 0.0001$). After six weeks supplementation with vitamin E the plasma α -tocopherol levels had plateaued ($36.4 \pm 1.7 \text{ mg l}^{-1}$ vs $20.4 \pm 0.99 \text{ mg l}^{-1}$, $p < 0.0001$) (Figure 4.3). Over the trial period there was a substantial increase in plasma α -tocopherol levels in most of the subjects (range 3-188%) (Figure 4.4).

Table 4.2 Effect of six weeks placebo and six weeks vitamin E supplementation (400 IU per day) on plasma lipoprotein profiles. Values expressed as mean \pm SEM or mean (range).

	Total Cholesterol mmol l ⁻¹	LDL Cholesterol mmol l ⁻¹	HDL Cholesterol mmol l ⁻¹	Triglycerides mmol l ⁻¹
Basal	7.1 \pm 0.15	4.9 \pm 0.15	1.3 \pm 0.05	1.7 (0.8,4.1)
Placebo	7.0 \pm 0.17	4.8 \pm 0.16	1.3 \pm 0.06	1.7 (0.7,6.9)
3 Wks Vit E	7.2 \pm 0.16	4.9 \pm 0.19	1.3 \pm 0.06	1.6 (1.1,4.4)
6 Wks Vit E	7.0 \pm 0.15	4.7 \pm 0.15	1.3 \pm 0.05	2.0 (0.9,3.1)

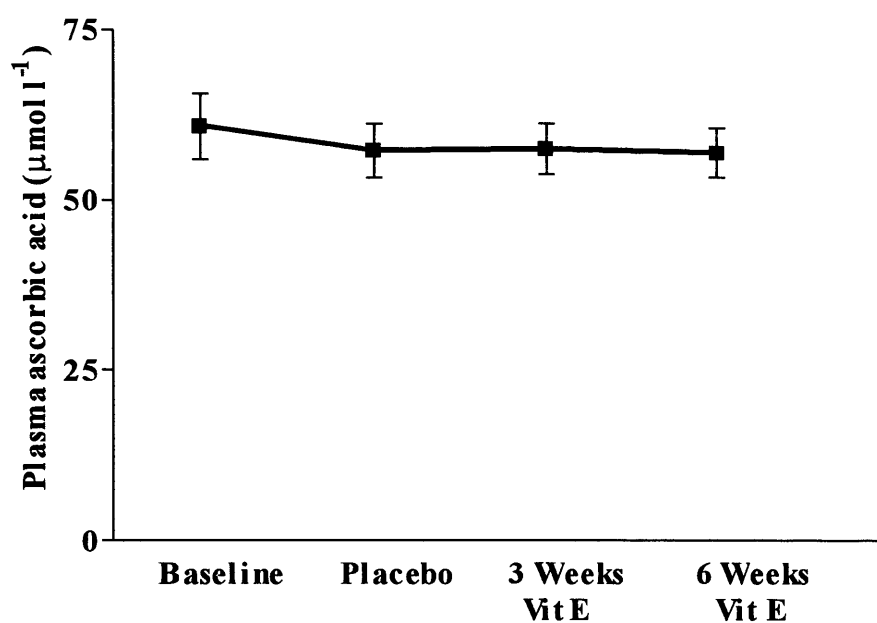


Figure 4.1 Effects of six weeks placebo (soybean oil) and six weeks vitamin E supplementation (400 IU per day) on mean plasma ascorbic acid levels ($\mu\text{mol l}^{-1}$). Data points are means, with SEM shown by vertical error bars.

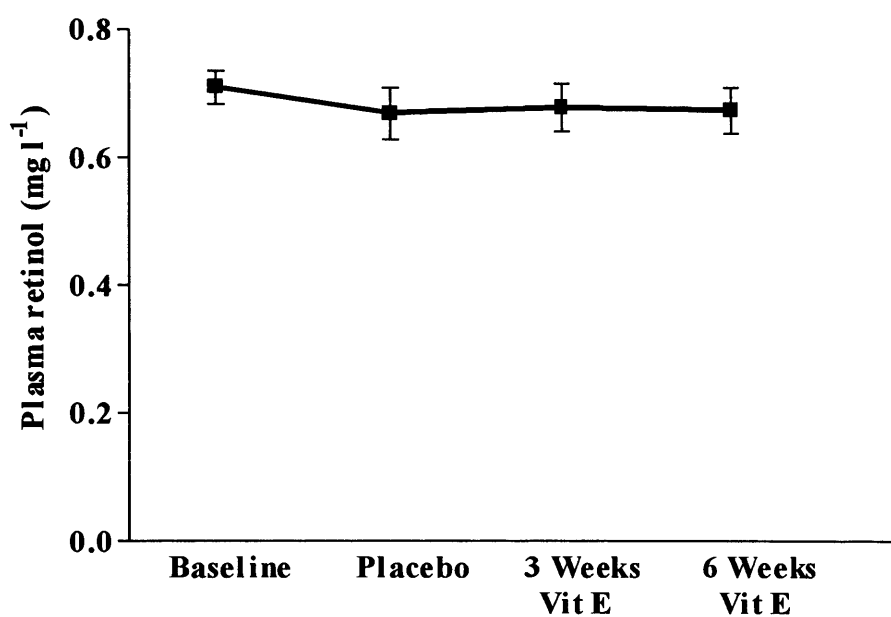


Figure 4.2 Effect of six weeks placebo (soybean oil) and six weeks vitamin E supplementation (400 IU per day) on mean plasma retinol levels (mg l⁻¹). Data points are means, with SEM shown as vertical error bars.

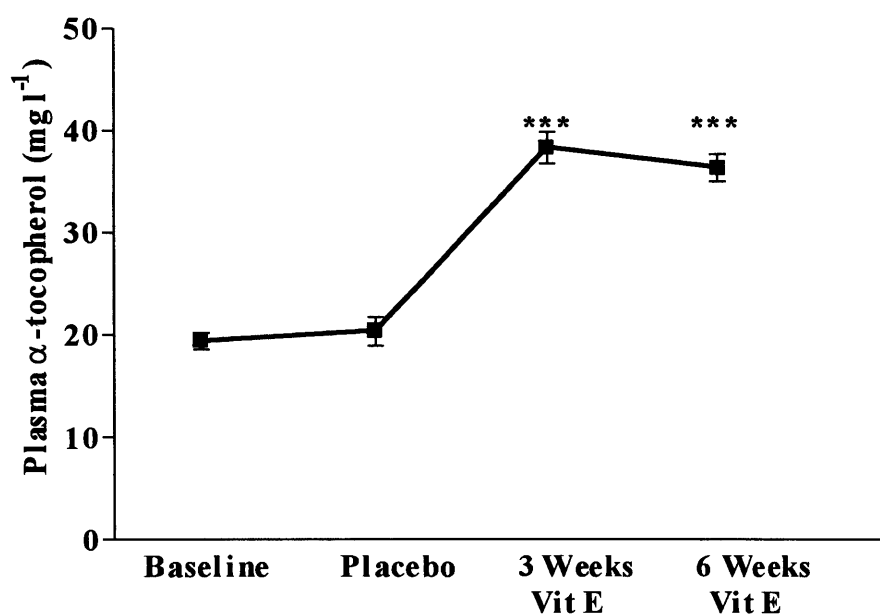


Figure 4.3 Effects of six weeks placebo (soybean oil) and six weeks vitamin E supplementation (400 IU per day) on mean plasma α -tocopherol levels (mg l⁻¹). Data points are means, with SEM shown by vertical error bars. ***p<0.001 vs baseline or placebo

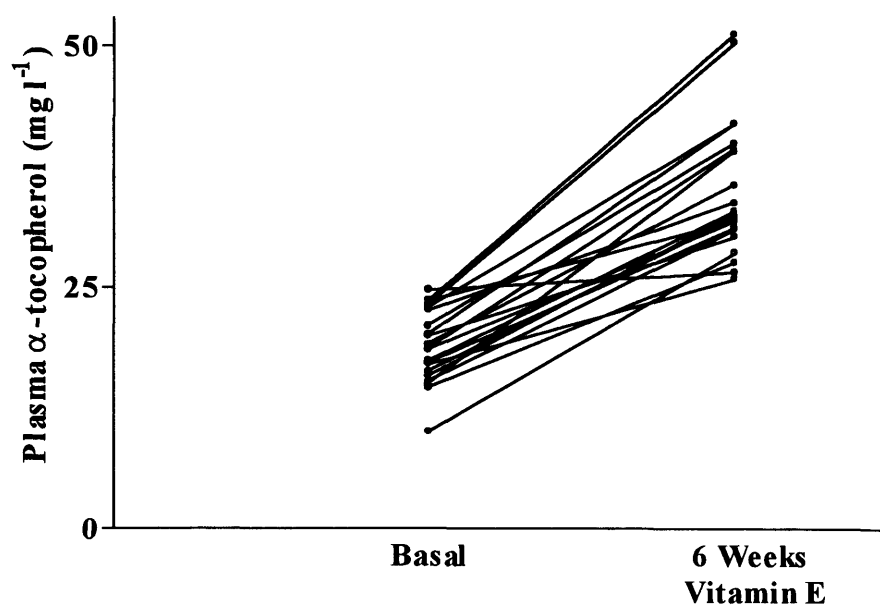


Figure 4.4 Individual plasma α -tocopherol responses (mg l^{-1}) to six weeks vitamin E supplementation at 400 IU per day.

4.3.3 Effect Of Vitamin E Supplementation On Platelet Aggregation *Ex Vivo*.

There was no significant change in the mean EC_{50} ($U\ l^{-1}$) following placebo treatment. After 3 weeks of vitamin E supplementation, the EC_{50} increased by approximately 47% ($421 \pm 102.9\ U\ l^{-1}$ vs. $278 \pm 44.1\ U\ l^{-1}$) but this failed to reach significance. However, after 6 weeks of treatment with vitamin E a further increase in EC_{50} ($U\ l^{-1}$) of 132% was observed ($664 \pm 103.0\ U\ l^{-1}$ vs. $278 \pm 44.1\ U\ l^{-1}$, $p < 0.05$) (Figure 4.5). Over the trial period vitamin E was found to have a significant inhibitory effect on thrombin-induced platelet aggregation ($p < 0.01$, ANOVA, $n=12$).

4.3.4 Effects Of Vitamin E Supplementation On Monocyte Adhesion *Ex Vivo*.

The basal adherence of mononuclear cells to confluent EA-hy-926 endothelial cells was approximately $12.15 \pm 1.36\%$ and $70.99 \pm 3.16\%$ to tissue culture plastic. Neither placebo, nor vitamin E treatment significantly affected mononuclear cell adhesion over the duration of the study (Figures 4.6a and b)

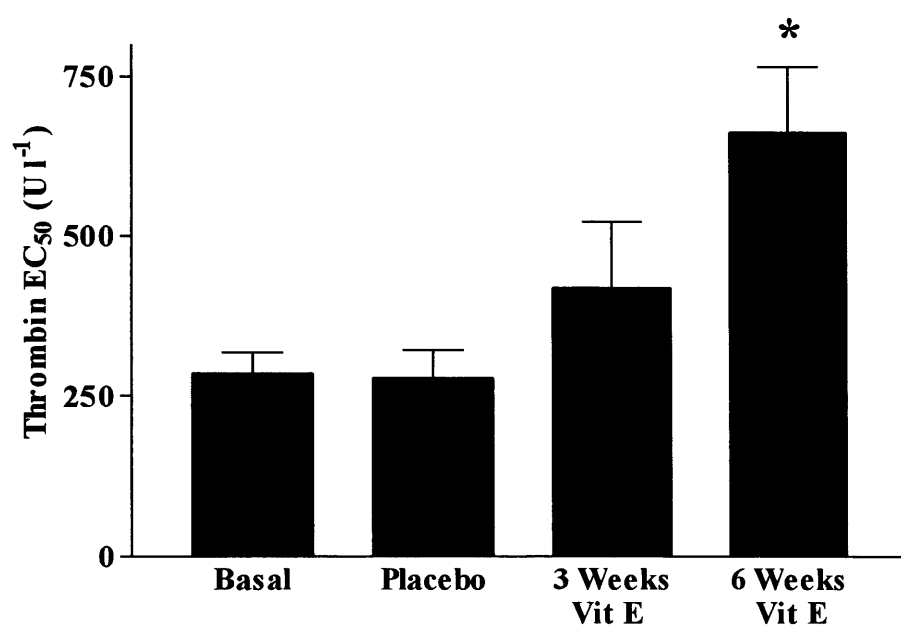
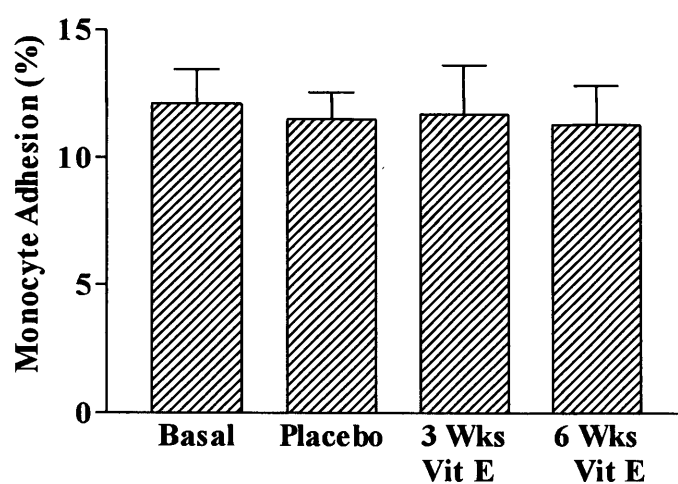


Figure 4.5 Effect of six weeks placebo (soybean oil) and six weeks vitamin E supplementation (400 IU per day) on the EC₅₀ (U l⁻¹) of thrombin-induced platelet aggregation in a group of patients with hypercholesterolaemia (n=12). Data are expressed as means, with SEM shown by vertical error bars. *p<0.05 vs baseline or placebo.

a)



b)

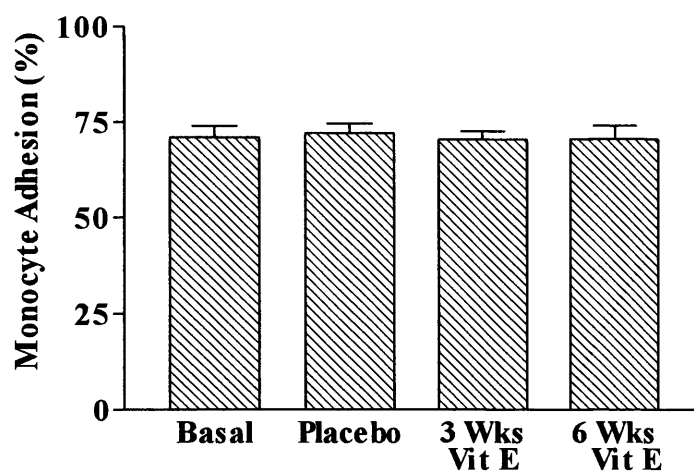


Figure 4.6 Effect of six weeks placebo (soybean oil) and six weeks vitamin E supplementation (400 IU per day) on monocyte adhesion (%) to a) EA-hy-926 endothelial cells and b) tissue culture plastic microwells. Data are expressed as means, with SEM shown by vertical error bars.

4.4 DISCUSSION

4.4.1 Effect Of Vitamin E Supplementation On Plasma Lipid And Antioxidant Profiles.

Treatment with placebo or vitamin E at a dose of 400 IU per day was not associated with any significant effects on plasma total-, LDL- or HDL-cholesterol, or triglycerides. These findings are consistent with previous reports (Szczeklik *et al.* 1985; Salonen *et al.* 1991), although others have reported that vitamin E supplements decrease plasma cholesterol levels (Cloarec *et al.* 1987).

Mean plasma α -tocopherol levels increased two-fold following vitamin E supplementation at a dose of 400 IU per day, which is similar to that reported by Princen *et al.* (1995) using the same dose. Mean plasma levels of the other antioxidant vitamins (retinol and ascorbic acid) were not affected by vitamin E supplementation at this dose.

4.4.2 Vitamin E Supplementation Inhibits Thrombin-Induced Platelet Aggregation *Ex Vivo*.

Vitamin E supplementation, at a dose of 400 IU per day for six weeks caused a significant increase in the EC₅₀ of thrombin-induced platelet aggregation, indicative of decreased sensitivity to the effects of this agonist. Srivastava (1986) and Fong (1976) have previously shown that vitamin E inhibits platelet aggregation *in vitro*, whilst the effects of vitamin E supplements on platelet aggregation *ex vivo* are less clear. Although Steiner (1983) has reported a small but significant inhibition of platelet aggregation at high levels of supplementation (up to 1200 IU per day), other groups (Stampfer *et al.* 1988; Salonen *et al.* 1991) failed to observe any significant inhibitory effect. However, the latter studies were performed on healthy volunteers whose basal platelet reactivity may be less marked. A recent double blind, randomised, placebo-controlled trial in healthy volunteers has reported an inhibitory effect of vitamin E on platelet function

(Caldaza *et al* 1997). In this study they examined the effect of antioxidant supplementation on collagen-, arachidonic acid- (AA) and adenosine diphosphate- (ADP) induced aggregation using a dose-response assessment as employed in our study. Vitamin E significantly inhibited AA- and ADP-induced aggregation. Other aspects of platelet function were significantly decreased following vitamin E supplementation, apparent by the increased sensitivity to inhibition by PGE₁, the decreased ATP secretion and the decreased plasma β -thromboglobulin (β -TG) concentration. Interestingly, they failed to see any significant effects following supplementation with other antioxidant vitamins, vitamin C and β -carotene.

Another study of the effects of vitamin E supplementation at a dose of 600 IU per day for 14 days in patients with hyperlipidaemia demonstrated that vitamin E inhibited AA- and ADP-induced aggregation (Szczeklik *et al.* 1985). However, the subjects in their study were more heterogeneous, several having overt clinical disease. Only four of the sample population were asymptomatic, while in our study, none of the patients had evidence of heart disease. We have not established the mode of action by which vitamin E inhibits thrombin-induced aggregation or whether this effect is observed when other agonists such as collagen, arachidonic acid or adenosine diphosphate are employed. Though Szczeklik *et al* (1985) and Calzada *et al* (1997) have both reported an inhibition of arachidonic acid- and adenosine diphosphate-induced aggregation by vitamin E. It is possible that vitamin E has a stabilising effect on the platelet membrane (Steiner 1981) through its interaction with polyunsaturated fatty acids (Diplock & Lucy 1973). Vitamin E may also alter the activity of cyclooxygenase (Ali *et al.* 1980) or lipooxygenase (Mower & Steiner 1983), key enzymes in the aggregatory process. A further mechanism by which vitamin E may inhibit platelet aggregation is via its ability to scavenge reactive oxygen species. Previous reports indicate that these are released during platelet aggregation, and that antioxidants can inhibit aggregation *in vitro* (reviewed by Salvemini & Botting 1990).

4.4.3 Vitamin E Supplementation Did Not Affect Mononuclear Cell Adhesion *Ex Vivo*.

Over the duration of the present study, vitamin E supplementation at 400 IU per day had no significant effect on monocyte adhesion *ex vivo*. This was so whether examining their adherence to the EA hy-926 endothelial cell line, or to tissue culture plastic. A recent paper by Devaraj *et al.* (1996) reported that supplementation with 1200 IU per day vitamin E led to a significant decrease in monocyte adhesion to human umbilical vein endothelial cells. This difference could be explained by the use of a much larger dose of vitamin E in this study.

CHAPTER 5**VITAMIN C SUPPLEMENTATION OF ELDERLY
HYPERTENSIVE AND NORMOTENSIVE SUBJECTS****5.1 INTRODUCTION**

Hypertension is a well-known risk factor for atherosclerosis (Kannel *et al.* 1969; Vogt *et al.* 1993) with the coronary, carotid and peripheral arteries being particularly susceptible to hypertension-enhanced atherosclerosis. The Framingham data demonstrated that higher levels of blood pressure are related to an increased incidence of morbid events (Kannel *et al.* 1981). The Multiple Risk Factor Intervention Trial (MRFIT) showed that an increase in the diastolic blood pressure (DBP) of 5 mmHg was independently, of cholesterol and smoking habit, able to increase the risk of coronary artery disease (MRFIT research group 1982).

The mechanism(s) by which hypertension enhances atherosclerosis is unclear. In many hypertensive subjects, the vascular wall is thickened with a decreased internal diameter, thus increasing peripheral resistance (Swales 1994). The coronary vasomotor response may also be abnormal (Panza *et al.* 1990; Treasure *et al.* 1992). Enhanced monocyte adhesion and increased endothelial adhesion molecule expression have been observed in animal models of hypertension (McCarron *et al.* 1994a, 1994b; Tropea *et al.* 1996; Haller *et al.* 1997). This may be a consequence of the endothelial dysfunction and activation that has been proposed in hypertension (Blann *et al.* 1993; 1994), or may be a result of monocyte activation, though the activation state of monocytes has received less attention. Platelet reactivity may also be enhanced in hypertension. Increased plasma levels of beta-thromboglobulin (β TG) and platelet factor-4 (PF4) (Mehta & Mehta 1981; Yamanishi *et al.* 1985), greater platelet aggregability (Coccheri & Fiorentini 1971; Vlachakis & Aledort 1980; Nyrop & Zweifler 1988), enhanced platelet adhesiveness (Poplawski *et al.* 1968; Andrioli *et al.* 1996) and elevated intracellular free calcium concentrations (Erne *et al.* 1984; Le Quan-Sang *et al.* 1995) have all been observed in

hypertension. Through one or a combination of these effects hypertension may contribute to the progression of atherosclerosis.

Low plasma and tissue concentrations of ascorbate have also been identified as risk factors for atherosclerosis (Gey *et al.* 1987; Riemersma *et al.* 1990). In a large epidemiological study, a cohort of over 11,000 participants were followed for 10 years, and the cardiovascular mortality was 34% lower in the group with the highest intake of vitamin C (Enstrom *et al.* 1992). The protective properties of vitamin C in cardiovascular disease may be a result of its inhibitory effect on platelet adhesion and aggregation (Cordova *et al.* 1982; Bordia & Verma 1985), improvement of the endothelium-dependent vasomotor capacity (Levine *et al.* 1996) and the reduction of monocyte adhesion (Weber *et al.* 1996).

Several strands of evidence suggest that hypertension is a state associated with increased free radical activity (Kumar & Das 1993; Lacy *et al.* 1998). Blood pressure is inversely related to vitamin C intake (McCarron *et al.* 1984; Salonen *et al.* 1987) and the fall in plasma vitamin C concentrations in the winter is associated with an increase in blood pressure (Brennan *et al.* 1982; Dobson *et al.* 1984). Small supplementation trials of vitamin C (Koh 1984, Trout 1991; Ghosh *et al.* 1994) and combined antioxidants (500mg ascorbic acid, 600mg α -tocopherol, 30mg β -carotene and 200mg zinc sulphate) (Galley *et al.* 1997) have reported a slight fall in blood pressure, though not all these reductions in blood pressure have attained statistical significance (Ghosh *et al.* 1994).

In this present study the effect of vitamin C supplementation at a dose of 500mg per day for 3 months on clinic blood pressure, 24-h blood pressure, platelet adhesion, monocyte adhesion and circulating levels of soluble E-selectin, soluble L-selectin, soluble ICAM-1 and von Willebrand factor (vWF) was examined in a group of newly diagnosed elderly hypertensive and normotensive subjects using a double blind placebo-controlled crossover design. Subjects with a history of symptomatic vascular disease, diabetes mellitus, current smokers or subjects using medication or vitamin supplements were

excluded due to their reported effects on platelet and monocyte function (Lederman *et al* 1982; Beswick *et al* 1991; Winocour *et al* 1994)

5.2 STUDY DESIGN.

5.2.1 Materials And Methods.

Materials and their suppliers are listed in appendix I

Details of methods employed during this study are described in section 2.2

5.2.2 Subjects.

Newly diagnosed untreated elderly hypertensive (HT) and normotensive (NT) volunteers were screened at Glenfield Hospital, Leicester, UK. Exclusion criteria were subjects with a history of symptomatic vascular disease; known hypertension; diabetes mellitus; current smokers or subjects using medication including aspirin and vitamin supplements. All subjects gave written informed consent and ethical approval was granted by the hospital's Ethics Committee. Conventional blood pressure was recorded in triplicate on three occasions, taking the mean of 3 sitting blood pressures. Home 24-h blood pressure monitoring was undertaken with the SpaceLabs 90207 ambulatory blood pressure device (SpaceLabs Inc, Washington, USA) programmed to take readings every 15 minutes; daytime blood pressure was defined as the mean of readings from 0700-2200 h. Subjects with a clinic systolic blood pressure of ≥ 160 mmHg and/or diastolic blood pressure of ≥ 90 mmHg were classified as hypertensive. Along with subjects with an ambulatory daytime systolic blood pressure of ≥ 140 mmHg and/or an ambulatory daytime diastolic blood pressure of ≥ 85 mmHg (Mancia *et al* 1995; Manning *et al* 1998).

5.2.3 Vitamin Supplementation.

Patients recruited on to the supplementation study were then randomly and double blindly allocated to a crossover trial of vitamin C 500mg daily versus placebo, each for 3 months separated by a 1 week washout period.

5.2.4 Blood Sampling.

Blood samples for baseline lipid levels (total cholesterol, triglycerides and high density (HDL) cholesterol), antioxidant vitamin (ascorbate and α -tocopherol) concentrations and assessment of platelet and monocyte adhesiveness and measurement of soluble adhesion molecules ICAM-1, E-selectin, L-selectin and vWF were taken between 0900 and 1100h, following a 12h, overnight fast.

5.2.5 Statistical Analysis.

Statistical analysis was performed using the method of Hills and Armitage (1979) for a 2 period cross-over trial, paired and unpaired Student's t-test or Mann-Whitney U test. To evaluate correlation between variables, Pearson's r correlation test was performed. $p < 0.05$ was considered statistically significant. Data is presented as mean \pm SEM, median and range or actual numbers. Analyses were performed using Instat software (Graphpad Inc, USA) on a PC.

5.3 RESULTS

5.3.1 Basal Subject Characteristics

A total of 56 subjects (male:female ratio 29:27); mean age 72 ± 4 years (range 64-80 years) underwent 24-h blood pressure monitoring, assessment of monocyte and platelet adhesion *ex vivo* and baseline measurement of plasma total-, HDL-cholesterol, total triglycerides, vitamins C and E, soluble ICAM-1, E-Selectin, L-Selectin and vWF. The group of subjects exhibited a wide range of blood pressure: daytime ambulatory SBP 102-162 mmHg and DBP 57-103 mmHg; clinic SBP 100-204 mmHg and DBP 65-112 mmHg.

Of these subjects, 40 were recruited onto the supplementation study. 16 were defined as hypertensive. Selected clinical and biochemical characteristics are summarised in Table 5.1. There were no significant differences in age, gender distribution, body mass index (BMI), plasma lipids, plasma vitamins C and E and cell function parameters between the hypertensive and normotensive subjects. The hypertensive subjects exhibited significantly elevated clinic SBP, 24-h mean arterial pressure (MAP), 24-h SBP and DBP, daytime SBP and DBP and night-time SBP and DBP compared to their normotensive counterparts (Table 5.1).

Table 5.1. Basal characteristics of hypertensive and normotensive subjects. Values are expressed as mean \pm SEM or mean (range) or actual numbers.

	Hypertensive	Normotensive	p value
N	16	24	
Mean Age (years)	73 \pm 1	71 \pm 1	0.261
M:F ratio	10:6	11:13	0.341
BMI (kg m ⁻²)	27.35 \pm 0.87	25.84 \pm 0.67	0.173
Clinic blood pressure measurements			
Clinic Supine SBP (mm Hg)	158 \pm 4	146 \pm 3	0.012
Clinic Supine DBP (mm Hg)	89 \pm 3	86 \pm 2	0.511
Clinic Sitting SBP (mm Hg)	148 \pm 3	138 \pm 3	0.043
Clinic Sitting DBP (mm Hg)	90 \pm 3	87 \pm 2	0.443
24 hour blood pressure monitoring			
24 hour MAP (mm Hg)	103 \pm 2	89.3 \pm 1	<0.0001
24 hour SBP (mm Hg)	144 \pm 4	122 \pm 2	<0.0001
24 hour DBP (mm Hg)	80 \pm 3	72 \pm 1	0.004
Daytime SBP (mm Hg)	149 \pm 3	125 \pm 2	<0.0001
Daytime DBP (mm Hg)	84 \pm 3	76 \pm 1	0.007
Night-time SBP (mm Hg)	134 \pm 4	115 \pm 2	0.0001
Night-time DBP (mm Hg)	73 \pm 3	66 \pm 1	0.03
Lipoprotein profile			
Mean total plasma cholesterol (mmol l ⁻¹)	6.1 \pm 0.22	6.4 \pm 0.22	0.30
Mean LDL cholesterol (mmol l ⁻¹)	4.8 \pm 0.30	5.4 \pm 0.31	0.15
Mean HDL cholesterol (mmol l ⁻¹)	1.6 \pm 0.11	1.5 \pm 0.08	0.55
Mean total triglycerides (mmol l ⁻¹)	1.9 (1.2,6.3)	1.4 (0.7,4.2)	0.07
Plasma antioxidant vitamins			
Vitamin C (μ mol l ⁻¹)	54.28 \pm 3.98	45.91 \pm 2.43	0.06
Vitamin E (mg l ⁻¹)	13.49 \pm 0.88	13.93 \pm 0.63	0.68
Cell function			
Monocyte adhesion to collagen (%)	64.5 \pm 5.5	57.3 \pm 3.8	0.27
Monocyte adhesion to plastic (%)	77.6 \pm 2.7	71.2 \pm 3.2	0.16
Platelet adhesion to collagen (%)	41.7 \pm 1.3	46.3 \pm 2.7	0.19
Platelet adhesion to plastic (%)	34.1 \pm 1.8	39.1 \pm 2.9	0.20
Plasma adhesion molecules			
ICAM-1 (ng ml ⁻¹)	389 \pm 20	327 \pm 10	0.005
E-Selectin (ng ml ⁻¹)	69 \pm 7	48 \pm 4	0.01
L-Selectin (ng ml ⁻¹)	1403 \pm 60	1441 \pm 67	0.70
vWf	1.26 \pm 0.05	1.25 \pm 0.05	0.89

5.3.2 Effect Of Vitamin C Supplementation On Plasma Lipids And Antioxidant Vitamins

During treatment with either placebo or vitamin C no significant changes in mean plasma total-, calculated LDL-, HDL-cholesterol or triglycerides were observed in either the normotensive or hypertensive subjects (Table 5.2 and 5.3, respectively). Mean plasma ascorbic acid levels were not affected by placebo treatment. A significant increase was observed in both the normotensive ($82.78 \pm 3.78 \mu\text{mol l}^{-1}$ vs $45.91 \pm 2.43 \mu\text{mol l}^{-1}$, $p < 0.0001$) and hypertensive subjects ($91.28 \pm 7.13 \mu\text{mol l}^{-1}$ vs $54.28 \pm 3.98 \mu\text{mol l}^{-1}$, $p < 0.0001$) following 3 months vitamin C supplementation at 500 mg per day (Figure 5.1). Following vitamin C supplementation there was a substantial increase in plasma ascorbic acid levels in most of the normotensive (range 1% - 257%) and hypertensive subjects (18% - 290%) (Figure 5.2a and b). Neither placebo nor vitamin C supplementation had a significant effect on mean plasma α -tocopherol levels (mg l^{-1}) (Figure 5.3).

5.3.3 Effect Of Vitamin C Supplementation On Blood Pressure.

Mean daytime and sitting blood pressure in the hypertensive and normotensive groups for each study phase are shown in Table 5.4 and 5.5 respectively. There was no significant period or treatment period interaction for either group. Daytime systolic and mean arterial pressure (MAP) were significantly reduced by $5 \pm 2 \text{ mmHg}$ ($p = 0.025$) and $3 \pm 1 \text{ mmHg}$ ($p = 0.047$), respectively following vitamin C supplementation in the hypertensive subjects. While mean daytime and sitting blood pressure in the normotensive group did not change significantly between placebo and vitamin C supplementation phases.

Table 5.2. Effect of 3 months of placebo and vitamin C supplementation (500 mg per day) on plasma lipoprotein profiles in normotensive subjects. Values are expressed as mean \pm SEM or mean (range).

	Total Cholesterol mmol l ⁻¹	LDL Cholesterol mmol l ⁻¹	HDL Cholesterol mmol l ⁻¹	Triglycerides mmol l ⁻¹
Baseline	6.4 \pm 0.22	5.4 \pm 0.31	1.5 \pm 0.08	1.4 (0.7,4.2)
Placebo	6.4 \pm 0.31	4.9 \pm 0.48	1.4 \pm 0.10	1.6 (0.1,5.0)
Active	6.6 \pm 0.36	5.8 \pm 0.49	1.2 \pm 0.09	1.5 (0.8,4.5)

Table 5.3. Effect of 3 months of placebo and vitamin C supplementation (500 mg per day) on plasma lipoprotein profiles in hypertensive subjects. Values are expressed as mean \pm SEM or mean (range).

	Total Cholesterol mmol l ⁻¹	LDL Cholesterol mmol l ⁻¹	HDL Cholesterol mmol l ⁻¹	Triglycerides mmol l ⁻¹
Basal	6.1 \pm 0.22	4.8 \pm 0.30	1.6 \pm 0.11	2.0 (1.2,6.3)
Placebo	6.1 \pm 0.19	4.2 \pm 0.29	1.6 \pm 0.08	1.9 (0.7,5.8)
Active	6.0 \pm 0.22	4.3 \pm 0.26	1.6 \pm 0.09	2.2 (0.8,6.3)

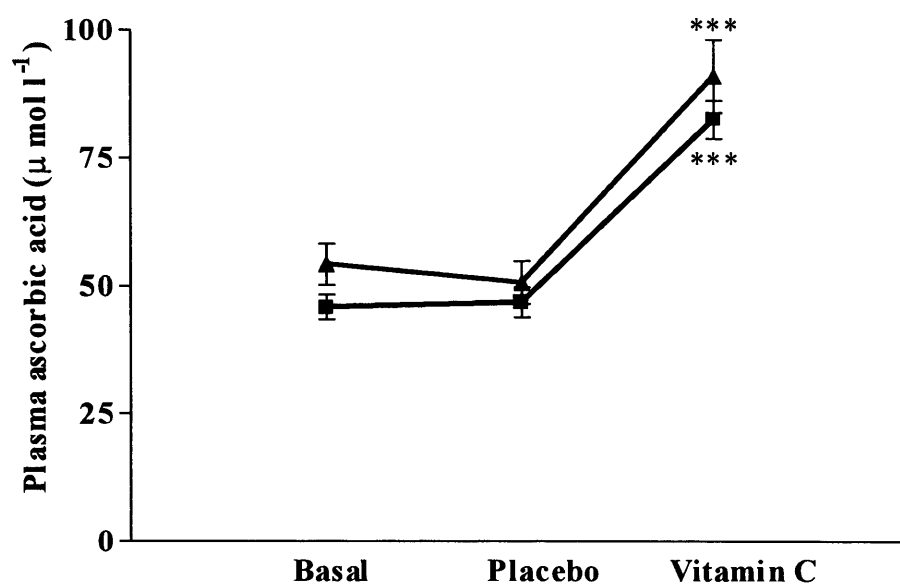
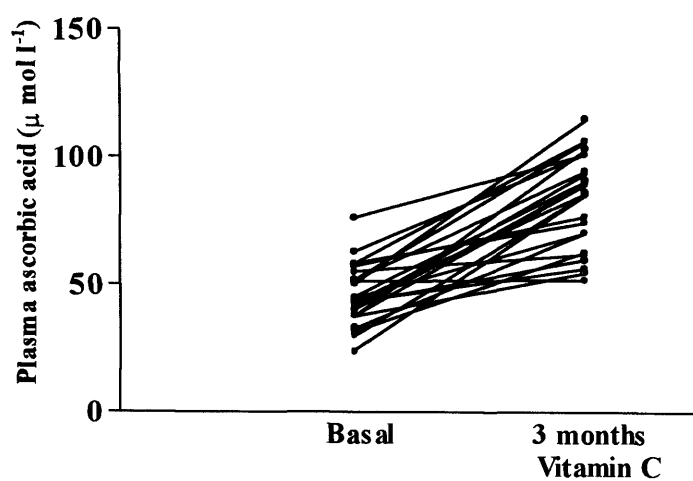


Figure 5.1. Effects of 3 months of placebo and vitamin C supplementation (500 mg per day) on plasma ascorbic acid levels ($\mu\text{mol l}^{-1}$) in hypertensive (▲) and normotensive (■) subjects. Data points are means, with SEM shown by vertical error bars. *** $p < 0.001$ vs basal or placebo.

a)



b)

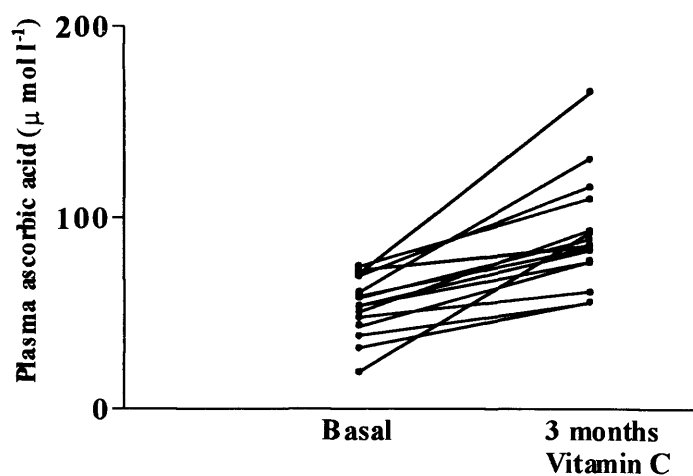


Figure 5.2. Graphs showing the individual plasma ascorbic acid responses ($\mu\text{mol l}^{-1}$) to three months supplementation of vitamin C at 500 mg per day in a) normotensive and b) hypertensive subjects.

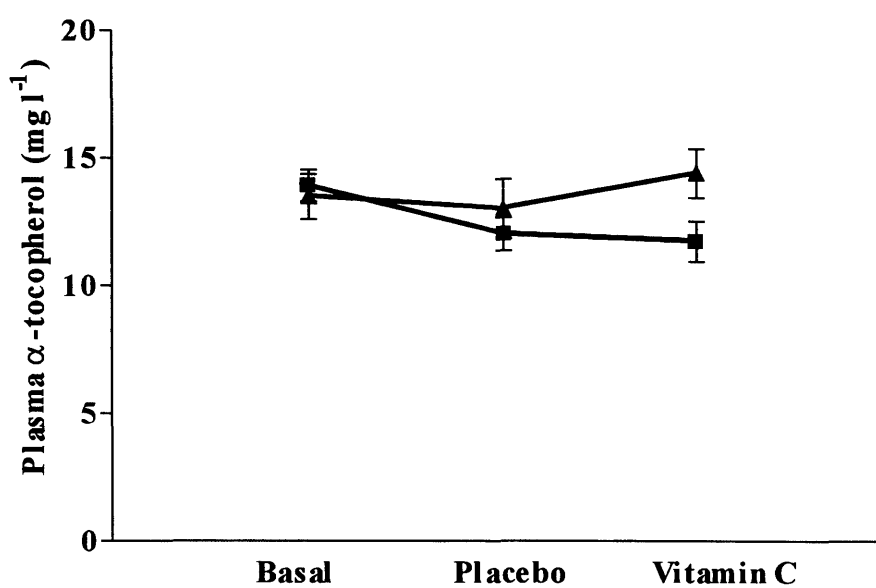


Figure 5.3. Effects of 3 months of placebo and vitamin C supplementation (500 mg per day) on plasma α -tocopherol levels (mg l⁻¹) in hypertensive (▲) and normotensive (■) subjects. Data points are means, with SEM shown by vertical error bars.

Table 5.4. Results of daytime ambulatory blood pressure and clinic sitting blood pressure during baseline, placebo and vitamin C phases of the study in the hypertensive subjects. Values are expressed as mean \pm SEM.

	Baseline (mmHg)	Placebo (mmHg)	Vitamin C (mmHg)	Difference Placebo – Vitamin C	p value
Daytime SBP	148 \pm 3	150 \pm 3	145 \pm 2	5 \pm 2	0.025
Daytime DBP	83 \pm 3	83 \pm 2	81 \pm 2	2 \pm 1	0.08
Daytime MAP	106 \pm 2	106 \pm 2	103 \pm 1	3 \pm 1	0.047
Sitting SBP	148 \pm 3	152 \pm 4	149 \pm 3	3 \pm 3	0.64
Sitting DBP	90 \pm 3	88 \pm 3	88 \pm 3	0 \pm 1	0.40

Table 5.5. Results of daytime ambulatory blood pressure and clinic sitting blood pressure during baseline, placebo and vitamin C phases of the study in the normotensive subjects. Values are expressed as mean \pm SEM.

	Baseline (mmHg)	Placebo (mmHg)	Vitamin C (mmHg)	Difference Placebo- Vitamin C	p value
Daytime SBP	126 \pm 2	125 \pm 2	125 \pm 2	0 \pm 2	0.74
Daytime DBP	76 \pm 1	76 \pm 1	76 \pm 1	0 \pm 2	0.52
Daytime MAP	93 \pm 1	93 \pm 1	93 \pm 1	0 \pm 2	0.49
Sitting SBP	138 \pm 3	140 \pm 2	137 \pm 3	3 \pm 2	0.78
Sitting DBP	87 \pm 2	88 \pm 1	86 \pm 1	2 \pm 1	0.66

5.3.4 Effect Of Vitamin C Supplementation On Platelet Adhesion *Ex Vivo*.

No significant correlation was found between platelet adhesion and the following clinical and laboratory characteristics: age, BMI, systolic and diastolic blood pressures, pulse pressure, cholesterol, soluble ICAM-1, soluble E-Selectin, soluble L-Selectin, vWF and vitamins C and E.

Basal platelet adherence to collagen coated (41.7 ± 1.3 % vs 46.3 ± 2.7 %, $p=0.19$) or tissue culture plastic microwells (34.1 ± 1.8 % vs 39.1 ± 2.9 %, $p=0.20$) was not significantly different in hypertensive compared to normotensive subjects. In the normotensive subjects, platelet adhesion to collagen coated or tissue culture plastic microwells was unaffected by supplementation with placebo. Vitamin C supplementation did produce a significant decrease in adhesion to collagen coated (38.1 ± 2.3 % vs 49.5 ± 3.5 %, $p<0.05$) and plastic microtitre wells (28.5 ± 2.5 % vs 40.5 ± 4.3 %, $p<0.01$) (Figure 5.4a). Placebo or vitamin C supplementation had no significant effect on platelet adhesion to collagen coated or tissue culture plastic microwells in the hypertensive subject group (Figure 5.4b). There was no significant period effect or treatment period interaction for platelet adhesion to either collagen coated or tissue culture plastic microwells in the hypertensive or normotensive subjects.

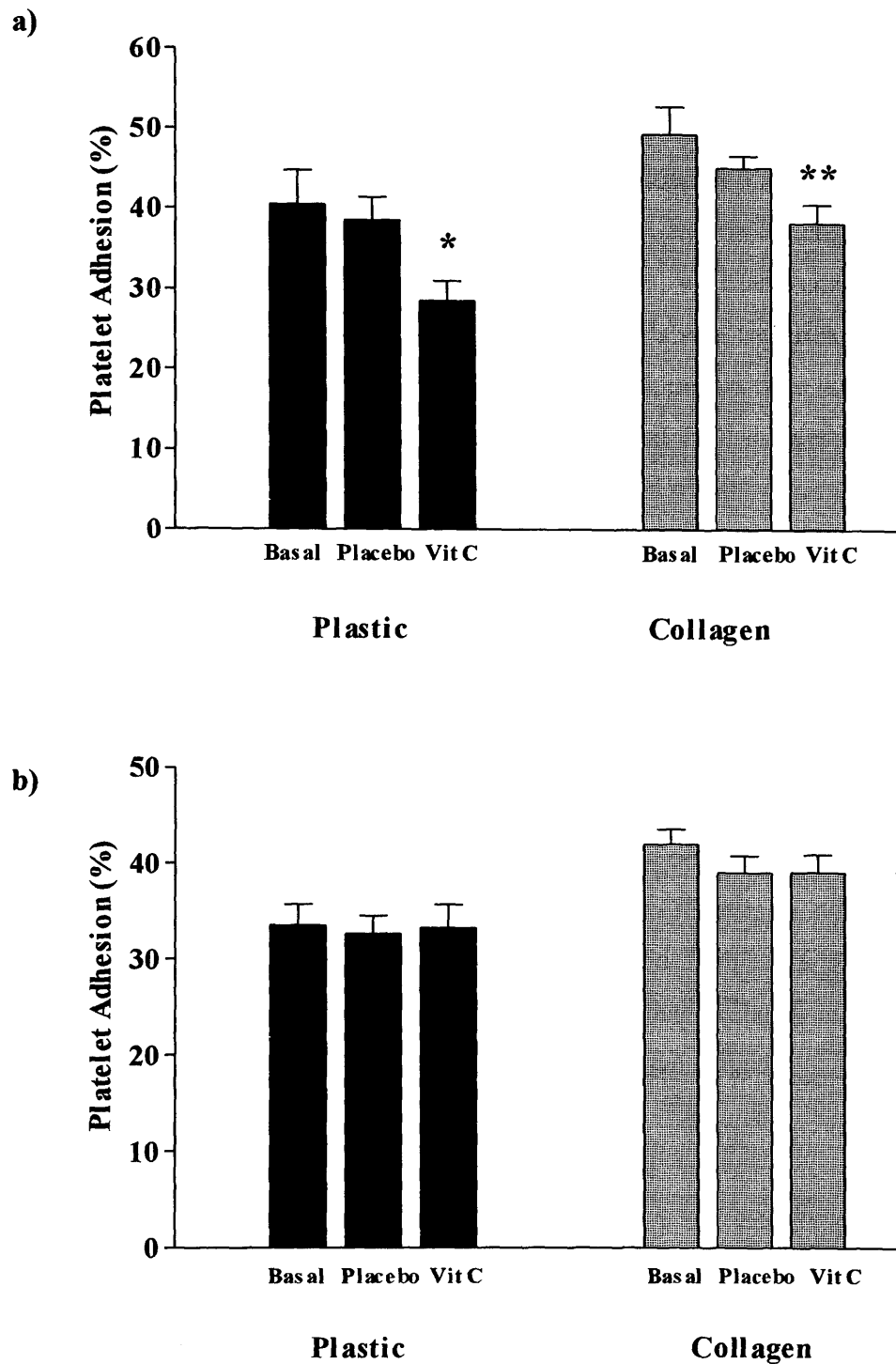


Figure 5.4. Effect of 3 months of placebo and vitamin C supplementation (500 mg per day) on platelet adhesion (%) to collagen coated and tissue culture plastic microwells in a) normotensive subjects and b) hypertensive subjects. Data are expressed as means, with SEM shown by vertical error bars. * $p < 0.05$ vs placebo, ** $p < 0.01$ vs placebo).

5.3.5 Effect Of Vitamin C Supplementation On Monocyte Adhesion *Ex Vivo*.

No significant correlation was found between monocyte adhesion to either collagen coated or tissue culture plastic microwells and the following clinical or laboratory characteristics: age, BMI, systolic and diastolic blood pressures, cholesterol, fibrin, factor VII, soluble E-Selectin, soluble L-Selectin, soluble ICAM-1, vWF and vitamins C and E. However, monocyte adhesion to collagen coated microwells was significantly correlated with daytime pulse pressure ($r=0.38$, $p=0.005$) (Figure 5.5); on multiple linear regression this relationship was independent of age, total-, LDL- and HDL-cholesterol ($R^2=0.08$, $p=0.016$). Monocyte adhesion to tissue culture plastic microwells was not significantly correlated with daytime or clinic pulse pressure $r=0.21$, $p=0.13$, $r=0.24$, $p=0.11$, respectively.

Basal monocyte adherence to collagen coated (64.5 ± 5.5 % vs 57.3 ± 3.8 %, $p=0.27$) or tissue culture plastic microwells (77.6 ± 2.7 % vs 71.2 ± 3.2 %, $p=0.16$) was not significantly different in hypertensive compared to normotensive subjects. Placebo or vitamin C supplementation failed to produce any significant difference in the adhesion of monocytes to collagen coated or tissue culture plastic microwells in normotensive or hypertensive subjects (Figures 5.6a and b). There was no significant period effect or treatment period interaction for monocyte adhesion to either collagen coated or tissue culture plastic microwells in the hypertensive or normotensive subjects.

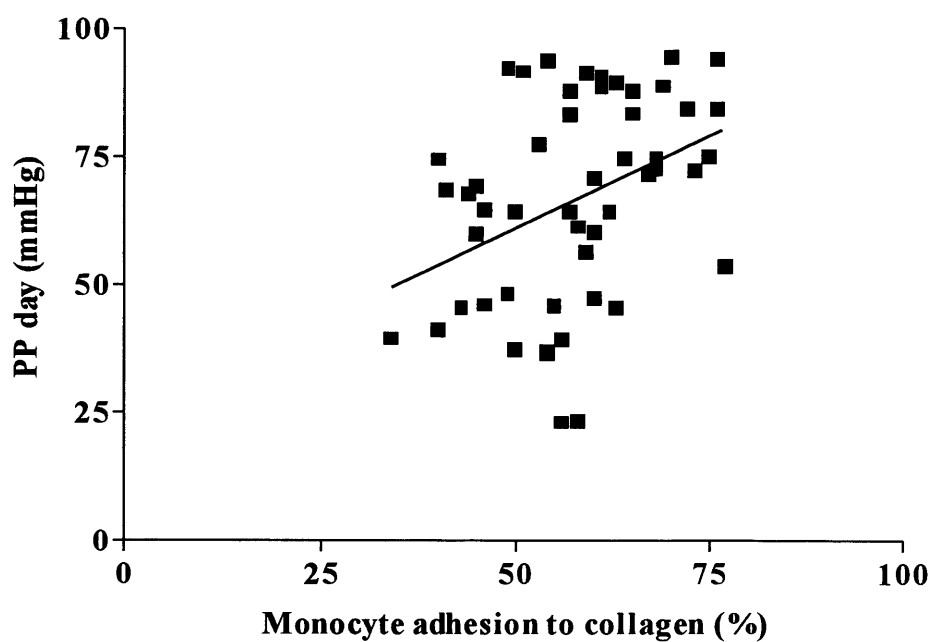


Figure 5.5. Correlation between monocyte adhesion to collagen (%) and daytime pulse pressure (PP day) (mmHg); $r=0.3796$, $p=0.0051$.

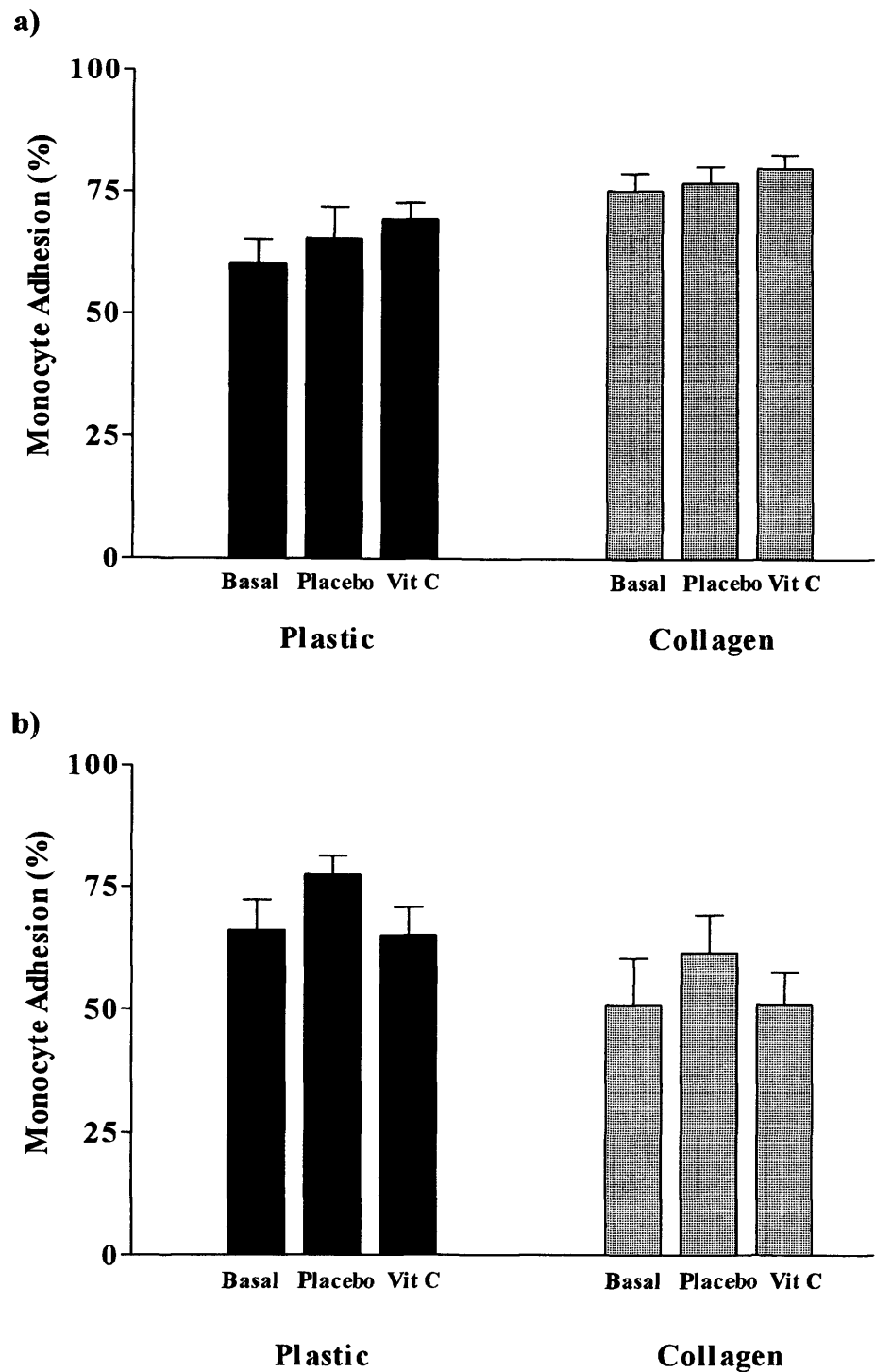


Figure 5.6. Effects of 3 months of placebo and vitamin C supplementation (500 mg per day) on monocyte adhesion (%) to collagen coated and tissue culture plastic microwells in a) normotensive subjects and b) hypertensive subjects. Data are expressed as means, with SEM shown by vertical error bars.

5.3.6 Soluble Adhesion Molecules In Hypertension And The Effect Of Vitamin C Supplementation.

Circulating levels of soluble ICAM-1 ($389 \pm 20 \text{ ng ml}^{-1}$ vs $327 \pm 10 \text{ ng ml}^{-1}$, $p < 0.01$) and soluble E-Selectin ($69 \pm 7 \text{ ng ml}^{-1}$ vs $48 \pm 4 \text{ ng ml}^{-1}$, $p < 0.05$) were significantly raised in the hypertensive compared to normotensive subjects (Table 5.1). While basal levels of vWF and soluble L-selectin were not significantly different between hypertensive and normotensive subjects. A significant correlation existed between plasma levels of soluble E-selectin and clinic DBP ($r = 0.396$, $p = 0.0005$, Figure 5.7). The regression equation used to predict plasma sE-selectin ($r^2 = 0.157$, $p = 0.0005$) was

$$\text{E-Selectin (ng ml}^{-1}\text{)} = 79.6 + 0.131 (\text{Clinic DBP [mmHg]}).$$

No significant correlation existed between any of the blood pressure parameters measured and soluble ICAM-1, soluble L-Selectin and vWF.

Neither placebo (1.25 ± 0.06 vs 1.26 ± 0.05 , $p = 0.90$; 1.25 ± 0.05 vs 1.25 ± 0.05 , $p = 0.92$) nor vitamin C supplementation (1.22 ± 0.06 vs 1.26 ± 0.05 , $p = 0.87$; 1.26 ± 0.06 vs 1.25 ± 0.05 , $p = 0.99$) produced any significant changes in mean plasma vWF levels in either hypertensive (Figure 5.8a) or normotensive subjects (Figure 5.8b), respectively. Also placebo (59 ± 6 vs 69 ± 7 , $p = 0.29$; 44 ± 21 vs 49 ± 4 , $p = 0.61$) or vitamin C supplementation (63 ± 6 vs 69 ± 7 , $p = 0.49$; 39 ± 3 vs 48 ± 4 , $p = 0.17$) did not produce any significant changes in mean plasma soluble E-Selectin levels in either the hypertensive subjects (Figure 5.9a) or normotensive subjects (Figure 5.9b) respectively.

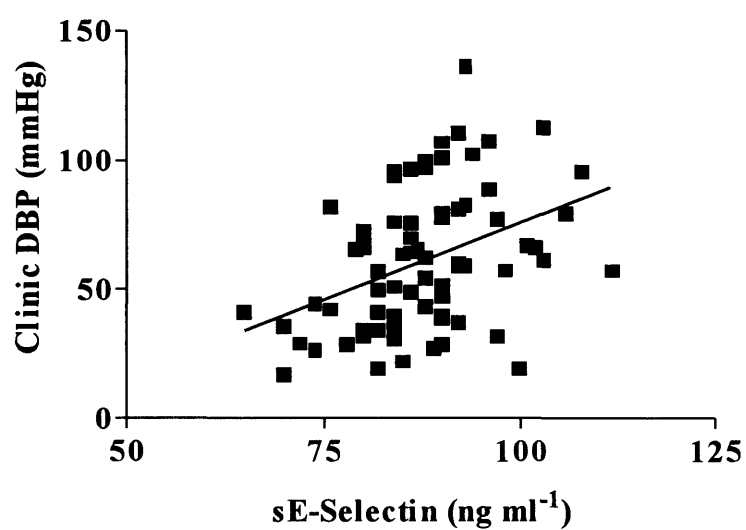
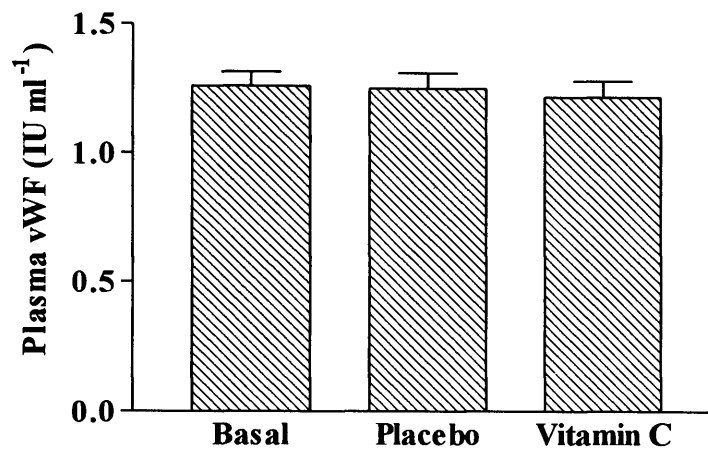


Figure 5.7. Correlation between soluble E-Selectin (sE-Selectin) and clinic diastolic blood pressure (Clinic DBP); $r=0.396$, $p=0.0005$.

a)



b)

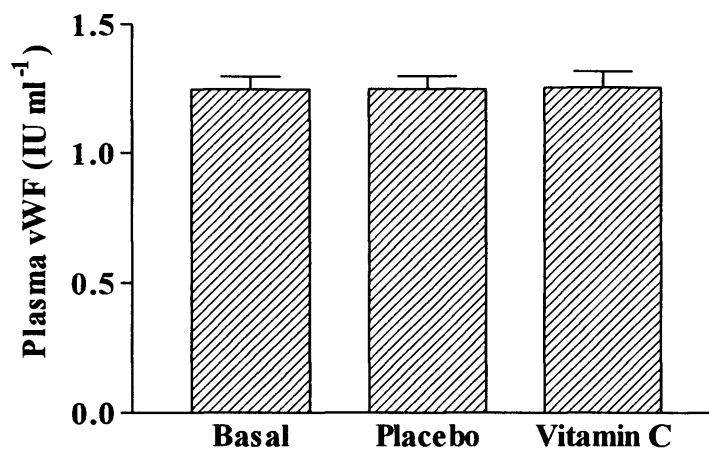


Figure 5.8. Effects of 3 months of placebo and vitamin C supplementation (500 mg per day) on mean plasma levels of vWF (IU ml⁻¹) in a) hypertensive and b) normotensive subjects. Values are expressed as mean \pm SEM.

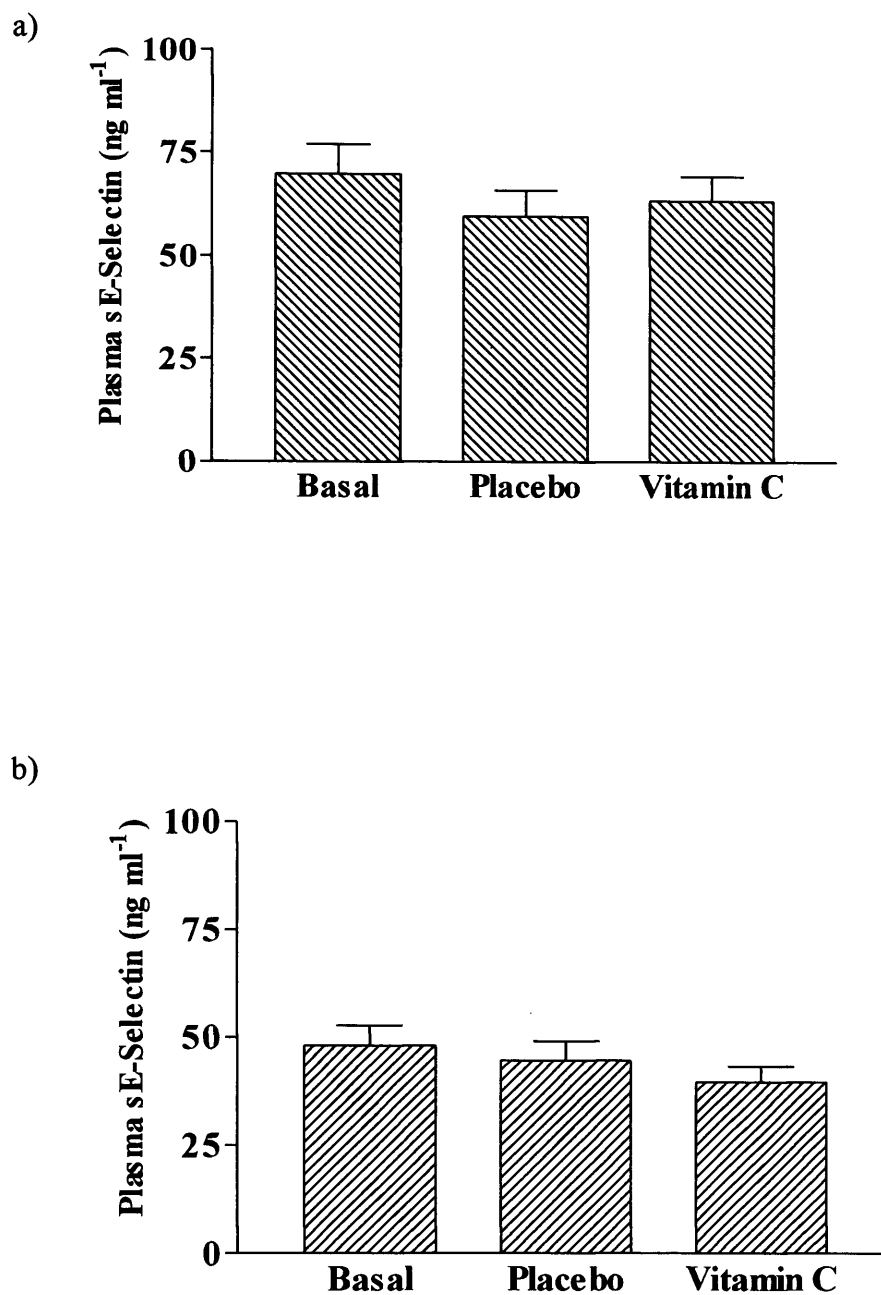


Figure 5.9. Effects of 3 months of placebo and vitamin C supplementation (500 mg per day) on mean plasma levels of soluble E-Selectin (ng ml⁻¹) in a) hypertensive and b) normotensive subjects. Values are expressed as mean \pm SEM.

5.4 DISCUSSION

5.4.1 Effect Of Vitamin C Supplementation On Plasma Lipid And Antioxidant Vitamin Profiles.

The role of ascorbic acid in lowering blood lipids is still controversial, with some studies demonstrating an improved lipoprotein profile with supplementation (Peterson *et al.* 1975; Ginter E 1976) and others reporting no change (Aro *et al.* 1988). In this study supplementation with placebo or vitamin C at a dose of 500 mg per day did not produce any significant effects on plasma total-, calculated LDL-, HDL-cholesterol or total triglycerides. Vitamin C is a water-soluble vitamin, which is readily eliminated from the body by excretion in the urine. A one-week washout period was incorporated into the study design to attempt to eliminate any carry-over effects that may occur, following vitamin C supplementation. This period was sufficient to return the mean plasma ascorbic acid levels to basal levels. Analysis for treatment-period interaction examined the possibility of a carry-over effect, no significant treatment period interaction was found. Mean plasma ascorbic acid levels increased approximately two-fold following vitamin C supplementation at 500 mg per day, reaching the level of tissue saturation achieved at plasma concentrations $\geq 80 \mu\text{mol l}^{-1}$ (Gey 1995). This degree of increase is similar to the levels achieved in a study by Ghosh *et al.* (1994) using the same dosage and another study in which a higher dose for a shorter duration was employed (Weber *et al.* 1996). Vitamin C has been demonstrated to regenerate α -tocopherol from its chromanoxyl radical to yield tocopherol (Packer *et al.* 1979) and as a result may have a 'sparing' effect on vitamin E. High levels of plasma ascorbate are associated with increased levels of vitamin E (Fidanza *et al.* 1982), however, in this study no effect on mean plasma α -tocopherol levels was seen following supplementation with 500 mg per day for 3 months.

5.4.2 Vitamin C Lowers Ambulatory Blood Pressure In Elderly Hypertensive Subjects.

A 500 mg vitamin C supplement administered for 3 months significantly lowered daytime systolic and mean arterial blood pressure in elderly hypertensive subjects. Several small ascorbic acid supplementation trials have also reported a slight drop in blood pressure (Koh 1984; Trout 1991; Ghosh *et al.* 1994). For the most part, however, these changes did not reach statistical significance. These studies examined the effect on clinic measurements of blood pressure, while we examined both clinic measurements and 24-h blood pressure monitoring. A significant fall in blood pressure was only observed in measurements obtained from this 24-h blood pressure monitoring, which is potentially a more accurate method for the measurement of blood pressure and eliminates any possible 'white-coat' effect that may be seen as a result of clinic measurements.

5.4.3 Vitamin C Supplementation Inhibits Platelet Adhesion *Ex Vivo* In Normotensive But Not Hypertensive Subjects.

Elevated platelet activation has been demonstrated in hypertensive subjects (Coccheri & Fiorentini 1971; Mehta & Mehta 1981; Yamanishi *et al.* 1985). Increased platelet adhesiveness has also been reported in hypertensive subjects compared to healthy normotensive controls (Andrioli *et al.* 1996). In the present study no significant differences in basal platelet adhesion between our hypertensive and normotensive subjects were observed.

The first step of the functional response of platelets is their adhesion to the vessel wall. Platelet adhesion is a complex event involving a series of plasma and subendothelial tissue components, which bind to specific platelet membrane glycoproteins. In this study and throughout this thesis platelet adhesion to collagen coated microwells and tissue culture plastic microwells has been examined by quantitation of the enzyme acid phosphatase. This method which examines static adhesion is not as informative as those

methods in which perfusion chambers are employed, allowing conditions of laminar flow that occurs *in vivo* to be mimicked.

The estimated threshold plasma vitamin C concentration of 50 to 60 $\mu\text{mol l}^{-1}$ for effective protection from cardiovascular disease (Gey *et al.* 1993) may be a possible explanation for the differing effects of vitamin C supplementation between our hypertensive and normotensive subjects. The hypertensive subjects had plasma vitamin C levels above this threshold ($54.28 \pm 3.98 \mu\text{mol l}^{-1}$), while the normotensive subjects had a level of vitamin C ($45.91 \pm 2.43 \mu\text{mol l}^{-1}$) which falls below this threshold. The difference between mean plasma vitamin C levels in these two groups just failed to reach significance ($p=0.064$). On this basis the hypertensive subjects may have had sufficient vitamin C levels to provide adequate protection, while in the normotensive subjects supplementation may have compensated for the reduced level at baseline. However, vitamin C supplementation at a dosage much higher than administered in our study (3 g per day), has been demonstrated to reduce platelet adhesion in healthy volunteers and patients with coronary artery disease (Bordia & Verma 1985) regardless of basal plasma vitamin C levels.

5.4.4 Vitamin C Supplementation Did Not Affect Mononuclear Cell Adhesion *Ex Vivo*.

Increased monocyte adhesion in hypertension has been demonstrated in both in vitro studies (McCarron *et al.* 1994a, 1994b; Kim *et al.* 1996) and an animal model (Tropea *et al.* 1996). This increased adhesion has been attributed to increased endothelial cell adhesion molecule expression including VCAM-1 and ICAM-1 (McCarron *et al.* 1994b; Tropea *et al.* 1996), while angiotensin II has also been suggested to increase monocyte adhesion independent of endothelial adhesion molecule expression (Kim *et al.* 1996). In our study monocyte adhesion in hypertensive subjects was not significantly different to that exhibited in matched normotensive subjects, however, we did not examine adhesion to endothelial cells as in the previous studies. Whether the increased monocyte adhesion demonstrated by others can be attributed to properties of the monocyte itself or is merely

a reflection of increased activation of the endothelium remains to be conclusively determined.

Pulse pressure, the difference between systolic and diastolic blood pressure, is strongly correlated with increased risk for cardiovascular disease (Madhavan *et al.* 1994; Flack *et al.* 1995). A wide pulse pressure may reflect the increased peripheral vascular resistance, whereby large arteries become increasingly stiff and less compliant (Madhavan *et al.* 1994; Bots *et al.* 1996). We have found a significant correlation between monocyte adhesion to collagen and pulse pressure. Whether this increased adhesiveness of monocytes with increasing pulse pressure contributes to the increased risk of cardiovascular disease is debatable.

Co-existing coronary risk factors may alter MNC adhesiveness and confound for effects of hypertension. For example monocytes isolated from patients with hypercholesterolaemia adhered to cultured human umbilical vein cells to a greater extent than those from subjects with normal cholesterol levels (Bath *et al.* 1991a, Lösche *et al.* 1992, Stragliotto *et al.* 1993). In the present study an attempt was made to control for possible confounding variables. Hypertensive and normotensive subjects were screened to exclude those with clinical evidence of vascular disease, diabetes mellitus and those who smoked. Despite entering other possible confounding variables including blood lipids into the regression equation, pulse pressure remained significantly correlated with monocyte adhesion to collagen coated microwells.

Monocyte adhesion to collagen coated and tissue plastic microwells was not affected by vitamin C supplementation of 500 mg per day. Vitamin C at a dose of 2g per day has been demonstrated to inhibit the increased adherence of monocytes in a group of smokers to human umbilical vein endothelial cells (Weber *et al.* 1996). This inhibition of monocyte adhesion was attributed to the reversibility of the upregulated monocyte CD11b expression observed in this group of smokers. As CD11b activation has been demonstrated to enhance monocyte adhesion to the endothelium (Weber *et al.* 1995), in

the present study examination of monocyte adhesion to HUVECs as opposed to collagen coated may have been more appropriate. Or our failure to observe any inhibition may be a result of the smaller dose of vitamin C administered, or that the groups of subjects studied did not exhibit enhanced monocyte adhesiveness.

5.4.5 Soluble Adhesion Molecules In Hypertension Are Not Affected By Vitamin C Supplementation.

Adhesion molecules that participate in endothelial: monocyte interactions include ICAM-1, E-Selectin and L-Selectin. Biologically active forms of these adhesion molecules are found in plasma and are referred to as soluble adhesion molecules. These soluble adhesion molecules are thought to result from proteolytic cleavage from the cell surface. The mechanism by which levels of soluble adhesion molecules are increased is unknown, but their levels are increased in conditions in which expression on the cell membrane has been shown to be increased (Adams *et al* 1993; Ballantyne *et al* 1994). Therefore, it is possible that a raised blood pressure causes endothelial cell activation *in vivo*, this in turn results in increased adhesion molecule expression, which has been demonstrated *in vivo* in an animal model (Tropea *et al* 1996) and *ex vivo* upon cytokine stimulation (McCarron *et al* 1994a; 1994b).

Significantly elevated levels of soluble ICAM-1 and E-selectin were observed in hypertensive compared to normotensive subjects, this is in accordance with Gearing *et al.* (1992) and Blann *et al.* (1994) who have also observed that hypertensive subjects exhibit increased plasma levels of soluble ICAM-1 and E-Selectin respectively. A significant correlation was found between soluble E-selectin and clinic DBP, similar to that also observed by Blann *et al* (1994), who have suggested that the increased levels of soluble E-Selectin may indicate endothelial activation. A failure to demonstrate an increase in the levels of vWF, an established marker of endothelial cell damage, in our study and that of Blann *et al* may suggest that the endothelium is activated as opposed to damaged in

hypertension. Or it could indicate that the proposed activation resulting in elevated E-selectin is brought about by a different mechanism to that which results in vWF release.

Soluble adhesion molecules may influence leucocyte adhesion (Schleiffenbaum *et al* 1992; Lo *et al* 1991), however, in this study no correlation between any of the soluble adhesion molecules and monocyte adhesion was observed. If increased levels of soluble ICAM-1 and E-selectin truly represent increased expression of these molecules on the endothelium, increased monocyte adhesion may occur *in vivo*. Adhesion and transmigration of monocytes do not necessarily correspond and once adhered to the endothelium, monocytes may become detached (Takahashi *et al* 1994). Although the detachment mechanism is unclear, perhaps this transient adhesion may result in activation of monocytes, which is reflected by increased monocyte adhesion *ex vivo*.

Vitamin C supplementation has been demonstrated to improve endothelial dysfunction in hypertensive patients (Solzbach *et al* 1997). Following vitamin C supplementation in either normotensive or hypertensive subjects there was no difference in the circulating levels of either vWF, the established marker of endothelial damage, or soluble E-selectin a proposed marker of endothelial activation. Despite the elevated levels of soluble E-selectin which may reflect endothelial activation in these patients vitamin C supplementation did not result in decreased endothelial activation as indicated by plasma levels of vWF or soluble E-selectin.

CHAPTER 6**VITAMIN E SUPPLEMENTATION OF PATIENTS UNDERGOING
ROUTINE PERCUTANEOUS TRANSLUMINAL CORONARY
ANGIOPLASTY.****6.1 INTRODUCTION**

Percutaneous transluminal coronary angioplasty (PTCA) is often used as a more economical and less invasive alternative to coronary bypass surgery. However, a persistent problem following angioplasty is that of restenosis which occurs in every patient undergoing PTCA to a greater or lesser extent, reaching clinical significance in about 30 % of cases within the first 6 months of the PTCA procedure (Holmes *et al.* 1984). Restenosis appears to be a result of two processes: an accelerated form of atherosclerosis induced by arterial injury and a wound healing response to severe intimal and medial damage.

The majority of PTCA procedures result in cracks or dissections of plaques (Farb *et al.* 1990). Highly thrombogenic subendothelial tissue is exposed and local thrombin generation occurs as a result of atherosclerotic plaque rupture (Ip *et al.* 1991). This promotes platelet adhesion, aggregation and thrombus formation. Early platelet deposition after angioplasty may be of major importance for the process of restenosis (Steele *et al.* 1985), as thrombocytopenic rabbits, compared to normal rabbits, do not develop marked intimal hyperplasia (Friedman *et al.* 1977).

Although platelets are capable of little or no protein synthesis, they contain within their granules a variety of agents, which can be released upon activation, including platelet-derived growth factor (PDGF). PDGF may contribute to atherogenesis in a number of ways including its chemotactic and mitogenic properties for smooth muscle cells (Ross *et al.* 1974; Grotendorst 1982). This may account for the migration of smooth muscle cells from the media to the intima seen in atherogenesis in response to arterial injury. PDGF

also induces binding of LDL by increasing the number of LDL receptors (Chait *et al.* 1980) and may enhance foam cell formation.

Clinical studies have reported increased leucocyte activation following coronary angioplasty (Ikeda *et al.* 1994a; Mickelson *et al.* 1996). While increased leucocyte adhesion *in vivo* has been observed after balloon angioplasty in pigs (Merhi *et al.* 1995). Expression of CD11b, a β_2 integrin that promotes leucocyte adhesion to the endothelium, on the leucocyte surface is also elevated following angioplasty (Ikeda *et al.* 1994a), as is the number of leucocytes with adherent platelets (Mickelson *et al.* 1996). The magnitude of leucocyte activation and platelet adherence may be instrumental in restenosis, as they were both higher in patients that experienced subsequent clinical events (Mickelson *et al.* 1996). The degree of leucocyte adhesion was influenced by the severity of the arterial injury. Together this evidence suggests that leucocyte activation and adhesion may be implicated in thrombogenesis and vascular responsiveness following angioplasty.

The acute release of reactive oxygen species (ROS) including superoxide and lipid hydroperoxides (Roberts *et al.* 1990; Coghlan *et al.* 1991) during PTCA may also result in predisposition to restenosis. Effects of ROS generated in the plasma, or at the intimal wall where the balloon catheter is acting, upon the endothelium include cytotoxicity (Ginsburg *et al.* 1989) and the inhibition of PGI₂ production (Moncada *et al.* 1976a). Platelet activation and adhesion are also promoted by ROS (Violi *et al.* 1988; Salvenimi *et al.* 1989). Antioxidants, including vitamin E and vitamin C, have been demonstrated to inhibit restenosis following balloon injury in several animal models (Ferns *et al.* 1992; Freyschuss *et al.* 1993; Nunes *et al.* 1993; Konneh *et al.* 1995; Lafont *et al.* 1995). In human supplementation studies probucol has also proved an effective inhibitor of restenosis post PTCA (Watanabe *et al.* 1996; Tardif *et al.* 1997). Whilst vitamin E has not proved as effective (DeMaio *et al.* 1992), though a trend towards a reduction of restenosis was observed in the study of DeMaio *et al.* (1992) in which supplementation did not begin until after the PTCA procedure. Whether these antioxidants inhibit restenosis via their protective effects on the endothelium (Kuzuya *et al.* 1991; Keaney *et*

al. 1993; Simon *et al* 1993; Stewart-Lee *et al.* 1994; Levine *et al.* 1996), anti-aggregatory and anti-adhesive effects on platelets (Steiner 1983; Bordia & Verma 1985; Jandak *et al.* 1988; Salonen *et al.* 1991) or effects on other cells including leucocytes (Martin *et al* 1997) and smooth muscle cells (Boscoboinik *et al* 1991a), remains to be elucidated.

In a randomized, double blind, placebo-controlled trial we have examined whether vitamin E supplementation, at 800 IU per day in patients undergoing routine PTCA, would alter platelet and monocyte adhesiveness, prior to and following the first six months of angioplasty. The effect of vitamin E supplementation on circulating levels of the soluble adhesion molecules P-selectin and ICAM-1, immediately prior to and immediately post PTCA, was also examined.

6.2 STUDY DESIGN

6.2.1 Materials And Methods.

Materials and their suppliers are listed in appendix I

Details of methods employed during this study are described in section 2.2

6.2.2 Subjects.

Patients undergoing routine percutaneous transluminal coronary angioplasty (PTCA) were recruited, at Glenfield Hospital, in collaboration with the Department of Cardiology, University of Leicester. Each patient recruited had angiographically defined disease, with >70% stenosis of one or more major epicardial coronary arteries. Any patients taking vitamin supplements were excluded from this trial.

6.2.3 Vitamin Supplementation.

With informed consent, and approval of the local ethical committee, patients were randomly and blindly allocated to one of two groups. One group received 800 International Units (IU) per day vitamin E, the other placebo, for one month prior to PTCA and for 6 months afterwards.

6.2.4 Blood Sampling.

Blood samples were taken for baseline measurements of total and HDL cholesterol and triglycerides. LDL cholesterol was calculated according to the Friedewald formula (Friedewald *et al* 1972). Plasma retinol and α -tocopherol were measured by HPLC according to the method of Bieri *et al* (1979). Also platelet and monocyte adhesion were measured *ex vivo* by the methods of Bellavite *et al* (1994) and Bath *et al* (1989) respectively. Further blood samples were taken immediately before PTCA, immediately afterwards, and 6 months post-angioplasty. Plasma was obtained from these blood samples and stored at -70°C .

6.2.5 Statistical Analysis.

Statistical significance was analysed by the chi-square test, Fischer's exact test and unpaired Student's t-test and analysis of variance with Tukey's post hoc test for normally distributed data and by Mann-Whitney U test and the Kruskal-Wallis test for data which is not normally distributed. Data is presented as mean \pm SEM, median and range or actual numbers. $p < 0.05$ was considered statistically significant. Analyses were performed using Instat software (Graphpad Inc, USA) on a PC.

6.3 RESULTS

6.3.1 Basal Patient Characteristics.

Eighty seven patients were randomly allocated to receive either placebo or vitamin E: 45 received vitamin E (800 IU per day) and 42 placebo. Selected demographic, biochemical and clinical characteristics of the placebo and vitamin E group are shown in Table 6.1. The group assigned to receive vitamin E consisted of 34 men and 11 women with a mean age of 60 ± 1 years (range 44-73 years). The placebo group comprised 33 men and 9 women with a mean age of 60 ± 1 years (range 40-72 years). There were no significant differences at baseline between the two groups.

6.3.2 Effect Of Placebo And Vitamin E Supplementation On Plasma Lipid And Antioxidant Vitamin Levels.

There were no significant changes observed in mean plasma total, LDL- or HDL-cholesterol, or total triglycerides following supplementation in either the vitamin E group or placebo group (Table 6.2 and 6.3 respectively). Vitamin E supplementation at 800 IU per day produced a significant increase in mean plasma α -tocopherol levels at PTCA (20.67 ± 1.39 vs 12.41 ± 0.61 , $p < 0.0001$), a further significant increase following 7 months of supplementation (25.66 ± 1.95 vs 12.41 ± 0.61 , $p < 0.0001$) (Figure 6.1). No significant effect on mean plasma retinol levels was observed following vitamin E supplementation (Figure 6.2). Placebo supplementation was not associated with any significant changes in either mean plasma α -tocopherol or plasma retinol levels (Figure 6.1 and 6.2).

Table 6.1 Basal demographic, clinical and biochemical characteristics of patients with angiographically defined coronary artery disease randomly allocated to receive either vitamin E (800 IU per day) or placebo. Values are expressed as mean \pm SEM, mean (range), or actual numbers (%).

	Vitamin E	Placebo	p value
n	45	42	
Mean age	60 \pm 1	60 \pm 1	0.83
M:F ratio	34 : 11	33 : 9	0.93
Current or former smoker no. (%)	31 (69)	21 (50)	0.11
Current smoker no. (%)	4 (9)	4 (10)	0.92
Diabetes no. (%)	4 (9)	2 (5)	0.74
Hypertension no. (%)	16 (36)	7 (17)	0.08
Exertional angina no. (%)			
I	7 (16)	3 (7)	0.37
II	19 (42)	16 (38)	0.86
III	17 (38)	22 (52)	0.25
IV	2 (4)	1 (2)	0.60
Previous MI no. (%)	21 (47)	19 (45)	0.89
Previous PTCA no. (%)	2 (4)	3 (7)	0.94
Previous CABG no. (%)	1 (2)	1 (2)	0.96
Lipoprotein profiles			
Mean total plasma cholesterol (mmol l ⁻¹)	5.6 \pm 0.19	5.9 \pm 0.18	0.36
Mean LDL cholesterol (mmol l ⁻¹)	3.6 \pm 0.18	3.8 \pm 0.16	0.57
Mean HDL cholesterol (mmol l ⁻¹)	1.2 \pm 0.05	1.2 \pm 0.05	0.64
Mean total triglycerides (mmol l ⁻¹)	1.9 (1.0,4.4)	2.1 (0.6,9.3)	0.56
Plasma antioxidant vitamins			
Vitamin E (mg l ⁻¹)	12.41 \pm 0.61	12.27 \pm 0.44	0.85
Vitamin A (mg l ⁻¹)	0.71 \pm 0.02	0.76 \pm 0.03	0.16
Cell Function			
Monocyte adhesion to cells (%)	5.69 \pm 1.30	7.40 \pm 0.93	0.28
Monocyte adhesion to collagen (%)	68.9 \pm 9.0	57.5 \pm 5.2	0.25
Monocyte adhesion to plastic (%)	75.8 \pm 3.4	67.5 \pm 3.8	0.14
Platelet adhesion to collagen (%)	30.0 \pm 2.2	33.4 \pm 2.7	0.36
Platelet adhesion to plastic (%)	28.8 \pm 1.5	28.5 \pm 2.0	0.90

Table 6.2 Effect of vitamin E supplementation at 800 IU per day, on plasma lipoprotein profiles. Values are expressed as mean \pm SEM or mean (range).

	Total cholesterol mmol l ⁻¹	LDL cholesterol mmol l ⁻¹	HDL cholesterol mmol l ⁻¹	Triglycerides mmol l ⁻¹
At Baseline	5.6 \pm 0.19	3.6 \pm 0.18	1.2 \pm 0.05	1.9 (1.0,4.4)
At PTCA	5.2 \pm 0.17	3.1 \pm 0.17	1.1 \pm 0.04	1.8 (0.6,4.2)
At Follow-up	5.3 \pm 0.16	3.2 \pm 0.18	1.2 \pm 0.06	1.7 (0.7,9.9)

Table 6.3 Effect of placebo supplementation on plasma lipoprotein profiles. Values are expressed as mean \pm SEM or mean (range).

	Total cholesterol mmol l ⁻¹	LDL cholesterol mmol l ⁻¹	HDL cholesterol mmol l ⁻¹	Triglycerides mmol l ⁻¹
At Baseline	5.9 \pm 0.19	3.8 \pm 0.16	1.2 \pm 0.05	2.1 (0.6,9.3)
At PTCA	5.4 \pm 0.23	3.4 \pm 0.18	1.1 \pm 0.04	1.9 (0.7,5.8)
At Follow-up	5.4 \pm 0.18	3.2 \pm 0.17	1.2 \pm 0.04	1.9 (0.9,4.9)

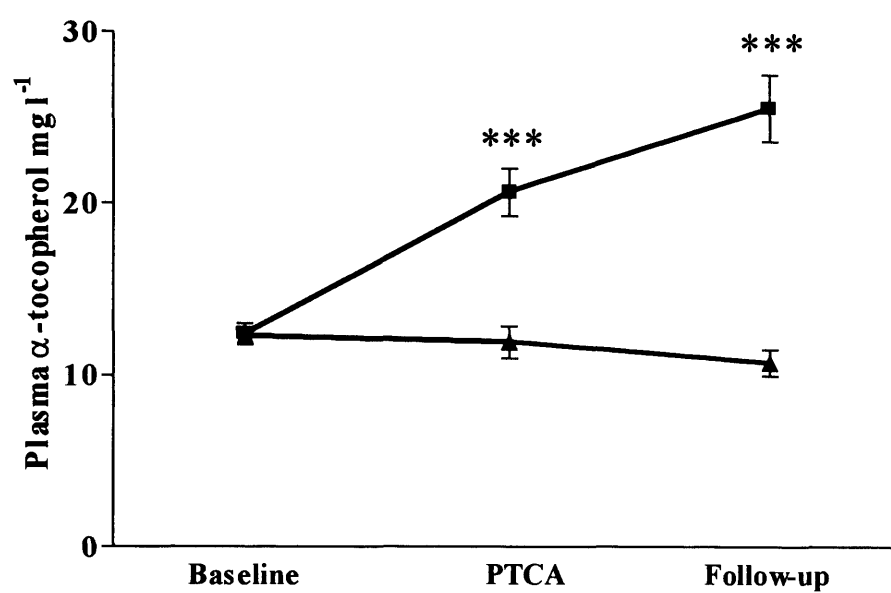


Figure 6.1 Effect of placebo (▲) and vitamin E (800 IU per day) (■) supplementation on mean plasma α -tocopherol levels (mg l⁻¹). Data points are means, with SEM shown by vertical error bars. ***p<0.001 vs baseline.

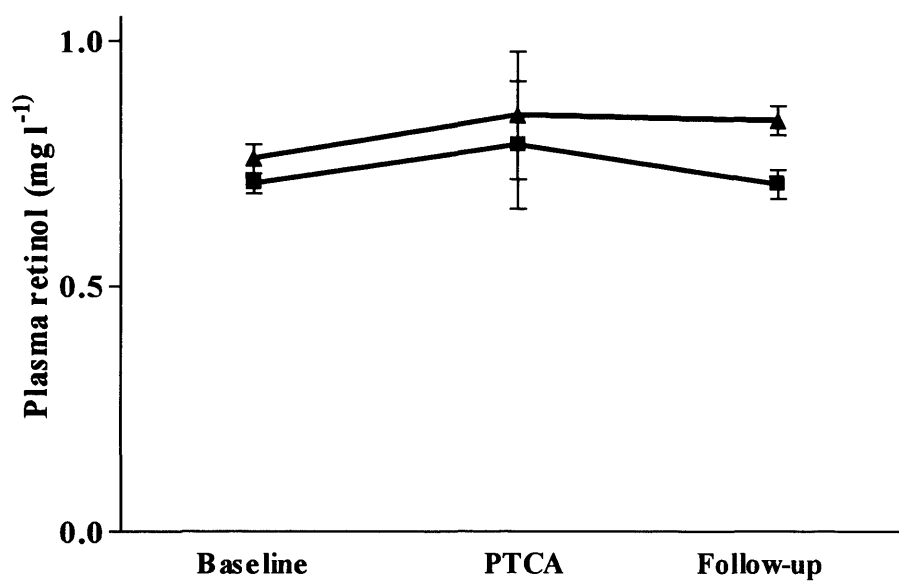


Figure 6.2 Effect of placebo (▲) and vitamin E (800 IU per day)(■) supplementation on mean plasma retinol levels (mg l⁻¹). Data points are means, with SEM shown by vertical error bars.

6.3.3 Effect Of Placebo And Vitamin E Supplementation On Platelet Adhesion *Ex Vivo*.

Neither basal resting platelet adherence to collagen coated (30.0 ± 2.2 vs 33.4 ± 2.8 , $p=0.36$) and tissue culture plastic microwells (28.8 ± 1.5 vs 28.5 ± 2.0 , $p=0.90$), nor stimulated platelet adherence to collagen coated (61.2 ± 3.9 vs 62.3 ± 2.5 , $p=0.81$) and tissue culture plastic microwells (59.0 ± 5.0 vs 59.3 ± 3.7 , $p=0.96$) were significantly different between vitamin E and placebo groups. Following supplementation with either placebo or vitamin E there was no significant effect on either resting (Figure 6.3a and b) or stimulated (Figure 6.4a and b) platelet adhesion *ex vivo* to collagen coated and tissue culture plastic microwells.

6.3.4 Effect Of Placebo And Vitamin E Supplementation On Monocyte Adhesion *Ex Vivo*.

Basal monocyte adherence to confluent EA-hy-926 endothelial cells (5.69 ± 1.30 vs 7.40 ± 0.93 , $p=0.28$), collagen coated (68.9 ± 9.0 vs 57.5 ± 5.2 , $p=0.25$) and tissue culture plastic microwells (75.8 ± 3.4 vs 67.5 ± 3.8 , $p=0.14$) was not significantly different between vitamin E and placebo groups. Following supplementation with either placebo or vitamin E there was no significant effect on monocyte adhesion *ex vivo* to endothelial cells, collagen coated or tissue culture plastic microwells (Figure 6.5a and b).

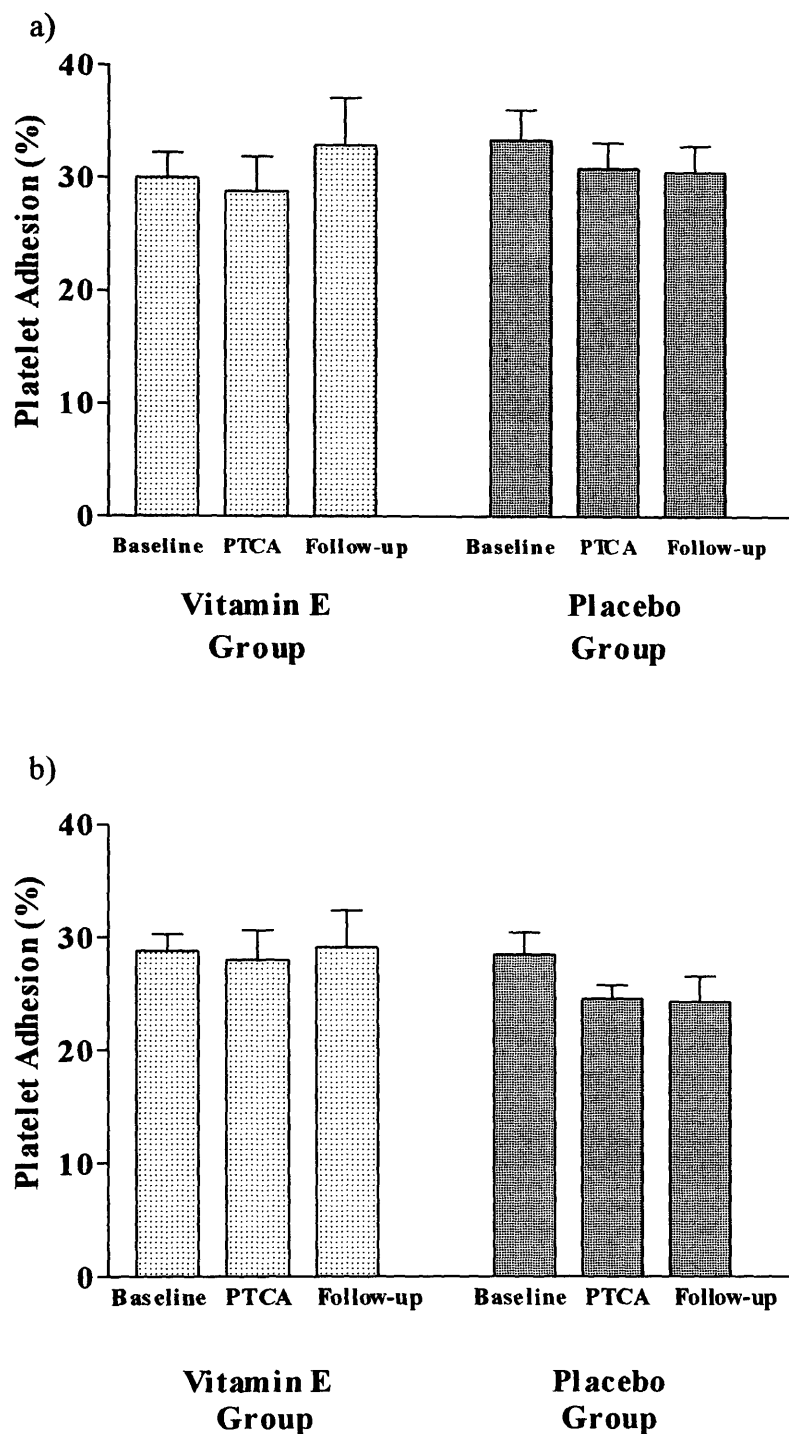
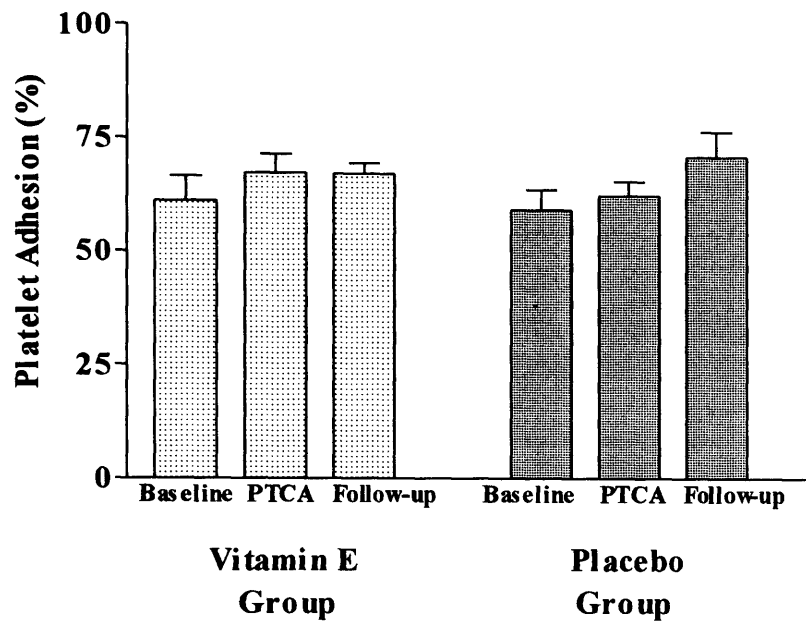


Figure 6.3 Effect of placebo and vitamin E (800 IU per day) supplementation on resting platelet adhesion to a) collagen coated and b) tissue culture plastic microwells. Data are expressed as means, with SEM shown by vertical error bars.

a)



b)

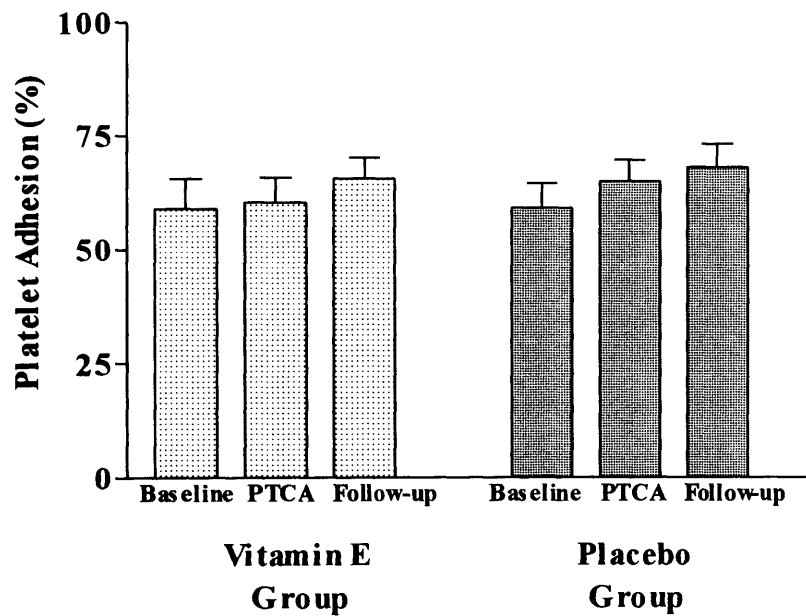


Figure 6.4 Effect of placebo and vitamin E (800 IU per day) supplementation on stimulated platelet adhesion to a) collagen coated and b) tissue culture plastic microwells. Data are expressed as means, with SEM shown by vertical error bars.

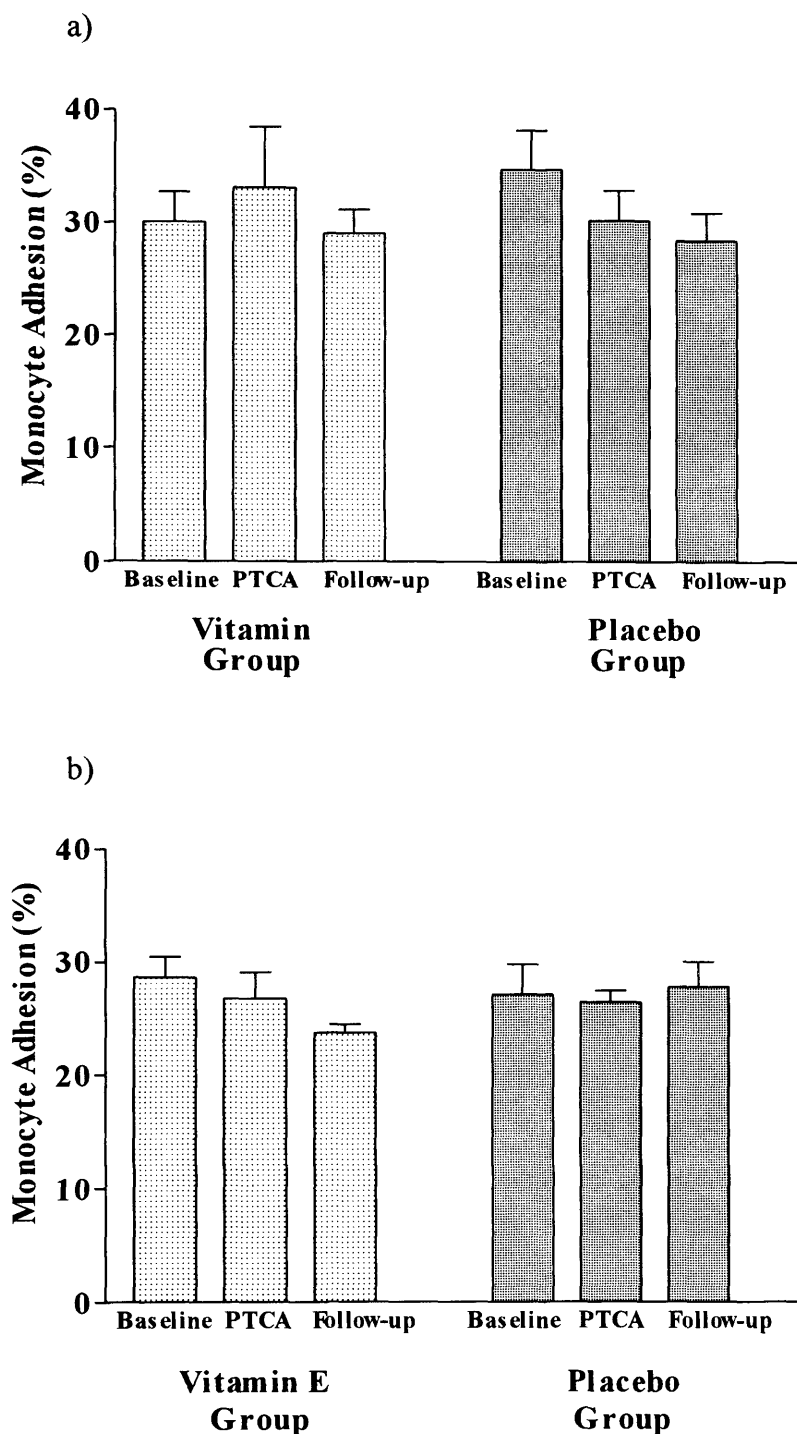


Figure 6.5 Effect of placebo and vitamin E (800 IU per day) supplementation on monocyte adhesion (%) to a) collagen coated and b) tissue culture plastic microwells. Data are expressed as means, with SEM shown by vertical error bars.

6.3.5 Effect Of Placebo And Vitamin E Supplementation On Soluble Adhesion Molecules

Plasma levels of soluble P-Selectin, soluble ICAM-1 and vWF were measured from blood samples taken from the femoral artery via the catheter immediately before and immediately after the PTCA procedure. All plasma soluble adhesion molecules were corrected for plasma protein levels, determined by the method of Lowry *et al* (1951), and expressed as ng mg⁻¹ protein. Soluble P-selectin levels were determined in an unselected group of 47 patients, of these 23 had been randomly allocated to receive vitamin E (800 IU per day) prior to the PTCA procedure, while the remainder received placebo. Soluble ICAM-1 and vWF levels were determined in a group of 30 patients, of these 15 had been randomly allocated to receive vitamin E (800 IU per day) prior to the PTCA procedure, the remainder received placebo. There was no significant difference in pre-PTCA levels of soluble P-Selectin (9.35 ± 0.81 vs 7.46 ± 0.80 , $p=0.10$), soluble ICAM-1 (2.20 ± 0.69 vs 2.18 ± 0.77 , $p=0.95$) and vWF (0.11 ± 0.05 vs 0.15 ± 0.05 , $p=0.11$) between the vitamin E and placebo groups. Mean plasma levels of soluble P-Selectin were significantly elevated post-PTCA (7.46 ± 0.81 vs 9.70 ± 0.78 , $p=0.02$) in the placebo group, while there was no significant change observed in the group which received vitamin E (9.35 ± 0.81 vs 8.85 ± 0.91 , $p=0.54$) (Figure 6.6). Mean plasma levels of ICAM-1 (2.18 ± 0.77 vs 2.16 ± 0.62 , $p=0.95$, 2.20 ± 0.69 vs 1.97 ± 0.38 , $p=0.39$) or vWF (0.15 ± 0.05 vs 0.13 ± 0.04 , $p=0.31$, 0.11 ± 0.05 vs 0.11 ± 0.06 , $p=0.92$) were not significantly altered post-PTCA in either the placebo or vitamin E group respectively (Figures 6.8 and 6.9)

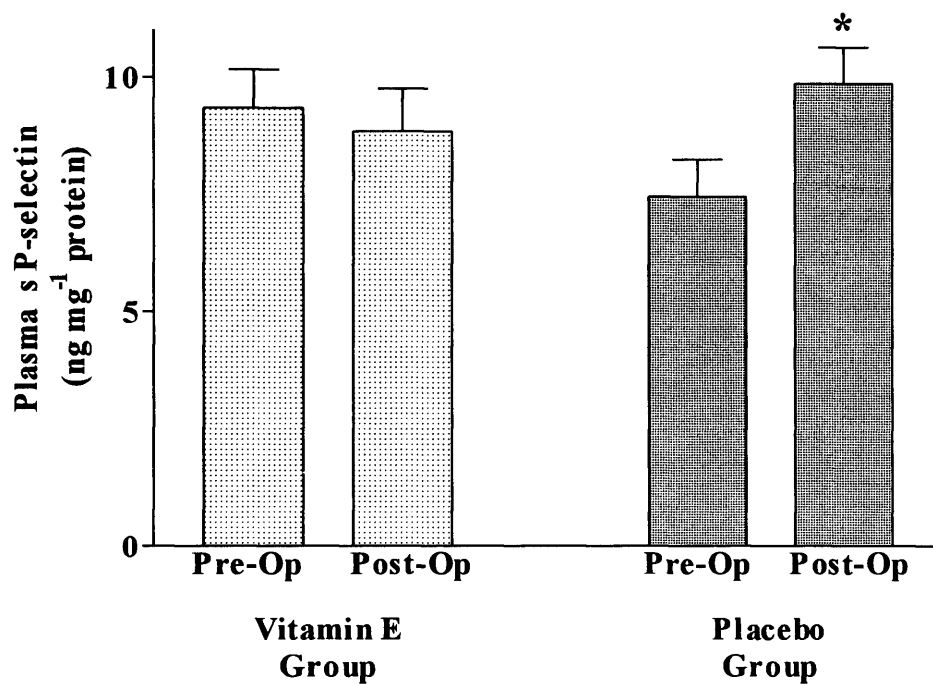
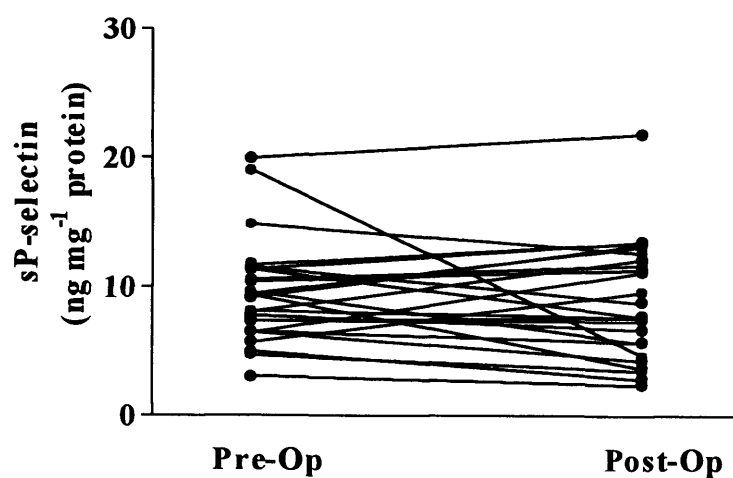


Figure 6.6 Mean plasma levels of soluble P-Selectin (ng mg^{-1} protein) immediately prior to and post PTCA in patients receiving either vitamin E (800 IU per day) or placebo. Values are expressed as mean \pm SEM. * $p < 0.05$ vs pre-op.

a)



b)

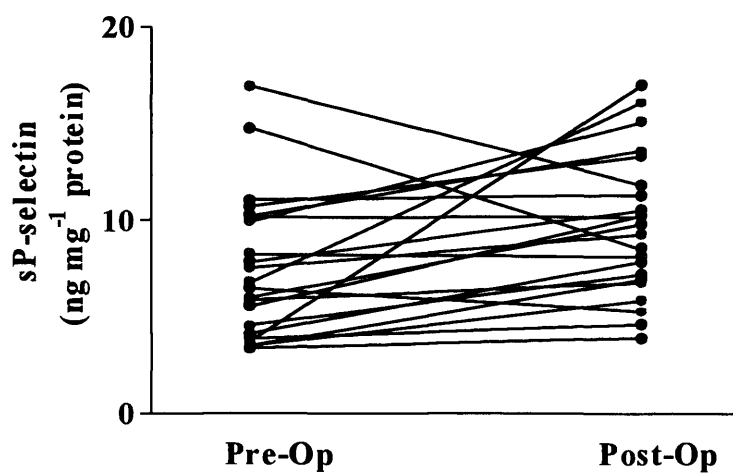


Figure 6.7 Individual plasma soluble P-selectin levels immediately before and immediately after the PTCA procedure in patients receiving a) vitamin E (800 IU per day) and b) placebo.

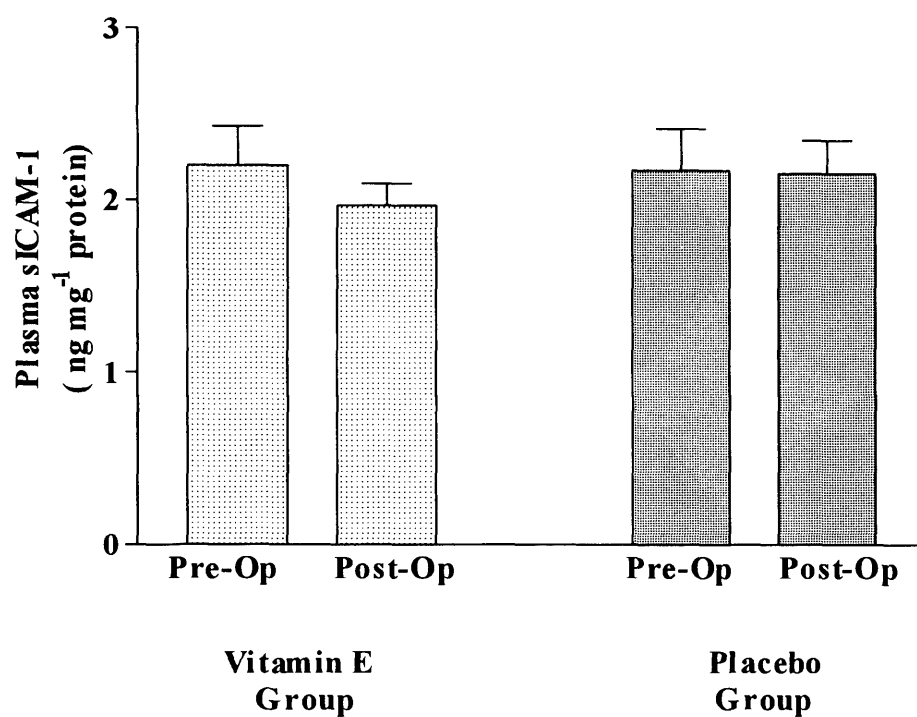


Figure 6.8 Mean plasma levels of soluble ICAM-1 (ng mg⁻¹ protein) immediately prior to and post PTCA in patients receiving either vitamin E (800 IU per day) or placebo. Values are expressed as mean \pm SEM.

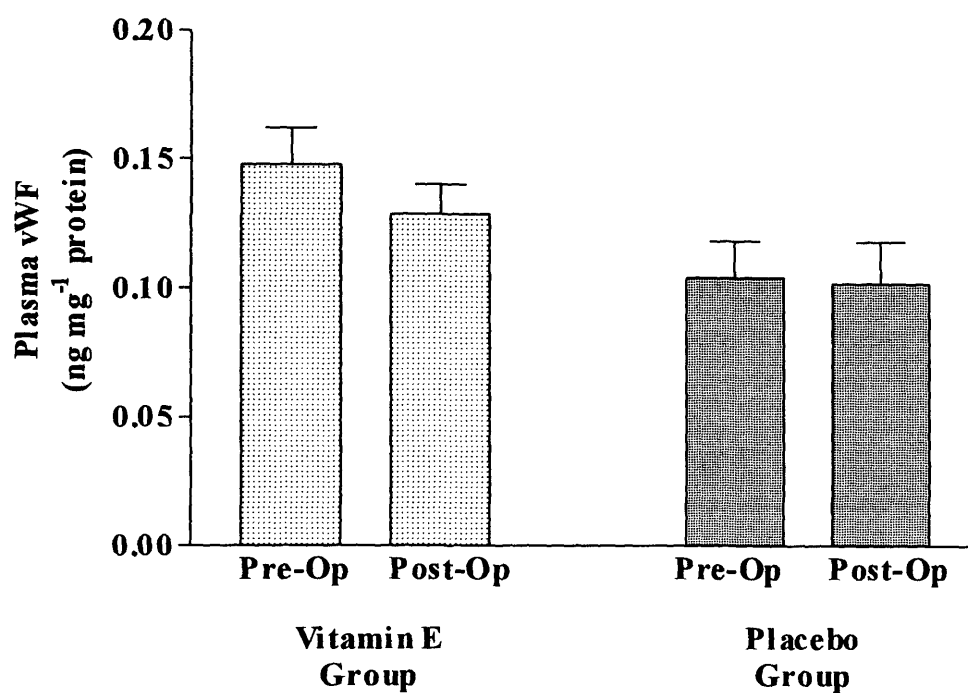


Figure 6.9 Mean plasma levels of vWF (IU mg⁻¹ protein) immediately prior to and post PTCA in patients receiving either vitamin E (800 IU per day) or placebo. Values are expressed as mean \pm SEM.

6.4 DISCUSSION

6.4.1 Effect Of Vitamin E Supplementation On Plasma Lipid And Antioxidant Vitamin Profiles.

Despite the previously reported cholesterol lowering properties of α -tocopherol (Cloarec *et al.* 1987), supplementation with placebo or vitamin E at a dose of 800 IU per day was not associated with any significant effects on plasma total-, LDL- or HDL-cholesterol, or triglycerides. These findings are in agreement with previous reports (Szczeklik *et al.* 1985; Salonen *et al.* 1991).

Supplementation with plasma α -tocopherol at a dose of 800 IU per day produced a two-fold increase in mean α -tocopherol levels (mg l^{-1}), which is similar to that reported by Princen *et al.* (1995). Mean plasma levels of retinol were not affected by vitamin E supplementation at this dose.

6.4.2 Platelet Adhesion *Ex Vivo* Is Unaffected By Vitamin E Supplementation.

Platelet adhesion may be regarded as a crucial step in both the haemostatic process and in restenosis following vessel injury. Following PTCA the subendothelial tissue is exposed, this promotes the adhesion of platelets to its components, which include collagen. Vitamin E supplementation at a dose of 800 IU has been demonstrated to inhibit platelet adhesion (Steiner 1983; Jandak 1988). In this study no alteration in resting or stimulated platelet adhesiveness to either collagen coated or tissue culture plastic microwells was observed following supplementation with either vitamin E at a dose of 800 IU per day or placebo.

Previous studies employed a dynamic system (Steiner 1983; Jandak 1988), in which platelet adhesion to particulate collagen was observed in a flow chamber, unlike our static system. Also in this study the effect of vitamin E supplementation on adhesion to one component of the subendothelium and non-specific adhesion to tissue culture plastic was examined. Platelets possess receptors for various proteins of the subendothelium including fibronectin, thrombospondin and vWF. Therefore, it cannot be assumed that supplementation with vitamin E would not have effected platelet adhesion to microwells coated with other protein components of the subendothelium. Healthy volunteers were employed in previous studies as opposed to this present study in which patients with angiographically defined coronary artery disease were recruited. A variety of medications were prescribed to the subjects within our study, such as lipid lowering drugs, aspirin, beta-blockers, nitrates and anti-coagulants. Therefore, this failure to observe an inhibition of platelet adhesion in the present study may be as a result of the different method by which adhesion was measured and the different subjects studied.

6.4.3 Vitamin E Supplementation Fails To Alter Monocyte Adhesion *Ex Vivo*.

Supplementation with vitamin E at a dose of 800 IU per day for 7 months failed to inhibit monocyte adhesion to EA-hy-926 endothelial cells, collagen coated or tissue culture plastic microwells in a group of patients with angiographically defined coronary artery disease. Previous studies have demonstrated the capacity of vitamin E to inhibit monocyte adhesion both *in vitro* (Faruqi *et al.* 1994; Martin *et al.* 1997) and *ex vivo* following supplementation (Devaraj *et al.* 1996). In the supplementation study of Devaraj and colleagues a much higher dose of vitamin E (1200 IU per day) was employed and monocyte adhesion in both resting and activated states to lipopolysaccharide (LPS) treated human umbilical vein endothelial cells (HUVEC) was examined. This difference in dosage administered and their use of stimulated endothelial cells may in part explain the lack of effect of vitamin E supplementation in our study.

6.4.4 Vitamin E Supplementation Prevents A Rise In Soluble P-Selectin Levels Post-PTCA.

Elevated levels of s-P-selectin have been observed in conditions such as ischaemic heart disease, peripheral atherosclerosis, stroke and hypertension (Ikeda *et al* 1994b; Blann *et al* 1995; Ikeda *et al* 1995; Lip *et al* 1995; Zarifis *et al* 1996). P-selectin is located in both endothelial cells and platelets, where, upon activation it may be expressed on the cell surface and secreted into the plasma. The origin of s-P-selectin may be one or both of these cell types, however, it has been suggested that platelets are the major source of circulating s-P-selectin (Blann *et al* 1997; Fijnheer *et al* 1997).

Platelet activation has been demonstrated to occur following PTCA (Peterson *et al* 1986; Scharf *et al* 1992; Gasperetti *et al* 1993; Marmur *et al* 1994). The observed increase in sP-selectin levels post-PTCA in the placebo supplemented group may support the idea that PTCA produces activation of platelets. With no such increase being observed in the vitamin E supplemented group, this may suggest that vitamin E has the ability to limit the activation of platelets during PTCA. The failure to observe any alteration in either soluble ICAM-1 or vWF levels post-PTCA in either the placebo or vitamin E supplemented group may indicate that any endothelial activation produced is not immediate enough to result in alterations in the levels of these two glycoproteins, or that the mechanism by which activation or damage occurs does not result in increased levels of either of these two molecules. Vitamin E supplementation at 600 IU per day has been demonstrated to reduce sP-selectin in hypercholesterolaemic patients (Davi *et al* 1998). This reduction may also represent a reduction of the enhanced activation, which occurs in hypercholesterolaemic subjects (Carvalho *et al* 1974; Oppen *et al* 1995). In support of this theory is the lower excretion of urinary 11-dehydro-thromboxane B₂, an established marker of platelet activation, which also occurred following vitamin E supplementation (Davi *et al* 1998).

A possible mechanism by which vitamin E prevents the elevation of soluble P-selectin post-PTCA is through its inhibitory action on protein kinase C (PKC) (Mahoney & Azzi 1988; Boscoboinik *et al* 1991a). PKC activation may induce P-selectin expression (Geng *et al* 1990), further, lysophosphatidylcholine induced P-selectin expression can be significantly reduced by PKC inhibitors, such as 7-hydroxystaurosporine and N,N,N-trimethylsphingosine (Murohara *et al* 1996). Therefore the previously reported ability of vitamin E to inhibit PKC (Boscoboinik *et al* 1991a, 1991b) may in part explain the unaltered sP-selectin levels post-PTCA in the group supplemented with vitamin E.

CHAPTER 7**GENERAL DISCUSSION**

Epidemiological studies in humans and supplementation studies in animal models of atherosclerosis have indicated that antioxidants may have the ability to prevent or reduce atherosclerosis. Antioxidants may have the potential to exert beneficial effects on risk factors of atherosclerosis, on atherosclerotic lesion development and its complications and on the surgical treatment of coronary heart disease.

7.1 ANTIOXIDANTS & RISK FACTORS**7.1.1 Hypercholesterolaemia**

Hypercholesterolaemia is an important risk factor for the development of atherosclerosis (Keys 1980; Rose & Shipley 1986) and studies have demonstrated the beneficial value of lowering cholesterol in patients with coronary artery disease (Shepherd *et al* 1995). In hypercholesterolaemia the function of a number of cell types is altered, including the endothelial cells (Thomas *et al* 1968; Ross & Harker 1976), monocytes (Bath *et al* 1991a; Lösche *et al* 1992) and platelets (Carvalho *et al* 1974; Opper *et al* 1995). The increased propensity to atherosclerosis may result from the alteration to these and other cells or from the increased presence of LDL, which may become oxidatively modified.

In 1979 Hermann and colleagues first proposed the idea that vitamin E supplementation may increase the HDL cholesterol and by this means exert an anti-atherosclerotic effect. Increased levels of HDL may be beneficial in the prevention or reduction of atherosclerotic lesion progression through its ability to accept excess cholesterol from foam cells and deliver this cholesterol to the liver via the reverse cholesterol transfer pathway (Glomset 1968). A review of two dozen clinical studies reported that 60% of

these studies support the possibility of a beneficial influence of vitamin E on HDL cholesterol, the remainder documented no effect (Muckle & Nazir 1989). Vitamin E has also been reported to reduce total plasma cholesterol (Hermann *et al* 1979; Howard *et al* 1982; Stampfer *et al* 1983) and triglyceride levels (Pritchard *et al* 1986b). In both our studies in which hypercholesterolaemic subjects and patients with angiographically defined coronary artery disease were supplemented with vitamin E at a dosage of 400 IU and 800 IU per day respectively, there was no significant alteration in any of the lipid parameters measured. The routes through which vitamin E could physiologically modulate HDL cholesterol may be via its effect on enzymatic cholesterol catabolism in the liver (Chupukcharoen *et al* 1985) and on levels of plasma cholesterol-ester transfer protein (Tollefson *et al* 1988).

Vitamin C intake and plasma levels have been positively associated with HDL cholesterol (Cerna & Ginter 1978; Ness *et al* 1996). There have been reports of negative (Cerna & Ginter 1978; Greco & La Rocca 1982) or no association between vitamin C and total and LDL cholesterol (Elwood *et al* 1970; Hooper *et al* 1983). Existing evidence suggests that vitamin C can elevate plasma HDL cholesterol (Horsey *et al* 1981; Buzzard *et al* 1982; Salonen *et al* 1988), though not all studies have observed this alteration in lipoprotein profile (Bishop *et al* 1985; Aro *et al* 1988). Despite the previously reported ability of vitamin C to increase HDL cholesterol levels, in our study we did not observe any significant alterations in total-, LDL- or HDL-cholesterol or triglyceride levels in elderly hypertensive and normotensive subjects following supplementation at 500 mg per day for 3 months. Reported elevations of HDL cholesterol may be a result of the enhancement of P450 activity by ascorbate, resulting in increased conversion of cholesterol to bile acids and increased production of HDL cholesterol (Ginter 1973). The ability of antioxidant vitamins to favourably alter lipoprotein profiles may in part explain the inverse relationship between the intake of these vitamins and incidence of coronary heart disease.

7.1.2 Hypertension

Hypertension is associated with greater mortality from cardiovascular disease (MRFIT research group 1982). Oxygen free radicals and related intermediates have been implicated in hypertension and the development of disorders of the cardiovascular system, such as atherosclerosis and myocardial infarction, thereby contributing to the higher morbidity and mortality (Sagar *et al* 1992; Halliwell 1993; Kumar & Das 1993; Lacy *et al* 1998). Hypertension, as well as being a state of increased free radical activity is also associated with lower than normal plasma levels of antioxidant enzymes (Kumar & Das 1993) and antioxidant vitamins (McCarron *et al* 1984; Salonen *et al* 1987). The relationship between vitamin C intake and blood pressure (reviewed by Bulpitt 1990) is particularly strong. Reductions in blood pressure have been observed in small trials following vitamin C supplementation (Koh 1984; Trout 1991), though not all studies have reported a significant reduction (Ghosh *et al* 1994). In our study we also witnessed a reduction in the blood pressure of elderly hypertensive subjects but not their normotensive counterparts following vitamin C supplementation at 500 mg per day. This decrease in blood pressure was only significant in the 24 hour ambulatory blood pressure measurement, which, is believed to be a more accurate and precise measure of blood pressure.

Vitamin C may exert its anti-hypertensive action, through its effect on the vasodilator, nitric oxide (NO), the activity and/or synthesis of which may be impaired in essential hypertension (Cawgan & Benjamin 1993; Panza *et al* 1990). Inactivation of nitric oxide by superoxide may occur *in vivo*, thereby reducing its vasodilatory effect (Gryglewski *et al* 1986). Vitamin C has been shown to be an efficient scavenger of many reactive species including superoxide (Frei *et al* 1989). Along with other antioxidants including the vitamin E analogue (Trolox), vitamin C has been shown *in vitro* to regulate the activity of nitric oxide synthetase in endothelial cells, probably through removal of superoxide (Galley *et al* 1996). The impaired endothelium-dependent relaxation present in both hypertensive subjects and patients with coronary artery disease has improved

upon administration of a one-off bolus of ascorbic acid (Levine *et al* 1996; Solzbach *et al* 1997).

Therefore, vitamin C may prevent the NO deficiency in hypertension through the removal of superoxide, and by enhancing the vasodilatory action of nitric oxide cause a reduction in blood pressure. Results from a recent study by Galley and colleagues (1997) support this idea. They witnessed a reduction in blood pressure following antioxidant therapy, which was only significant in hypertensive subjects. These hypertensive subjects also exhibited a significant increase urinary nitrite excretion at the end of the antioxidant treatment; neither the normotensive nor the control group had any significant alteration in urinary nitrite excretion. One must bear in mind that NO is not the only contributor to nitrite/nitrate excretion, as dietary sources must also be considered. In the study by Galley *et al* (1997) no attempt was made to control or monitor any dietary changes which may have occurred. However, antioxidant enhancement of NO activity and/or synthesis may be a mechanism by which blood pressure can be reduced following supplementation with vitamin C.

7.2 ATHEROSCLEROTIC LESION DEVELOPMENT

Animal studies have supported the proposed anti-atherogenic effects of antioxidants, such as probucol (Carew *et al* 1987; Kita *et al* 1987; Daugherty *et al* 1989) and vitamin E (Verlangieri *et al* 1992; Konneh *et al* 1995; Lafont *et al* 1995) which have been shown to inhibit the development of atherosclerosis. However, not all studies have confirmed an inhibitory effect (Dam 1944; Godfried *et al* 1989). The progression of an atherosclerotic lesion may be limited at a number of stages by antioxidants. Even before the atherosclerotic lesion is visible, the endothelium may already be altered and its vasodilatory capacity impaired (Ludmer *et al* 1986; Simon *et al* 1993). This may be as a result of reduced NO activity and/or synthesis (Freiman *et al* 1986) or a consequence of decreased PGI₂ production (Verbeuren *et al* 1990). Antioxidants including β -carotene, α -tocopherol and vitamin C can improve the impaired endothelium-dependent relaxation (Keaney *et al* 1993; Stewart-Lee *et al* 1994; Levine *et al* 1996; Solzbach *et al* 1997). This may be a result of the ability of antioxidants to enhance endothelial production of prostacyclin (Kunisaki *et al* 1992) or through their ability to prevent superoxide or oxidised LDL inactivation of NO (Galley *et al* 1996).

Reduced NO production and activity may also result in the increased monocyte adhesion, which is evident in lesion prone areas (Hansson *et al* 1980, 1981). NO produced by the endothelium may modulate leucocyte adherence *in vivo* (Kubes *et al* 1991) and *in vitro* (Bath *et al* 1991b). Therefore by preventing the inactivation of NO, antioxidants may have the capacity to reduce leucocyte adhesion. Administration of antioxidants, including probucol, vitamin C and vitamin E have been shown to inhibit monocyte adhesion to endothelial cells *in vitro* (Faruqi *et al* 1994), *in vivo* (Ferns *et al* 1993) and *ex vivo* (Devaraj *et al* 1996; Weber *et al*. 1996).

The action of antioxidants on NO may not be the only mechanism by which leucocyte adhesion is inhibited. Faruqi and colleagues (1994) observed that the inhibition of agonist-induced monocyte adhesion to endothelial cells correlated with a decrease in

steady state levels of E-selectin mRNA and cell surface expression of E-selectin. The intracellular events involved in mediating monocyte adhesion are not fully elucidated and may require PKC activation. α -tocopherol has been shown to inhibit PKC (Boscoboinik *et al* 1991a, 1991b; Ozer *et al* 1993), however, the PKC inhibitor, Calphostin C, had no significant effect on monocyte-endothelial cell adhesion. An alternative mechanism by which monocyte adhesion may be enhanced is the activation of the nuclear transcription factor, NF κ B. NF κ B can be activated by oxidative stress, and modifiers of monocyte-endothelial cell adhesion including IL-1, PMA and LPS may act by the generation of ROS. Antioxidants have been shown to prevent NF κ B activation (Schreck *et al* 1992; Suzuki & Packer 1993), therefore, by inhibiting the release of ROS, α -tocopherol may reduce monocyte adhesion. However, Faruqi *et al* (1994) failed to demonstrate an effect of α -tocopherol on NF κ B activation in endothelial cells. The *in vitro* studies reported within this thesis have demonstrated an inhibition of monocyte adhesion following the pre-incubation of either endothelial cells or the monocytes themselves with vitamin E. Despite these *in vitro* observations and the previous demonstrations of the capacity of antioxidants to reduce monocyte adhesiveness, in the studies reported within this thesis which examined the effect vitamin E and vitamin C supplementation no significant effect of these antioxidants upon monocyte adhesiveness *ex vivo* was observed.

Monocyte adhesion and subsequent transendothelial migration followed by the uptake of oxidised LDL results in foam cell formation, which is the basis of the fatty streak, visibly the initial lesion of atherosclerosis. Along with the contribution to foam cell formation, Ox LDL within the lesion may exert a variety of effects including: induction of adhesion molecule expression on endothelial cells (Berliner *et al* 1990; Frostegård *et al* 1991); secretion of MCP-1 (Cushing *et al* 1990); cytotoxicity to endothelial cells, smooth muscle cells and macrophages (Hessler *et al* 1979; Morel *et al* 1984; Hughes *et al* 1994; Reid & Mitchinson 1993); alteration of PGI₂ production (Triau *et al* 1988; Zhang *et al* 1990) and inactivation of EDRF (Chin *et al* 1992); modulation of growth factor (Fox *et al* 1987) and cytokine release (Hamilton *et al* 1990; Frostegård *et al* 1992); promotion of

monocyte adhesion (Couffinhal *et al* 1993) and platelet aggregation (Ardlie *et al* 1989). The ability of antioxidants including probucol and vitamins C and E (Jialal *et al* 1990; Esterbauer *et al* 1991a, 1991b; Jialal *et al* 1991; Reaven *et al* 1992) to inhibit LDL oxidation may prevent or reduce these effects and therefore slow lesion progression. Indeed antioxidants have been demonstrated to protect cells from the cytotoxic effects of Ox LDL (Kuzuya *et al* 1991), prevent enhanced adhesion molecule expression (Faruqi *et al* 1994), inhibit platelet aggregation (Srivastava 1986; Violi *et al* 1990) and reduce cytokine release (Devaraj *et al* 1996).

Within the lesion monocytes differentiate to become macrophages. Upon activation, macrophages can act as a source of growth factors such as PDGF (Glenn & Ross 1981) within the lesion. They are also able to release cytokines including IL-1 and TNF (Bevilacqua *et al* 1984, 1985; Pober *et al* 1988) and produce ROS (Cathcart *et al* 1985; Leake & Rankin 1990), hence they may contribute to the development of the lesion in this way also. Supplementation with α -tocopherol has resulted in the inhibition of monocyte cytokine release and production of ROS (Devaraj *et al* 1996). Previously it has been shown that α -tocopherol inhibits PKC activity (Ozer *et al* 1993) which may be crucial for superoxide release and LDL oxidation by activated monocytes (Li & Cathcart 1994). The PKC inhibitor, Calphostin C, decreased monocyte superoxide anion release and inhibited lipid oxidation (Devaraj *et al* 1996). Therefore, α -tocopherol could possibly inhibit monocyte function as a result of its ability to reduce PKC activity.

Release of growth factors such as PDGF within the evolving lesion can stimulate the proliferation of human smooth muscle cells (Ross *et al* 1974; Glenn & Ross 1981). *In vitro* α -tocopherol has been demonstrated to inhibit smooth muscle cell proliferation by mediators including PDGF (Boscoboinik *et al* 1991a), which is believed to be mediated by the reduction of PKC activity (Boscoboinik *et al* 1991a; Azzi *et al* 1995). As the lesion progresses further damage to the endothelium can result in platelet adhesion, activation and subsequent aggregation. Through the release of their contents including PDGF (Fuster *et al* 1978) platelets can contribute to the progression of the lesion.

Vitamin E has been demonstrated to inhibit the release reaction (Steiner & Anastasi 1976) and through this effect may slow lesion progression

The lesion may rupture or fissure resulting in platelet adhesion and subsequent thrombosis (Chandler *et al* 1974; Davies *et al* 1976). Antioxidant vitamins C and E can also inhibit platelet adhesion and aggregation (Cordova *et al* 1982; Bordia & Verma 1985; Szczeklik *et al* 1985; Jandak *et al* 1988), though not all studies have reported inhibitory effects (Steiner 1983; Stampfer *et al* 1988). Possible mechanism by which vitamin E may alter platelet adhesiveness and aggregability include increased membrane fluidity (Steiner 1981), inhibition of calcium release (Butler *et al.* 1979), inhibition of PKC activity (Freedman 1996) and inhibition of cyclooxygenase (Mower & Steiner 1983; Salonen *et al* 1991) and lipooxygenase (Gwebu *et al* 1980), enzymes of the arachidonic acid cascade. One or a combination of these effects may be responsible for the inhibitory effects of antioxidants on platelet function. In the *in vitro* studies reported within this thesis an inhibitory effect of vitamin E both *in vitro* and following supplementation in hypercholesterolaemic subjects on platelet aggregation was observed. The effects on platelet adhesion have not been so conclusive. *In vitro* ascorbic acid failed to affect platelet adhesiveness, while α -tocopherol inhibited thrombin stimulated adhesion at the higher concentration of 200 $\mu\text{mol l}^{-1}$. Following supplementation with vitamin E there was no effect on platelet adhesion. Vitamin C, however, inhibited platelet adhesion in normotensive but not their hypertensive counterparts.

Together the beneficial effects of antioxidants upon endothelial dysfunction, inhibition of monocyte adhesion, cytokine and ROS species release, inhibition of smooth muscle cell proliferation, inhibition of platelet adhesion and aggregation and prevention of oxidation of LDL may act in concert to reduce or prevent atherosclerotic lesion progression (Figure 7.1)

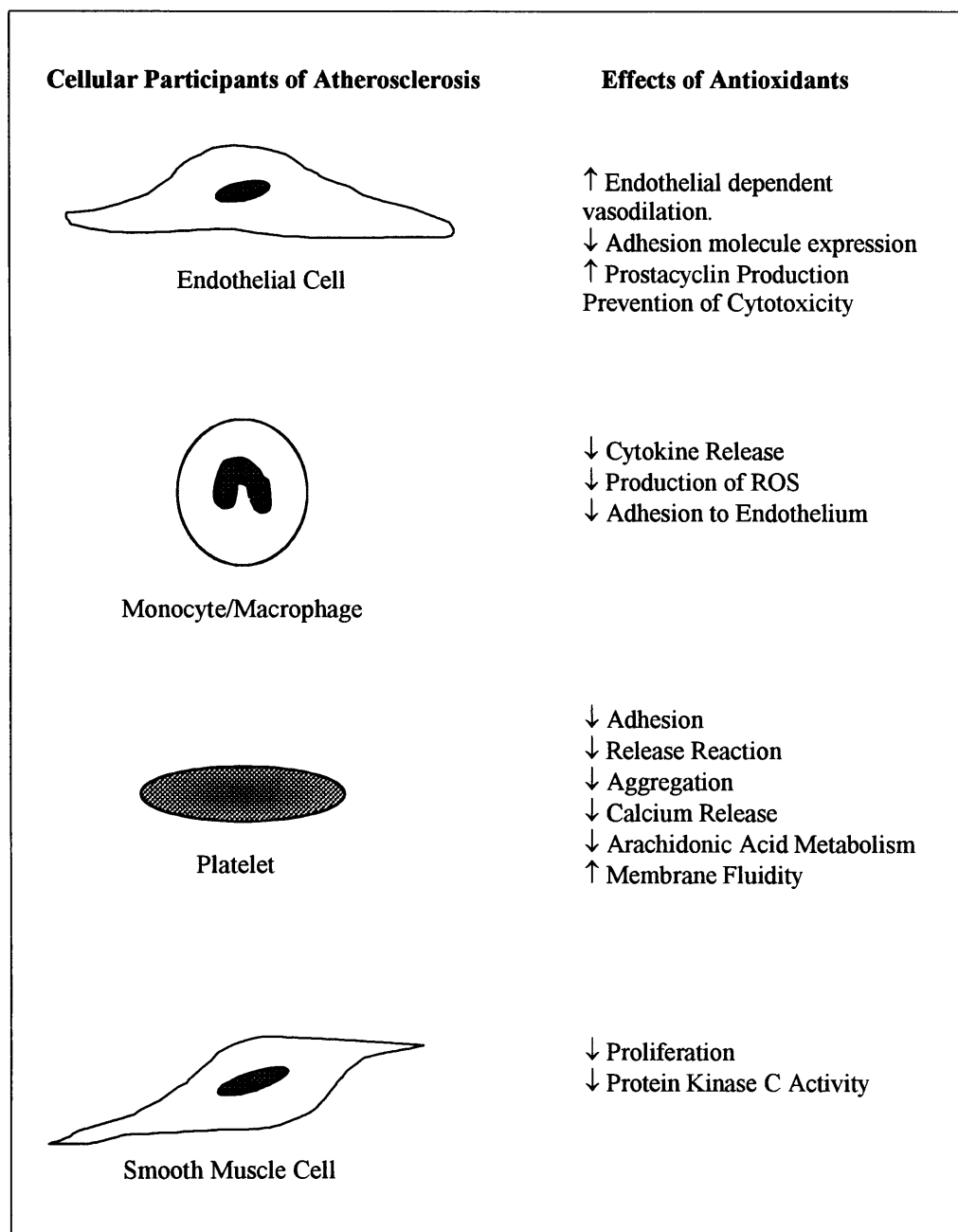


Figure 7.1 Potential anti-atherogenic effects of antioxidants on the cellular components of an atherosclerotic lesion.

7.3 CLINICAL SYMPTOMS OF ATHEROSCLEROSIS

Myocardial ischaemia is a well-recognised complication of atherosclerotic cardiovascular disease resulting from an imbalance between oxygen supply and demand in the working myocardium due to the diminished blood flow, a consequence of a narrowed lumen. Plaque fissuring, coronary vasospasm and platelet thrombus formation may all contribute to narrowing of the lumen. Transient ischaemia occurs in unstable angina, which may evolve to myocardial infarction. Re-establishment of blood flow may promote tissue injury through generation of oxygen free radicals and lipid peroxidation. Pre-supplementation with vitamin E in an animal model has been shown to reduce the extent of tissue lipid peroxidation, myocardial infarct size and tissue damage, following cardiopulmonary bypass (Axford-Gately & Wilson 1993). Although another free radical scavenger, SOD, failed to produce any beneficial effects (Uraizee *et al* 1987).

The risk of angina and myocardial infarction has been shown to be increased in subjects with antioxidant deficiency (Gey *et al* 1991; Riemersma *et al* 1991; Nyyssönen *et al* 1997). In a clinical study, SOD also failed to improve the outcome from myocardial infarction (Flaherty *et al* 1994). A recent study by Stephens *et al* (1996) reported that supplementation with α -tocopherol in patients with angiographically proven coronary atherosclerosis, produced a significant reduction in non-fatal MIs. Studies have also examined the effect of vitamin E on angina (Rinzler *et al* 1950; Anderson 1974; Anderson & Reid 1974; Gillilan *et al* 1974). Only two of these were randomised double blind trials and only small groups were investigated, little clinical benefit was demonstrated from these trials. However, a reduction in the requirement for nitroglycerine in patients has been reported following vitamin E supplementation (Toone 1973).

7.4 SURGICAL INTERVENTION

Coronary artery disease may proceed to such an extent that surgical intervention is required, whether it is coronary angioplasty or coronary bypass surgery.

7.4.1 Percutaneous Transluminal Coronary Angioplasty

Percutaneous transluminal coronary angioplasty is a widely accepted treatment of stenotic lesions resulting from atherosclerosis. The success of this treatment still remains compromised by restenosis, despite mechanical (Serruys *et al* 1991; Kakuta *et al* 1994) and pharmacological intervention. Pharmacological approaches include antiplatelet agents (Knudtson *et al* 1990; Topol *et al* 1994), anticoagulants (Ellis *et al* 1987; Sarembrook *et al* 1990; Thornton *et al* 1984), cholesterol-lowering agents (Sahni *et al* 1991) and ω 3 fatty acids (Dehmer *et al* 1988; Reis *et al* 1989). Restenosis is initiated by endothelial and deep vessel injury leading to platelet aggregation, thrombus formation, inflammation and activation of leucocytes and smooth muscle cells (Lange *et al* 1993). Production and release of growth factors and cytokines also result, which may promote their own synthesis and release from target cells. Thus a self-perpetuating process is initiated (Libby *et al* 1992), resulting in the migration of smooth muscle cells from the media to the intima where they proliferate and produce extracellular matrix, the outcome of which is stenosis within the vessel lumen.

Reactive oxygen species are also released at the site of injury following PTCA (Roberts *et al* 1990; Coghlan *et al* 1991). Damaged endothelium (Rubuyani 1988), activated platelets (Finazzi-Agrò *et al* 1982; Salvenimi & Botting 1990) and activated neutrophils (Ikeda *et al* 1994a) at the angioplasty site can generate these reactive oxygen species. In turn these ROS can act upon various cell types including smooth muscle cells (Rao *et al* 1992), platelets (Violi *et al* 1988; Salvenimi *et al* 1989) and the endothelium (Ginsburg *et al* 1989). This interaction can result in smooth muscle cell proliferation (Rao *et al* 1992),

promotion of platelet aggregation (Violi *et al* 1988) and alteration in the release of vasoactive factors from the endothelium (Gryglewski *et al* 1986) which can contribute to restenosis following angioplasty.

Administration of antioxidants such as probucol (Ferns *et al* 1992; Schneider *et al* 1993) and vitamin E (Konneh *et al* 1995; Lafont *et al* 1995) have produced inhibitory effects on restenosis after angioplasty in animal models. Not all studies, however, have observed a favourable effect (Hsiang *et al* 1991). A beneficial effect of antioxidants on restenosis was mainly observed in studies where the animals were pre-medicated with the relevant antioxidant at least 2 days before angioplasty. This may allow sufficient time for incorporation of lipid soluble antioxidants into lipoproteins and membranes and also for optimisation of plasma and cytosol water soluble antioxidant vitamin concentrations.

A recent clinical study by Tardif and colleagues (1997) also reported a reduction in restenosis by 47% by probucol after PTCA. In this study a group receiving multivitamin treatment (30,000 IU β -carotene, 500 mg vitamin C and 700 IU vitamin E) failed to exhibit a significant reduction in restenosis. Each group was pre-treated for 30 days prior to angioplasty, unlike the study by O'Keefe *et al* (1996) in which probucol and lovastatin treatment only began 24 and 48 hours prior to angioplasty, no significant inhibition was observed. As mentioned Tardif and colleagues failed to observe any significant inhibition in the multivitamin group despite adequate supplementation prior to angioplasty. DeMaio and colleagues observed a trend toward reduction of restenosis following vitamin E supplementation at 1200 IU per day, however, this just failed to reach statistical significance ($p = 0.06$). In this study supplementation did not begin until 48 hours after angioplasty (De Maio *et al* 1992). Despite these studies the question of whether antioxidants may prevent restenosis in clinical studies of PTCA has not been answered conclusively.

Antioxidants may reduce restenosis following angioplasty via a number of mechanisms. Increased lipid uptake and reactive oxygen species generation post-angioplasty (Roberts

et al 1990) can result in enhanced oxidised LDL production. Although the role of oxidised LDL in restenosis has not been definitely resolved, vitamin E, vitamin C and β -carotene can reduce free radical formation and prevent oxidative modification of LDL (Jialal *et al* 1990; Esterbauer *et al* 1991a, 1991b; Jialal *et al* 1991). Through which they may slow oxidised LDL mediated plaque progression after angioplasty. The inflammatory response initiated following vessel injury during angioplasty could influence the amount of collagen and matrix formation produced as a result of vessel injury. Vitamin E has been shown to reduce inflammation, (Stuyvesant & Jolley 1967; Kamimura 1972) facilitate wound healing (Kim & Shklar 1983) and reduce collagen and scar tissue formation (Ehrlich *et al* 1972). Through these effects, vitamin E could theoretically reduce the amount of neointimal formation. Platelet aggregation, thrombosis and release of platelet-derived vasoactive and mitogenic agents are a consequence of vessel injury. Antioxidants including vitamin E and vitamin C may modulate platelet function through their inhibitory effects on platelet aggregation, (Fong 1976; Agradi *et al* 1981; Cordova *et al* 1982) platelet adhesion, (Steiner 1983; Bordia & Verma 1985; Jandak *et al* 1988), activity of enzymes of the arachidonic acid cascade (Toivanen *et al* 1987) and thromboxane synthesis (Karpen *et al* 1981; Toivanen *et al* 1987). Together these multiple sites of action may decrease platelet activation and platelet-mediated thrombosis after angioplasty. In our study α -tocopherol supplementation did not significantly affect platelet adhesion either prior to angioplasty or at the follow up visits. Nevertheless the prevention of a rise in soluble P-selectin levels post-angioplasty in the α -tocopherol supplemented group compared to the placebo group is indicative of an inhibition of platelet activation by α -tocopherol.

Smooth muscle cell proliferation is a major contributor to restenosis and may be induced by interleukin-1 (Raines *et al* 1989), oxidised LDL (Chatterjee 1992) and PDGF (Ross *et al* 1974). α -tocopherol can inhibit PDGF-, endothelin- and LDL-induced proliferation of vascular smooth muscle cells *in vitro* at physiologically relevant concentrations (Boscoboinik *et al* 1991a; Azzi *et al* 1995; Ozer *et al* 1993). This inhibition of smooth muscle cell proliferation may be due to inhibition of protein kinase C activity

(Boscoboinik *et al* 1991a; Azzi *et al* 1995). Oxidative conditions (Gopalakrishna *et al* 1989) and lipid oxidation products (O'Brian *et al* 1988) can activate PKC, therefore the inhibitory effect of α -tocopherol on protein kinase C may in part be antioxidant mediated. However, antioxidants such as trolox, BHT, and compounds structurally similar to α -tocopherol have failed to inhibit smooth muscle cell proliferation (Boscoboinik *et al* 1991a, 1991b). It is also possible that α -tocopherol may act as a site-specific ligand for protein kinase C and prevent its translocation to the membrane, which is required for protein kinase C activation (Boscoboinik *et al* 1991a).

7.4.2 Cardiopulmonary Bypass Surgery

Cardiopulmonary bypass during cardiac surgery is a clinical setting in which the myocardium is exposed to a brief period of ischaemia. Although ischaemia itself can result in cell injury, which depends upon the tissue in question and the period of oxygen deprivation, it is at the point of reperfusion that the majority of the injury occurs (Granger *et al* 1981). The attenuation of this injury by superoxide-dismutase (SOD) demonstrated that this was free radical mediated (Granger *et al* 1981; Parks *et al* 1982).

Controlled studies have shown that cardiac vitamin E content is reduced, leading to oxidative stress, during bypass surgery (Barsacchi *et al* 1992; Coghlan *et al* 1993). Therefore reperfusion injury may be reducible by antioxidants. Supplementation with a combination of vitamin E 400 mg and vitamin A 100,000 IU for 5 days prior to coronary artery bypass surgery produced diminished oxidative stress and membrane lipid peroxidation compared to the control group (Ferreira *et al* 1991). Pre-operative treatment with allopurinol before elective cardiopulmonary bypass has shown substantial benefit with regard to cardiac function (Johnson *et al* 1991).

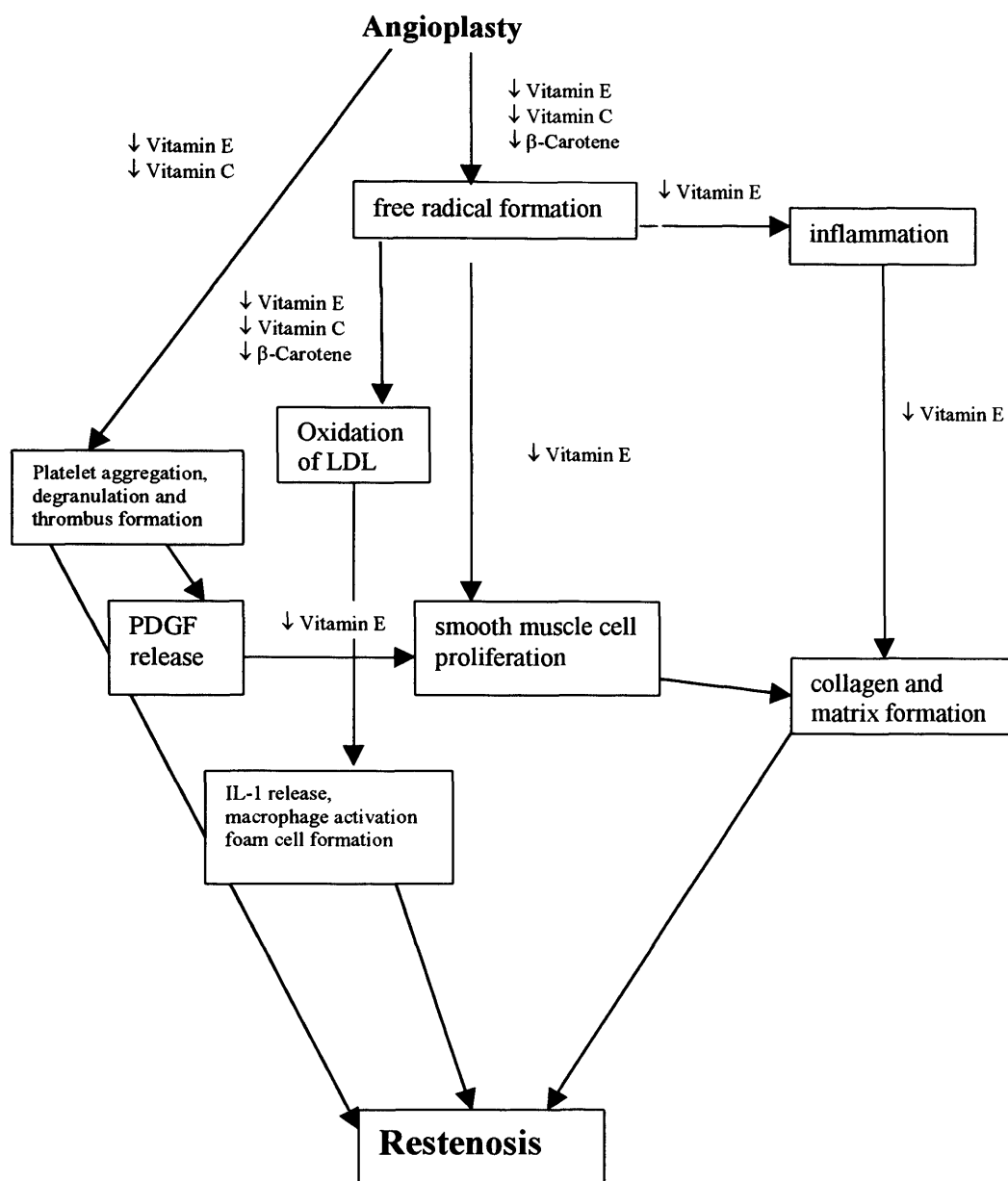


Figure 7.2 Mechanisms by which antioxidants may slow the progression of restenosis following angioplasty (adapted from Godfried & Deckelbaum 1995).

↓ = Inhibition

7.5 CLINICAL SUPPLEMENTATION STUDIES

Epidemiological studies have demonstrated an association between increased intake of antioxidant vitamins, such as vitamin E and vitamin C and both reduced clinical manifestations of atherosclerosis and reduced morbidity and mortality from coronary artery disease. To fully investigate this relationship randomised supplementation trials have been undertaken. The results of recent randomised trials have been mixed.

The Alpha Tocopherol Beta Carotene Cancer Prevention Trial (ATBC) was designed to look at the effects on vitamin E and β -carotene on lung cancer and later encompassed coronary artery disease (The ATBC Prevention Study Group 1994). A total of 29,133 male smokers were randomly assigned to receive one of four regimens: β -carotene (20 mg per day), α -tocopherol (50 mg per day), both α -tocopherol and β -carotene or placebo. Follow-up continued for five to eight years, during which time 3570 deaths occurred. Amongst participants assigned to α -tocopherol, there were fewer deaths from ischaemic heart disease and ischaemic stroke than there were amongst those who did not receive α -tocopherol, but more deaths due to cancer other than lung cancer or due to haemorrhagic stroke. Overall mortality was 2% higher in the α -tocopherol groups than in the groups that received no α -tocopherol. There were more deaths due to lung cancer, ischaemic heart disease, and ischaemic and haemorrhagic stroke among recipients of β -carotene. Overall mortality was 8% higher amongst participants who received β -carotene than amongst those not given β -carotene. The dose of α -tocopherol was probably too low but the dose of β -carotene was adequate. Another trial the Physicians' Health Study, also reported no reduction in deaths from cardiovascular causes among physicians receiving supplemental β -carotene over a 12 year period (Hennekens *et al* 1996).

In the Probucol Quantitative Swedish Regression Trial, 303 patients were assigned to receive either probucol or a cholesterol-lowering drug and the end point was angiographically defined atherosclerotic lesion size in femoral arteries (Walldius *et al* 1994). Despite a reduction in total and LDL cholesterol and a reduced susceptibility of

LDL from this group to oxidation, this group exhibited no beneficial effects regarding femoral atherosclerosis above that of the group assigned to the cholesterol-lowering drug alone. The authors suggest this may be due to the HDL lowering effect of probucol.

A recent study investigated the effect of supplementation of vitamin E at 800 or 400 IU per day or placebo in 2002 patients with angiographically proven coronary atherosclerosis (Stephens *et al* 1996). These patients were supplemented for a mean of 510 days (range 3-981) and the primary endpoints were a combination of cardiovascular death and non-fatal MI as well as non-fatal MI alone. There were 50 cardiovascular deaths and 55 non-fatal MIs during the study period. This study reported a significant reduction in the risk of non-fatal MI in the α -tocopherol group. There was a non-significant excess of cardiovascular deaths and total mortality was slightly but not significantly greater in the α -tocopherol group compared to the placebo group.

The results from these studies suggest that antioxidants may not be as beneficial in the treatment of cardiovascular disease as one would predict from the epidemiological evidence, animal supplementation studies and *in vitro* data and may actually produce deleterious effects. Overall the randomised therapeutic trials reported so far have shown no benefit with β -carotene or probucol and a possible benefit with vitamin E. This may suggest that antioxidant supplementation should not be prescribed to all but possibly confined to a subset of patients with sub-optimal antioxidant vitamin levels in which any favourable effects may be more clearly observed.

7.6 SUMMARY

Development of atherosclerosis is a complex process that involves a co-ordinated interplay between cellular elements, including monocytes, platelets, endothelial cells and smooth muscle cells, molecules such as cytokines, adhesion molecules and growth factors, and risk factors which may predispose an individual. The progression from initial insult through to complicated lesion is lengthy with a number of points at which interventions may act.

Throughout this thesis a number of stages at which antioxidant vitamins can act to prevent or slow atherosclerotic lesion progression have been highlighted. Risk factors include elevated plasma cholesterol concentration, cigarette smoking, high blood pressure, diabetes and obesity. This thesis while reporting no beneficial effect of supplementation of the antioxidant vitamin C and E on plasma lipoprotein profiles in hypercholesterolaemics, elderly normotensive and hypertensive subjects and patients with coronary artery disease has shown an ability of vitamin C to lower blood pressure. The observed reduction of blood pressure in elderly hypertensive subjects may suggest that antioxidants, and more specifically vitamin C may act to prevent or reduce the effects of the risk factor hypertension.

Monocyte adhesion is one of the earliest visible events of atherosclerotic lesion development (Gerrity 1981). *In vitro* studies supported the ability of antioxidants to reduce monocyte adhesion. This anti-adhesive action was not evident following supplementation of hypercholesterolaemic patients and those with undergoing routine PTCA with vitamin E, or elderly normotensive and hypertensive subjects with vitamin C. The examination of monocyte adhesion in hypertensive subjects did reveal a relationship between pulse pressure and monocyte adhesiveness to collagen. This correlation between monocyte adhesion and pulse pressure, may be one mechanism by which pulse pressure can contribute to increased risk of cardiovascular disease.

Though the role of platelets in early lesion development is disputed, they do play a crucial role in later stages and more specifically the thrombotic complications. Platelet adhesion and subsequent aggregation are vital to the haemostatic process. These studies have demonstrated the ability of vitamin E to inhibit both platelet adhesion and aggregation *in vitro*, this inhibition of platelet function may be a consequence of the effects of vitamin E on platelet membrane fluidity. While platelet aggregation was inhibited in hypercholesterolaemic subjects following vitamin E supplementation, the effects on platelet adhesion were not so conclusive, with no significant effect seen following supplementation in patients undergoing routine PTCA. However, the prevention of elevated levels of soluble P-selectin following PTCA in the group supplemented with vitamin E compared to the placebo group does suggest an limitation of platelet activation by vitamin E. Stephens and colleagues (1996) suggest that the favourable effects observed in the CHAOS study may be as a result of the effects of vitamin E on platelet function. Vitamin C may also have the capacity to inhibit platelet function (Cordova *et al* 1982; Bordia & Verma 1985), following vitamin C supplementation of elderly normotensive subjects an inhibition of platelet adhesion was observed.

These results are suggestive of a beneficial effect of the antioxidant vitamins, vitamin C and vitamin E, in CHD, especially with regard to platelet function. However, the true test of the therapeutic potential of antioxidant vitamins in CHD is the use of these vitamins in clinical supplementation studies. To date the results obtained from the limited numbers already completed are not as promising as one would hope and therefore bring in to doubt the benefit of antioxidant vitamin supplementation. Rather a more appropriate strategy may be their use in subjects with low antioxidant status or use as a preventative measure during early atherosclerotic lesion development.

APPENDICES

APPENDIX I--MATERIALS AND SUPPLIERS

Manufacturers of equipment are stated where appropriate in the text

Blood Collection

Lithium-Heparin Monovette® tubes (1.5 U heparin ml⁻¹) (Sarstedt, Leicester, UK)

Potassium EDTA Monovette® tubes (1.6 mg ml⁻¹) (Sarstedt, Leicester, UK)

21 gauge butterfly needles (Appleton Woods, Birmingham, UK)

Multi-Adapters (Sarstedt, Leicester, UK)

Chemicals and Solutions

α-tocopherol (Sigma Chemical Company, Poole, UK)

α-tocopherol acetate (Sigma Chemical Company, Poole, UK)

Adenosine 5'-diphosphate (ADP), aggregation reagent (Sigma Chemical Company, Poole, UK)

Apyrase (Grade 1) (Sigma Chemical Company, Poole, UK)

Ascorbic acid (Sigma Chemical Company, Poole, UK)

Bovine Serum Albumin (Sigma Chemical Company, Poole, UK)

Calcium chloride (Sigma Chemical Company, Poole, UK)

Citric acid (Sigma Chemical Company, Poole, UK)

Collagen (type IV) (Sigma Chemical Company, Poole, UK)

Collagen, aggregation reagent (Sigma Chemical Company, Poole, UK)

Copper (II) sulphate 5-hydrate (BDH Chemicals, Lutterworth, UK)

1,6-diphenyl-1,3,5-hexatriene (DPH) (Sigma Chemical Company, Poole, UK)

Folin-Ciocalteu reagent (BDH chemicals, Lutterworth, UK)

Glacial acetic acid (BDH chemicals, Lutterworth, UK)

Glucose (Sigma Chemical Company, Poole, UK)

HEPES (N-[2-Hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]) (Sigma Chemical Company, Poole, UK)

Hexadecyltrimethylammonium bromide (Sigma Chemical Company, Poole, UK)

Histopaque®-1077 (Sigma Chemical Company, Poole, UK)

Human thrombin (Sigma Chemical Company, Poole, UK)

Magnesium chloride (Sigma Chemical Company, Poole, UK)

Metaphosphoric acid (Sigma Chemical Company, Poole, UK)

Phosphate Buffered Saline tablets (Sigma Chemical Company, Poole, UK)

Phosphate citrate buffer (Sigma Chemical Company, Poole, UK)

p-nitrophenyl phosphate tablets (Sigma Chemical Company, Poole, UK)
 Potassium sodium tartrate (Sigma Chemical Company, Poole, UK)
 Prostacyclin (Sigma Chemical Company, Poole, UK)
 Retinol (Sigma Chemical Company, Poole, UK)
 Retinyl acetate (Sigma Chemical Company, Poole, UK)
 Sodium bicarbonate (Sigma Chemical Company, Poole, UK)
 Sodium chloride (Sigma Chemical Company, Poole, UK)
 Sodium citrate (Sigma Chemical Company, Poole, UK)
 Sodium hydrogen carbonate (Sigma Chemical Company, Poole, UK)
 Sodium hydroxide (Sigma Chemical Company, Poole, UK)
 di-Sodium hydrogen orthophosphate (Sigma Chemical Company, Poole, UK)
 Sulphuric acid (BDH chemicals, Lutterworth, UK)
 Tetramethylbenzidine (Sigma Chemical Company, Poole, UK)
 Triton-X-100 (Sigma Chemical Company, Poole, UK)

Solvents

Acetonitrile (Romil Ltd, Cambridge, UK)
 Dimethylsulfoxide (Sigma Chemical Company, Poole, UK)
 Ethanol (Romil Ltd, Cambridge, UK)
 Hexane (Romil Ltd, Cambridge, UK)
 Methanol (Romil Ltd, Cambridge, UK)
 Tetrahydrofuran (Romil Ltd, Cambridge, UK)

Tissue Culture

Cryotubes (Bibby Sterilin Ltd., Staffordshire, UK)
 Dulbecco's modified Eagle's medium with HAT (Life Technologies Ltd., Scotland, UK)
 EA-hy-926 cell line (Gift from Dr Cora-Jean Edgell, University of North Carolina, USA)
 Foetal calf serum (Life Technologies Ltd., Scotland, UK)
 RPMI-1640 (Life Technologies Ltd., Scotland, UK)
 Sterile flat bottomed 96 well microtitre plates (Bibby Sterilin Ltd., Staffordshire, UK)
 Tissue culture flasks 75mm² (Bibby Sterilin Ltd., Staffordshire, UK)
 Trypan blue (Sigma Chemical Company, Poole, UK)
 Trypsin EDTA solution (Life Technologies Ltd., Scotland, UK)

Vitamin Capsules

Vitamin C and placebo capsules (Nova Pharmaceuticals, Leicester, UK)
 Vitamin E and placebo capsules (gift from Dr Rupert Mason, Bioglan Ltd, Herts, UK)

Miscellaneous

Aggregometer Cuvettes (Eden Scientific, Richmond, Surrey, UK)

Aggregometer Stir Bars (Eden Scientific, Richmond, Surrey, UK)

Autosampler vials (Jones Chromatography Ltd., Mid Glamorgan, Wales, UK)

Chart Recorder Paper (Fisher Scientific, Loughborough, UK)

Nitrogen (BOC Ltd., Guildford, Surrey, UK)

Plastic Transfer pipettes (Appleton Woods, Birmingham, UK)

Sterilin flat bottomed 96 well microtitre plates (Bibby Sterilin Ltd., Staffordshire, UK)

APPENDIX II--BUFFERS AND SOLUTIONS

Ca²⁺ Free Tyrodes Buffer

10 mmol l⁻¹ HEPES, 145 mmol l⁻¹ NaCl, 2.7 mmol l⁻¹ K Cl, 1.8 mmol l⁻¹ MgCl₂, 5.55 mmol l⁻¹ glucose, 5.95 mmol l⁻¹ NaHCO₃ and 0.42 mmol l⁻¹ Na₂HPO₄, pH 7.4.

Phosphate Buffered Saline

10 mmol l⁻¹ phosphate buffer, 2.7 mol l⁻¹ KCl, 137 mmol l⁻¹ NaCl, pH 7.4 at 25°C

Phosphate Citrate Buffer

50 mmol l⁻¹ phosphate citrate buffer containing 0.03 % (w/v) sodium perborate, pH 5.0 at 25°C.

Platelet adhesion buffer

150 µl of 0.1 mol l⁻¹ citrate buffer, pH 5.4, containing 0.1% (v/v) Triton-x-100 and 5 mmol l⁻¹ p-nitrophenol phosphate

Monocyte adhesion buffers

Lysis Buffer

100 µL hexadecyltrimethyl-ammonium bromide (0.5% in PBS; pH 5.0).

Substrate Buffer

TMB (0.1 mg ml⁻¹ in 0.05M phosphate citrate buffer (pH 6.0) containing 0.03% sodium perborate)

Lowry Solution

2% Na₂CO₃ in 0.4% NaOH (w/v)/ 1% CuSO₄ (w/v)/ 2% potassium sodium tartrate (w/v) were mixed in the following ratio 10 ml/ 0.1 ml/ 0.1 ml respectively.

Folins Reagent

50% (v/v) Folin-Ciocalteu phenol reagent in dH₂O

Trypsin EDTA solution

0.05% (w/v) Trypsin (1:250) and 0.02% (w/v) EDTA per litre of Modified Puck's Saline A.

APPENDIX III--CALCULATIONS

Friedwald Formula

LDL cholesterol = total cholesterol – (HDL cholesterol + (triglycerides/2.2))

CALCULATION OF APPARENT MEMBRANE MICROVISCOSITY**Grating factor for Analyser**

Firstly, the correcting grating factor was determined by measurement of the emission intensity detected through an analyser orientated parallel $[(I_{\parallel})_H]$ and perpendicular $[(I_{\perp})_H]$ to the direction of polarisation of the horizontal excitation light:

$$G = \frac{(I_{\parallel})_H}{(I_{\perp})_H} \quad \text{Equ. 1}$$

Where:

G = correcting grating factor (Arbitrary);

$(I_{\parallel})_H$ = emission intensity detected through an analyser oriented parallel $[(I_{\parallel})_H]$ to the direction of polarisation of the horizontal excitation light.

$(I_{\perp})_H$ = emission intensity detected through an analyser oriented perpendicular $[(I_{\perp})_H]$ to the direction of polarisation of the horizontal excitation light.

Degree of fluorescence polarisation

The degree of fluorescence, P was determined. This was corrected using the instrument specific grating factor determined in equation 1:

$$P = \frac{(I_{\parallel} - I_{\perp}) \cdot G}{(I_{\parallel} + I_{\perp}) \cdot G} \quad \text{Equ.}$$

Where

P = degree of fluorescence polarisation;

I_{\parallel} = emission intensity detected through an analyser oriented parallel to the direction of polarisation of the excitation light;

I_{\perp} = emission intensity detected through an analyser oriented perpendicular to the direction of polarisation of the excitation light.

Apparent microviscosity

The apparent microviscosity was approximately determined, in systems labelled with DPH by measuring the fluorescence polarisation:

$$\bar{\eta} \approx \frac{\left(\frac{I_{\parallel}}{I_{\perp}}\right) - 1}{0.73 - 0.27 \cdot \left(\frac{I_{\parallel}}{I_{\perp}}\right)} \quad \text{Equ. 3}$$

Where:

η = apparent microviscosity [dyne s cm⁻² (poise, P)]

Equation 3., can also be expressed in terms of degree of fluorescence polarisation, P :

$$\bar{\eta} \approx \frac{2p}{0.46 - p} \quad \text{Equ. 4}$$

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